Bacterial profiles and ex vivo effects of *Salmonella* Heidelberg on leukocyte function in turkey purebred lines

By

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ABSTRACT

Escalating product recalls as a consequence of *Salmonella*-contaminated poultry products have resulted in detrimental economic impacts. One long-term alternative to *Salmonella* prevention, receiving increased attention, is selection to improve genetic resistance. This study evaluated the effects of an oral *Salmonella* Heidelberg (SH) challenge on bacterial colonization, and the ex vivo effects of SH on phagocytic and bactericidal leukocyte function in turkeys from six pedigree lines (A-F). Data were analyzed using JMP Pro (SAS) and differences were determined using Student's *t*-test following ANOVA with significance reported at $P \leq 0.05$. Interaction effects of treatment X gender X genetic line were significant on bacterial colonization in the ceca. Cumulatively, females exhibited higher phagocytosis potential than males. The main effect of genetic line was significant bactericidal activity of PBMCs. Microbial profiling of cecal DNA was performed to examine differences in colonization of *Salmonella*, *E. coli*, and *Enterococcus* species among the genetic lines. Results indicated line E having the highest *Enterococcus* but lowest *Salmonella* colonization than all other lines, while line A birds displayed the highest *Salmonella* colonization. These results suggest that gender and genetic line have a marked effect on susceptibility to *Salmonella* colonization, while genetic line X gender has a more eminent effect on *Enterococcus* cecal colonization. If able to determine genetic markers associated with these immune responses to *Salmonella*, genetic selection for increased resistance could be feasible in turkeys.

Keywords: Turkey, *Salmonella*, Resistance, Susceptibility, Heterophils

Dedication

I would like to dedicate this thesis to my mother and father, Leshia and Howard D. Bell Jr. Thank you for being my inspiration and for showing me that anything is possible. I will love you forever and always.

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List of abbreviations

APCs: antigen presenting cells

AvBD: avian β-definsins

BHI: brain Heart Infusion broth

CFU: colony forming unit

CMI: cell-mediated immunity

C_t: threshold cycle

D: day(s)

DC: dendritic cells

dpi: days post-inoculation

GALT: gut-associated lymphoid tissues

IELs: intra-epithelial lymphocytes

Ig: Immunoglobulin

ILK: interleukin

LB: Luri-Bertani broth

LPS: lipopolysaccharide

LS Means: least squares means

MALT: mucosa-associated lymphoid tissues

MAMPs: microbe-associated molecular patterns

MHC: major histocompatibility complex

NK cells: natural killer cells

PALS: periarteriolar lymphoid sheaths

PBMCs: peripheral blood mononuclear cells

PCR: polymerase chain reaction

P/L: polymorph/lymphocyte

PRRS: pattern recognition receptors

qRT-PCR: quantitative real-time polymerase chain reaction

SH: *Salmonella* Heidelberg

Tc cell: cytotoxic T cell

Th cell: helper T cell

TLRs: Toll-like receptors

Chapter I

Introduction

Salmonella enterica, subspecies *enterica*, are Gram-negative bacterial pathogens consisting of more than 2,500 different serovars, of which only a finite number is associated with poultry. The most commonly isolated serovars in poultry include *S.* Enteritidis, *S.* Typhimurium, *S.* Heidelberg, and *S.* Kentucky, all of which are non-host-adapted and can cause infections in a variety of animal hosts and humans (Desin et al., 2013). *Salmonella enterica* can cause a wide range of illnesses, ranging from gastroenteritis to acute, life-threatening enteric fever. The inflammatory response induced by *Salmonella* Typhimurium and *Salmonella* Enteritidis often restricts infection to the area of the gastrointestinal tract, where it may establish a carrier state and become a potential source of poultry-product contamination resulting in foodborne illness (Cheng et al., 2013). Salmonellosis is one of the most prevalent infectious foodborne diseases in the world (McCarthy et al., 2009). An estimated number of over one million cases of human infection with *Salmonella* species occur in the United States each year (Cheng et al., 2013). *S. enterica* Heidelberg was found to be the most prevalent serovar isolated from both chicken and turkey carcasses (Logue et al., 2007). An increasing number of *Salmonella* contaminated poultry products has resulted in widespread product recalls and detrimental economic impacts. Furthermore, consumer skepticism of the safety of poultry products has intensified. In order to alleviate this issue, researchers are striving to discover an advantageous and cost-effective *Salmonella* preventative.

Although biosecurity measures are functional in a *Salmonella* prevention plan, biosecurity alone does not negate the risk of contamination. Currently, researchers are

investigating the use of vaccines and dietary direct-fed microbials to mitigate *Salmonella*. Despite numerous studies, there has yet to be a cumulatively safe, cost-effective, and well-defined *Salmonella* vaccine available for use in the poultry industry. The development of such a vaccine for poultry faces numerous obstacles, the most critical being the cost as well as the ability for mass immunization (Desin et al., 2013). Studies of direct-fed microbials (e.g. PrimaLac) have proven to be effective at reducing *Salmonella* colonization in poultry flocks (Grimes et al., 2008); however, a more permanent method of prevention is needed in order to restore consumer confidence in the poultry industry and diminish the risk of ongoing economic impacts.

One long-term and cost-effective alternative to conventional methods of *Salmonella* prevention is selection for genetic resistance. Previous studies have focused on the genetics and genomics of resistance to *Salmonella* in chickens; however, few trials have focused on these aspects in turkey breeding flocks. The initial stage in such studies is to identify the phenotypic traits of interest and determine that a genetic basis actually exists for those specific traits. Estimated heritability of parameters of *Salmonella* response as well as differences observed between genetic lines of chickens suggests a partial genetic control of most response phenotypes. This implies that genetic selection to improve resistance to *Salmonella* carrier state is feasible in chickens (Cheng et al., 2013). Some studies suggest that particular major histocompatibility complex haplotypes and polymorphisms may serve as markers for genetic resistance to *Salmonella* enteritidis in young chicks (Liu et al., 2002).

The potential of genetic selection as a long-term method of *Salmonella* prevention has been well researched in chickens. Studies of genetically distinct chicken lines suggest a strong relationship between heterophils and resistance to systemic *Salmonella* enteritidis (Swaggerty et

al., 2005). Due to the fact that the avian heterophil is an important component in host defense against bacterial infections, this knowledge is critical in determining factors to consider when analyzing traits for genetic selection of *Salmonella* resistance. Further studies should consider observing aspects such as *Salmonella* colonization and heterophil functional differences in turkey purebred lines.

Only recently has research been published concerning factors related to the feasibility of genetic selection for *Salmonella* resistance in turkeys. Genovese and colleagues (2006) measured functional differences in heterophils isolated from a commercial turkey line and those isolated from wild-type Rio Grande turkeys. Results of this study suggested immunological advantages and disadvantages between genetic lines. Therefore, there is a need for further research into the differences and similarities between the innate immune response of commercial turkey lines and wild-type turkeys to improve genetic resistance and decrease pathogen contamination in commercial turkey lines (Genovese et al., 2006). A more effective alternative would be to improve genetic resistance to *Salmonella* at the pedigree level in order to ultimately improve resistance at the commercial flock level; therefore, diminishing the risk of *Salmonella* contamination and reducing its consequential economic impact.

The objective of this study was to determine the effects of a *Salmonella* Heidelberg challenge on bacterial colonization of the ceca, liver and spleen in six turkey pedigree lines differing broadly in disease susceptibility. Furthermore, microbial profiling and ex vivo effects of *Salmonella* Heidelberg on turkey leukocyte function were characterized in order to provide additional insight on the phenotypic differences between genetic lines.

Chapter II

Literature Review

Introduction

The avian immune system persistently battles foreign antigens, allowing the bird to remain healthy and produce a superior product for the future consumer. In certain instances, such as during situations of environmental stress, the immune system may become compromised and permit entry of opportunistic foreign antigens including disease-causing bacteria. *Salmonella* contaminated poultry products present a dire threat to the poultry industry and the economy. A long-term and cost effective prevention method is needed in order to reduce the occurrence of *Salmonella* contaminated products and restore consumer confidence in the poultry industry. In this chapter, the components of the avian immune system are briefly discussed along with disease fighting mechanisms. Pertinent aspects of the menacing bacteria *Salmonella* are particularly detailed. Methods of transmission, economic impact, increasing antibiotic resistance, short-term prevention, and potential long-term prevention methods such as genetic selection for increased resistance levels are illustrated throughout this literature review.

Avian Immune System

There are few distinct functional differences in the avian and mammalian immune systems. For all vertebrates, including avian species, the immune system is divided into two key components: innate and adaptive immunity.

Innate immunity and mucosal defenses

Innate immunity and mucosal defenses constitute the first line of defense when encountering antigens. The innate immune system is marked by non-specific immune responses that are available upon the day of hatch providing immediate protection against an imminent foreign challenge.

Physical and chemical barriers

The epithelial layers, mucous membranes, and body secretions create physical barriers to prevent entry of foreign antigens. However, hosts with a compromised immune system and lacking integrity in these areas are more susceptible to further break down of physical barriers by infectious agents. Mechanisms such as respiratory ciliary movement, fatty acids on the integument, peristaltic movement within the intestine, high gastric acid pH, bird's body temperature, mucus secretions, and antimicrobial peptides prove to be highly effective barriers to potential infection (Juul-Madsen et al., 2014).

The respiratory and gastrointestinal tracts are the major port of entry for foreign antigens. The respiratory tract uses ciliated epithelium and mucus membranes to filter the airways clear of invading threats. In most cases, cilia are able to remove the organism; however, if the cilia have sloughed due to current ongoing infection or disease, the host is then susceptible to inhaled organisms, which can present a potential threat to the immune system (Butcher and Miles, 2003).

The thick mucosal layer of the gastrointestinal tract blocks pathogens from invading host cells, therefore protecting the integrity and health of the gastrointestinal tract. The gut maintains a stable and dense microbial flora that prevents colonization of invading organisms through methods of competitive exclusion and production of toxic substances (Sharma, 2003). In order to

prevent *salmonella* infections, day-old chicks can be treated with mixtures of normal GI flora to enhance colonization with beneficial flora; therefore allowing commensal flora to compete with invading pathogens (Juul-Madsen et al., 2014). Chemical defense mechanisms, such as low pH of the gastrointestinal tract, are effective against ingested pathogens (Goldsby et al., 2000). Furthermore, the high body temperature of the avian species potentially eliminates many infectious agents from causing disease (Butcher and Miles, 2003).

Multifunctional epithelial cells play a key role in the function of the enteric immune system. A physical barrier between the host and intestinal contents is composed of mainly enterocytes and mucus layer. Specialized enterocytes, known as goblet cells, secrete numerous mucin proteins making up the mucus protective layer covering the epithelial layer. Mucins are commonly divided into those that are secreted (gel-forming) and those that are membrane-bound. Candidate mucins identified in the chicken include Muc1, Muc2, Muc4, Muc5ac, Muc5b, Muc6, Muc13, Muc16 and the bird-specific ovomucin. Muc2 is identified as the primary mucin produced in the large intestine and plays an indispensable role in maintaining the balance between the host and commensal microbes in the lower gut (Smith et al., 2014).

Antimicrobial peptides are critical elements of natural defense. These short peptides (approximately 10-50 amino acids in length) are enriched in hydrophobic and cationic amino acid residues, allowing them to be highly efficient in penetrating bacterial cell walls. Two major classes of antimicrobial peptides, cathelicidin-like proteins and defensins (β-defensins), have been identified in chickens. Cathelicidin-like proteins have been designated as CATH-1, 2, 3 and CATH-B1. CATH-1 and CATH-2 are primarily expressed in the bone marrow but CATH-2 is also highly expressed in heterophils. Both CATH-1 and CATH-2 peptides illustrate cytotoxic activity and binding capacity to lipopolysaccharide (LPS) (Juul-Madsen et al., 2014).

There are 14 β-defensins identified in the chicken, known as avian β-defensins (AvBD). The AvBD are not only highly produced by heterophils and macrophages, but are expressed at mucosal surfaces of the urogenital, respiratory, and intestinal tracts. These peptides are active against a wide range of microorganisms including *E. coli*, *S.* Enteritidis, and *S.* Typhimurium (Juul-Madsen et al., 2014).

Although the majority of pathogens are eliminated from the host by these physical and chemical barriers, infectious agents may gain access if these defense mechanisms are damaged or compromised. Increasing resistance of bacteria to commonly used antibiotics has resulted in a critical need to find alternative treatment methods. Long-term and cost-effective prevention is needed in order to minimize widespread economic losses.

Cellular barriers

Infectious agents that surpass physical and chemical barriers encounter cellular barriers designed to protect the host from further invasion. Antigen presenting cells (APCs) sample the environment for foreign antigens and convey with B and T lymphocytes of the adaptive immune system.

The major histocompatibility complex (MHC) is a genetic locus containing properties of dominantly expressed class I and class II molecules. The primary function of these surface molecules is to bind and present peptide fragments to T lymphocytes of the immune system. A foreign antigen undergoes either endogenous or exogenous processing resulting in peptides being expressed on the surface of the APCs. These peptides are bound to either MHC class I or MHC class II molecules depending on the source of the antigen. The type of MHC molecule determines the immune response to the antigen. Almost all cells express class I MHC molecules

on their outer surface and express peptides originating from endogenous antigens. Exogenous antigens reside within endosomes and can be phagocytosed by macrophages or taken up by dendritic cells (DC) and B cells. Digestion of exogenous antigens into antigenic peptides results in antigenic peptide presentation on an MHC class II complex. Antigen presentation results in either activation of lymphocytes to eliminate the foreign antigen or self-antigen tolerance. The MHC has been shown to determine resistance or susceptibility to certain infectious pathogens (Kaufman, 2014). Characteristic APCs of the innate immune system include phagocytic cells such as macrophages, heterophils (the avian equivalent to the mammalian neutrophil), and dendritic cells. The complement system assists antibodies and phagocytic cells in working side by side to eliminate invading pathogens. Pathogen elimination can be achieved through cell lysis, opsonization, chemotaxis, and/or phagocytosis (Kaspers and Kaiser, 2014).

Phagocytic cells, such as heterophils and peripheral blood mononuclear cells (PBMCs), play a significant role in innate immunity by initiating a strong immune response to foreign invaders. Heterophils are the most prominent granulated leukocytes in the acute inflammatory response. These cells are part of the first line of defense against invading microbial pathogens in areas where resident macrophages are lacking. Heterophils show a broad spectrum of antimicrobial activity and often accumulate in inflamed tissues. Due to a lack of myeloperoxidase, heterophils depend on oxygen independent mechanisms for antimicrobial activity. A wide variety of bacteria can be eliminated by the potent β-defensins found in heterophil granules. An identified group of heterophil-granule antimicrobial peptides have been identified as gallinacins (Gal 1- α, Gal 1, Gal 2), chicken heterophil antimicrobial peptides (CHP 1, CHP 2), and turkey heterophil antimicrobial peptides (THP 1, THP 2, THP 3) (Evans et al., 1994; Harwig et al., 1994). These defensins have a broad spectrum of activity against Gram-

positive and Gram-negative bacteria, protozoa, fungi, and some viruses (Lehrer et al., 1993; Aley et al., 1994).

A particularly significant cellular component of the innate immune system is natural killer (NK) cells. Chicken NK cells are distinguished as large lymphocytes containing electron dense granula. Unlike the B and T cells, NK cell development is bursa and thymus-independent. NK cell-mediated cytotoxicity has been identified in the spleen and in blood cell populations (Kaspers and Kaiser, 2014). These cytotoxic lymphocytes do not require activation in order to destroy foreign microbes and therefore are not restricted by the MHC. NK cells attack foreign microbes by releasing cytoplasmic granules containing toxic proteins such as perforin and proteases that are designed to kill virus infected cells and tumor cells (Sharma, 2003).

The dendritic cell (DC) is identified as an APC responsible for stimulating naïve T cells in an antigen-specific immune response. In some instances, antigen uptake occurs in an area that is anatomically distant from where antigen presentation to lymphocytes occurs; therefore, the DC has the ability to selectively migrate to areas that are optimal for antigen presentation (Juul-Madsen et al., 2014).

Pattern recognition receptors (PRRs) located on the surface of the immune cell recognize microbe-associated molecular patterns (MAMPs) on microbes and trigger the cells of the innate immune system to take action. The Toll-like receptors (TLRs) are an example of PRRs that recognize microbial patterns and trigger an immediate response by innate immune cells. Antigen presentation occurs post activation and phagocytosis of the foreign pathogen. The phagocyte presents a processed fragment of the pathogen to B and T lymphocytes of the adaptive immune system to stimulate a response to the pathogen. This method of presentation deems the innate immune cells as APCs and triggers both immediate innate immune defense mechanisms as well

as the activation of the adaptive immune response. Dendritic cells and macrophages process antigens and stimulate naïve lymphocytes to become effector cells that are capable of eliminating specific pathogens in the host (Lee et al., 2007).

Adaptive Immunity

When innate immunity is unable to stop the invasion of foreign pathogens, adaptive immunity intervenes to establish a specific and efficient host immune response. The antigen is processed by the cells of the innate immune system and then recognized by T- and B-lymphocytes of the adaptive immune system. Adaptive immunity is not only able to eliminate foreign antigens, but also builds a memory with to protect against subsequent challenges in a fast and efficient manner.

Adaptive immunity is developed through vaccination or natural occurrences and can be further divided into humoral or cell-mediated immunity. Pathogen specific characteristics will determine whether the immune system will utilize a humoral response, cell-mediated response, or a combination of the two in order to eradicate foreign antigens. Immune organs that play a vital role in these responses include the thymus, bursa of Fabricius, spleen and mucosal lymphoid tissues (Oláh et al., 2014).

The thymus consists of 7-8 lobes found on each side of the neck that extend from the third cervical vertebra to the upper thoracic segments. These lobes are enclosed in fibrous connective tissue and embedded in adipose tissues. In the epithelial anlage of the thymus, immature T lymphocytes proliferate in the subcapsular zone before migrating towards the medulla during T cell maturation. Medullary post-capillaries permit immunologically competent cells to exit the thymus for a cell-mediated immune response (Davison, 2014).

The bursa of Fabricius is a sac-like structure dorsal to the cloaca of the bird that is primarily essential for antibody-mediated immunity. It is here that somatic gene conversion produces antibodies that recognize specific conformational molecular shapes on their target antigen through the immunoglobulin (Ig) variable region. In the absence of a bursa, an antibody repertoire would not be generated; therefore, resulting in a non-functional humoral immune system (Oláh et al., 2014).

The round or oval structure lying dorsal to and on the left side of the proventriculus is known as the spleen. This immune organ has a closed circulation system and begins development post-hatch, following exposure to antigens. The spleen is composed of both red and white pulp with white pulp lacking erythrocytes and predominantly containing lymphocytes. Hematopoiesis is restricted to the red pulp; however, once this function ceases, the red pulp is responsible for filtering out old erythrocytes. Both lymphoid and non-lymphoid cells can be found in the red pulp of the spleen. Many phagocytic macrophages and heterophils are scattered throughout the red pulp sinuses. The spleen has the ability only to obtain antigens from blood circulation. The white pulp of the spleen contains distinct areas such as the periarteriolar lymphoid sheaths (PALS) that surround the central arteries and the periellipsoidal white pulp (PWP) that surround the penicillary capillaries. The spleen is considered to be an important organ for immune regulation because of its involvement in both the innate and adaptive immune response. The location of various lymphocytes and non-lymphoid cells in the spleen indicate that the PALS have strong involvement in adaptive immunity while PWP is involved in both the innate and adaptive immune response. Furthermore, B cell progenitors undergo rearrangement of their Ig genes in the spleen before colonizing the bursa of Fabricius (Oláh et al., 2014).

Once lymphocytes are developed in either the thymus or bursa of Fabricius, they differentiate into effector cells in secondary lymphoid organs such as the spleen and mucosal associated lymphoid tissues. It is here that effector cells come in contact with foreign pathogens or other antigens considered to be “non-self” (Dalloul and Lillehoj, 2006).

Humoral immune response

Humoral immunity involves specific Ig or antibody production and accessory processes such as cytokine production, helper T cell activation, phagocytosis promotion, classical complement activation, and memory cell generation. It is characterized by antibodies that are synthesized and secreted by plasma cells derived from B-lymphocytes originating from the bursa of Fabricius, which is avian specific. These B cells generate specific antibodies post stimulation by microbial or antigen exposure. Antibodies have three distinct mechanisms of ridding the host from foreign antigens. These mechanisms include opsonization, neutralization, and complement activation. Opsonization occurs when antibodies bind to antigen receptors and coat the surface of the pathogen in order to facilitate phagocytosis. Antibody neutralization takes place when antibodies react with epitopes on an infectious agent and inhibit attachment to the host, thereby, preventing the occurrence of an infection. When antibodies bind to the surface of the invading pathogen, complement is activated which aids in successful phagocytosis (Sharma, 2003).

A distinct difference in the mammalian and avian immune system is the aspect that birds only have three main classes of immunoglobulins: IgM, IgG (also known as IgY), and IgA, while mammals carry five main types of antibodies. IgM is the first antibody found in circulation post challenge and is commonly found on the surface of B lymphocytes. IgG is the most abundant immunoglobulin found in circulation and is produced during a secondary immune response,

while IgA is a secretory antibody that is primarily involved in mucosal immunity and can be found on numerous mucosal surfaces (Sharma, 2003).

Cell-mediated immune response

Cellular immunity is primarily controlled by the essential maturation of T lymphocytes into effector cells. Upon antigen exposure, a highly regulated immune response is generated through the stimulation of both humoral and cellular immunity. Antigen presenting cells process and present the antigen to immune cells, which initiate a complex series of events that create the ultimate immune response. T cells differentiate into subpopulations with multiple effector functions to effectively remove the foreign antigen. These subpopulations of avian T lymphocytes are categorized into CD4+ (helper T or Th cells) and CD8+ (cytotoxic T cells) (Davison et al., 2008). Activation of Th cells occurs through recognition of a class II MHC paired with processed antigen on an APC where they then divide and produce various cytokines in order to activate B and T lymphocytes. The profile of cytokines present in the environment will determine the Th cell subtype. Type 1 helper (Th1) cells produce cytokines that initiate inflammation and activate B and T lymphocytes and macrophages; furthermore, inhibiting the function of Th2 cells in order to generate immunity to intracellular pathogens. On the other hand, Th2 cells secrete cytokines that stimulate B-lymphocyte proliferation and antibody production and inhibit Th1 cell function to strengthen immunity to foreign pathogens (Tizard, 2009). Th17 produces the distinct pro-inflammatory cytokine IL-17. Cytotoxic T (Tc) cells recognize and perform lysis of infected cells with endogenous pathogens associated with class I MHC molecules (Dalloul and Lillehoj, 2006).

Gut-associated lymphoid tissue (GALT)

The gut associated lymphoid tissues (GALT) include the cecal tonsils, Meckel's diverticulum, Peyer's patches, and intra-epithelial and lamina propria lymphocytes. The gastrointestinal tract major function is to digest and absorb nutrients in order to meet metabolic demands for normal growth and development. Furthermore, the gastrointestinal tract acts as a barrier that prevents the penetration of harmful antigens and pathogens attempting to enter from the external environment (Beal et al., 2006) as described above. The GALT comprise the largest component of the mucosa-associated lymphoid tissues (MALT) and are subject to continuous exposure to microflora and ingested pathogens (Yun et al., 2000). Through both innate and adaptive immune responses, the gut is protected from infection caused by foreign antigens.

The mucosal layer of the gut is primarily composed of the epithelium and the lamina propria. The epithelial layers are highly populated with a specialized group of lymphocytes collectively known as the intra-epithelial lymphocytes (IELs). This population includes major subsets of NK and T cells; however, B cells are completely absent from the IELs. The lamina propria underlies the epithelial layer and contains a wide variety of different leukocytes including granulocytes, macrophages, DC, B and T cells. Along the luminal side of the lamina propria, there is a border of thick basement membrane lined with columnar epithelium. It is here that mucus-producing goblet cells can be found interspersed between epithelial cells (Smith et al., 2014). Antigen recognizing T lymphocytes can be found throughout the GIT in the intra-epithelium and lamina propria. Chickens, unlike mammals, have scattered lymphoid aggregates as well as organized lymphoid structures rather than lymph nodes. These structures include the cecal tonsils, Meckel's diverticulum, Peyer's patches, in addition to the bursa of Fabricius. The cecal tonsils contain both T and B lymphocytes and serve as a site of antibody production and

cell mediated immune function. The Meckel's diverticulum, also known as the remnant of the yolk sac, contains B cells and macrophages to enhance the cellular immune response to foreign antigens. The Peyer's patches are lymphoid cell accumulations commonly found at the ileocecal junction (Oláh et al., 2014). The epithelium of the Peyer's patches contains phagocytic antigen sampling cells known as microfold (M) cells (Muir et al., 2000). These cells remove antigens from the lumen and deliver them to APCs allowing B and T lymphocytes to generate a specific immune response to eliminate the obtruding antigen (Beal et al., 2006).

Microbial ecosystem.

The gut microbiota plays a critical role in host health by positively influencing the host's gastrointestinal development, immune responses, physiology, and nonspecific pathogen resistance (Gordan and Pesti, 1971). In avian species, microorganisms colonizing the gastrointestinal tract early post hatch form a mutually beneficial relationship with their host (Torok et al., 2007). Bacteria within the gut not only interact with the host but associate with each other (Angelis et al., 2006) and although typically studied as individual species, microbes in general appear as communities in the GIT. Microorganisms can attach to the GIT lining and alter the physiology and immunological health of the bird. Approximately 640 different bacterial species and 140 different genera have been identified in the chicken gastrointestinal tract (Apajalahti et al., 2004). Dietary manipulations significantly affect the gut microbial communities of the ileum and cecum. Furthermore, the presence of beneficial bacteria and absence of detrimental bacteria may lead to improved bird performance (Torok et al., 2007).

Effects of environmental stress on immunity

Genetics as well as environmental stress play a key role in disease resistance or susceptibility to a bacterial pathogen. A stress response is typically determined by previous experiences and genetic sensitivity. Stress response intensity may be measured through evaluation of the glucocorticoid hormones and the thyroid hormone T_3 of the host. Due to circulating polymorphonuclear cells (polymorph) and lymphocytes being efficient defenders against bacterial invaders, the polymorph/lymphocyte (P/L) ratio can also be used as a stress indicator (Gross and Siegel, 1983). Short-term stressors often result in increased P/L ratios and physiological effects including decreased antibody response to foreign pathogens, poor feed conversion, and a decreased resistance to viral infections; however, resistance to bacteria, parasites, and toxins increases (Gross and Siegel, 1965; Gross, 1990). Although lymphoid mass may potentially be decreased, antibody responsiveness may be normal and resistance to bacteria, parasites, and toxins is actually increased rather than decreased. The magnitude of the cell-mediated response is increased while its effectiveness is suppressed. Stresses such as these may lead to adrenal exhaustion. If a host in a relatively low stress environment receives an optimal dose of stress, then their resistance to bacterial infections will increase and may elevate host defenses to bacterial pathogens without the use of antibacterial drugs. In order to obtain transcription of DNA to mRNA at the most effective level and maximize feed conversion, a producer should strive for optimal stress levels for good husbandry which may vary among different genetic lines (Gross and Siegel, 1997).

Salmonella

Salmonella is a rod-shaped, Gram-negative, primarily motile, non-spore forming bacterium. The cell envelope structure of *Salmonella* is composed of a cytoplasmic membrane, a thin peptidoglycan layer, and an outer membrane containing LPS. The peptidoglycan layer is pinpointed as the loose network of peptidoglycan chains located in the space between the cytoplasmic membrane and outer membrane. This space is referred to as the periplasmic space. These unique characteristics of the cell envelope structure permit the classification of *Salmonella* as Gram-negative bacteria (Adair et al., 2003).

This facultative anaerobe is commonly found in poultry, swine and environmental sources such as water, animal feces, improperly cooked swine/poultry meat products, and dairy products. *Salmonella* invade the gastrointestinal tract of poultry and colonizes the intestinal mucosa, cecal tonsils, and Peyer's patches. The bacterium then has the capability of surviving and multiplying in macrophages and, in severe cases, colonizing in the liver and spleen via the blood stream or lymphatic system. Furthermore, *Salmonella* has the ability to penetrate and thrive inside the egg, gizzard, and yolk sac. Colonization may also be found in the ovary and oviduct resulting in contaminated eggs from layers. The serovars Typhimurium and Enteritidis are the most common forms of *Salmonella* found in the United States (Adair et al., 2003). *Salmonella enterica* subspecies *enterica* serovar Heidelberg is one of the most commonly detected serovars found in poultry and in the top five serogroups associated with human salmonellosis (Foley et al., 2011). In the United States, it is responsible for approximately 84,000 human infection each year and 7% of *Salmonella*-related deaths (Kennedy et al., 2004). Although mostly asymptomatic in poultry, a variety of *Salmonella* strains present a significant threat to the poultry industry and the average consumer.

Transmission of Salmonella

Salmonella can be transmitted in poultry flocks through a variety of methods including, but not limited to, direct infected bird-to-bird contact, ingestion of contaminated feces or litter, contaminated water, or personnel and equipment. A primary source of flock contamination results from *Salmonella* contaminated feeds, particularly those that contain animal products and do not withstand pelleting. The wide variety of *Salmonella* hosts creates an unlimited number of reservoirs of infectious organisms. For instance, insects, mice, wild birds, and even humans have the ability to carry and spread *Salmonella* infections. Furthermore, transmission of *Salmonella* to the progeny of infected breeder flocks can easily result from either internal or external contamination of eggs. This often occurs through fecal contamination during oviposition. Egg-borne or vertical transmission is a rising concern and plays a major role in the transmission of *S. arizonae* infections in turkeys. In fact, *Salmonella* serotypes isolated from naturally infected chicks and poults are often also isolated from parent flocks. It is apparent that *Salmonella* carried in or on eggs is spread throughout the hatchery. Newly hatched birds are highly susceptible to intestinal colonization by *Salmonella*. Furthermore, *Salmonella* is easily spread within and between flocks. Contaminated poultry house environments are often associated as the leading source of pathogenic *Salmonella* (Adair et al., 2003). Lahellec and Colin (1985) concluded that serotypes present in broiler houses during the rearing period were more likely to appear on processed carcasses than serotypes originating from the hatchery.

Clinical signs and pathology of Salmonella

Clinical signs of *Salmonella* in poultry are very rarely observed after the first two weeks of life; however, morbidity and mortality may be high during that time period followed by

significant growth retardation. Although clinical disease is not typically associated with pathogenic *Salmonella* in mature poultry, some strains may cause anorexia, diarrhea, and reduced egg production in infected laying hens. In chicks and young poults, *Salmonella* may cause drooped wings, ruffled feathers, anorexia, emaciation, shivering, huddling near heat sources, diarrhea, pasty vents, lameness and blindness in more severe cases. In newly hatched poultry, septicemia can cause high mortality with or without the presence of lesions. Long-term disease results in focal necrotic lesions in the mucosa of the small intestine. Spleen, livers, and kidneys are often found swollen and congested along with unabsorbed, coagulated yolk material remaining in the yolk sac (Adair et al., 2003).

Salmonella has the ability to overwhelm epithelial cells throughout the gastrointestinal tract; however, the ceca and the ileocecal junction are identified as areas of key interest. As *Salmonella* invades the epithelial cells at the tip of the villi, a series of pathologic changes occur affecting the intestinal fluids and regulation of electrolytes. These pathologic changes ultimately culminate in cell death and profuse diarrhea. Bacteria such as *S.* Enteritidis can produce inflammation of the epithelium, lamina propria, and ceca in response to heterophilic infiltration. As epithelial cells are invaded, *Salmonella* can be moved through the basement membrane and into the lamina propria by macrophages. Not only is *Salmonella* able to invade cells but also has the ability to survive and multiply in internal organs, particularly the liver, spleen, and reproductive tract (Adair et al., 2003).

Relationship of Salmonella colonization level of poultry with food safety

Salmonella species are estimated to cause over a million human infections annually in the U.S. alone, resulting in approximately 500 deaths and making it one of the most common enteric

pathogens (Scallon et al., 2011). Due to milder cases not being reported, the actual number of infections may be significantly higher. In the U.S. alone, disease resulting from paratyphoidal salmonellosis costs approximately $3.3 billion per year (Strugnell et al., 2014). Case-control studies identified chicken consumption as a major risk factor in *Salmonella* infections (Kimura et al., 2004). Changes in the Kaufmann-White scheme of *Salmonella* classification have resulted in the newly named *S. enterica* subspecies Enterica serovar Enteritidis. Recently, more than half a billion eggs from two U.S. farms were recalled due to *Salmonella* Enteritidis contaminated shell eggs (U. S. FDA, 2010). Furthermore, *Salmonella* Heidelberg contamination of ground turkey meat in 2011 resulted in one of the largest voluntary recalls in U.S. history, involving more than 36 million pounds of meat (Tarr, 2011). Thus, *Salmonella* in poultry presents a large and imminent threat to the overall economy.

The most recent estimate (2012) of turkeys produced by Virginia farmers is 17 million birds with an output of 449 million pounds (VPF, 2013). Turkeys are ranked as Virginia's fourth largest agricultural commodity in terms of cash receipts generating $285 million and eggs are ranked ninth with cash receipts of $77 million. Nationally, Virginia is ranked fourth among states for turkey production (Farm Bureau, 2012). When coupled with the numbers associated with a major recall due to bacterial contamination, the cost to Virginia's agricultural economy becomes significant at multiple levels.

The recent *Salmonella* Heidelberg contamination of ground turkey meat resulted in one of the largest recalls in U.S. history costing the turkey industry untold amounts of dollars (Tarr, 2011). Such product recalls also undermine consumer confidence in poultry products as a safe and wholesome food, thereby reducing the economic viability of turkey production, in Virginia and the nation. Thus, although mostly asymptomatic in poultry, *Salmonella* presents a large and

imminent threat to human health and the poultry industry. Therefore, reducing the load of *Salmonella* in poultry (turkeys) would significantly impact public health and the overall agricultural economy.

Salmonella Heidelberg and antibiotic resistance

Antibiotic resistance has become a major concern in salmonellosis. Zhao and colleagues (2008) observed the antimicrobial resistance in *Salmonella enterica* serovar Heidelberg isolates from chicken breast (*n = 5,075*), ground beef (*n = 5,100*), ground turkey (*n = 5,044*), and pork chops (*n = 5,706*) from 2002 to 2006. These studies indicated poultry meat being the dominant source of *Salmonella* Heidelberg, accounting for a total of 96.6% of all isolates. Approximately 2% of these isolates, all from turkey, were resistant to nine antimicrobials at minimum. When observing specific antimicrobial resistance, poultry isolates were most resistant to tetracycline (39.9%) and streptomycin (37.8%). Other common antimicrobials of resistance included sulfamethoxazole (27.7%), gentamicin (25.7%), kanamycin (21.5%), ampicillin (19.8%), amoxicillin-clavulanic acid (10.4%), and ceftiofur (9.0%). These data indicate multi-drug resistance among a variety of *Salmonella* Heidelberg strains (Zhao et al., 2008).

Salmonella prevention

In 2011, the USDA strengthened safety standards through a *Salmonella* initiative program designed to directly reduce *Salmonella* in raw meat and poultry. Due to new policies, *Salmonella* in young chickens has dropped over 75 percent since 2006 (Tarr, 2013). Although processing facilities and producers are constantly working to lower *Salmonella* contamination of consumer products, the number of *Salmonella* outbreaks has steadily risen since the 1920s.

Cautious biosecurity measures are supportive in a *Salmonella* prevention plan; however, biosecurity alone cannot completely eliminate the risk of contamination.

Previous studies have looked into alternate methods of *Salmonella* prevention. Grimes and colleagues (2008) analyzed the effect of a dietary direct-fed microbial (Primalac) on turkey poult *Salmonella* challenge. *Salmonella* populations were reduced one log when feeding Primalac; therefore, suggesting that direct-feeding of Primalac may be a feasible method of reducing *Salmonella* colonization in poultry flocks (Grimes et al., 2008). Similar studies evaluated the ability of a lactic acid commercial probiotic culture (FloraMax, IVS-Wynco LLC, Springdale, AR) to reduce colonization of *Salmonella* Heidelberg in neonatal broiler chickens and young turkey poults. Probiotic treatment proved to significantly reduce cecal colonization levels, further suggesting the effectiveness of probiotic products in controlling *Salmonella* colonization (Menconi et al., 2011). Although dietary probiotic supplements may reduce the risk of *Salmonella* colonization in poultry flocks, the solution is merely a temporary fix.

Despite the fact that temporary methods have been investigated to reduce the risk of *Salmonella* colonization in poultry flocks, a long-term alternative would permanently reduce detrimental economic impacts of *Salmonella* contaminated products. A more effective, long-term strategy is needed in order to guarantee a safe product for consumers and maintain consumer confidence in the poultry industry. Determining factors of genetic resistance to *Salmonella* colonization is an important step in developing a cost-effective solution to this overwhelming issue.

Genetics of resistance to Salmonella colonization in poultry

Genetic improvements are permanent and cumulative manner in which to reduce bacterial burdens in poultry. To determine the feasibility of using host genetics to help improve resistance to *Salmonella*, there must be at least partial genetic control of the *Salmonella*-response traits.

Previous studies have shown that genetics play a key role in *Salmonella* resistance and susceptibility levels. Genetic resistance to systemic *Salmonella* infection has been extensively studied and linked to a variety of factors including the MHC (Mariani et al., 2001), TLR-4 (O'brien et al., 1980; Poltorak et al., 1998), and the natural resistance associated macrophage protein (Nramp1 [currently known as SLC11A1]) (Blackwell et al., 2001). Resistance to systemic infection has also been associated with a novel gene known as *SAL1* that leads to increased macrophage activity against *Salmonella* (Wigley et al., 2004). Ample evidence indicates that genetic selection to improve resistance to *Salmonella* colonization is feasible in chickens (Kaiser and Lamont, 2001; Kaiser et al., 2002). Young layer chicks post *Salmonella enterica* serovar Enteritidis (SE) exposure have shown significant genetic line differences when observing 6-day survival post challenge and SE burden in cecal content (Kaiser and Lamont, 2001). Estimated heritability of resistance to cecal carrier state is reported as 0.20 in laying hens (Berthelot et al., 1998), the number of bacteria persisting in internal organs reported as 0.02 (liver and ovary) and 0.13 (spleen), and cecal contamination of laying hens at 0.53 (Beaumont et al., 1999). This further indicates a genetic influence that permits *Salmonella* resistance or susceptibility passed from parent to offspring. This is particularly important because carriage in the ceca may result in zoonotic infection through shell contamination by feces or through ovarian infection. These findings suggest that differences in levels of resistance and susceptibility to

Salmonella colonization of the ceca and internal organs are affected by genetic traits. Traits such as these could potentially be manipulated in order to improve *Salmonella* resistance levels in poultry; therefore reducing consumer worries and the harsh economic impacts that result from *Salmonella* contaminated product recalls. Breed-specific influences have also been observed on the ability of an egg to resist *Salmonella* Heidelberg penetration due to eggshell thickness. Higher *Salmonella* Heidelberg cfu/eggshell can be observed in eggs originating from layer breeds that produce thin eggshells when compared to those originating from breeds that produce thicker eggshells. These results suggest that eggs of certain genetic breeds show greater resistance to *S*. Heidelberg while others are more susceptible (Rathgeber et al., 2013).

The preceding evidence, therefore, indicates that genetic selection to improve resistance to *Salmonella* colonization is feasible. Many types of gene families have been demonstrated to be associated with specific aspects of response to *Salmonella* (Cheng and Lamont, 2008; Lamont, 2008; Lamont, 2010). However, most genes account for only a small fraction of the genetic variance in the traits, indicating the highly polygenic nature of host response to *Salmonella*, and the need for more detailed studies to fully understand the genetic mechanisms of resistance.

Heterophil affiliation with resistance to Salmonella infection

Heterophils are the avian equivalent to mammalian neutrophils. They are one of the most common granulated leukocytes involved in an acute inflammatory response and occur alongside monocytes, basophils, eosinophils, and lymphocytes. These phagocytic cells are capable of a broad spectrum antimicrobial activity, accumulate in inflamed tissue, and form the first line of cellular defense against invading microbial pathogens in the lungs and air sacs where resident

macrophages are lacking. Within the cytoplasmic granules of heterophils, there is lysozyme along with other proteins necessary for bactericidal activity. Swaggerty and associates (2005) studied the susceptibility of four genetically distinct broiler lines (two parent lines [A and B] and the F1 reciprocal crosses [C = A hen x B rooster; D = B hen x A rooster]). Birds were subjected to an intra-abdominal injection of either 0.5 mL sterile phosphate buffered saline (PBS) or 5 x 10^3 cfu/chick SE. Phenotypic characteristics, such as modulation of leukocytes and survivability post PBS or SE injection, were evaluated. Seventy-two hours post injection, genetic lines A and D showed a significantly lower percent mortality in comparison to lines B and C. Abdominal exudates from each genetic line were used for leukocyte isolation and the number of heterophils and macrophages were determined. Birds within each genetic line that were injected with sterile PBS showed no variation in heterophil influx post injection; however, SE intra-abdominal injection resulted in significant heterophil influx into the abdominal cavity. Although statistical analysis was only performed comparing parental lines alone and F1 reciprocal crosses alone, results indicated a significantly higher abdominal heterophil influx in line A in comparison the line B. Similar results were observed when comparing genetic lines C and D, with line D having a significantly higher abdominal heterophil influx than line C (Swaggerty et al., 2005). These findings indicate that lines A and D are immunologically more responsive and more resistant to SE infections than lines B and C. This further suggests that the host-innate immune response to *Salmonella* infection could potentially be controlled through genetic selection for increased heterophil levels post-infection; therefore, potentially reducing colonization levels.

Immunoprophylactic administration of *Salmonella* Enteritidis immune lymphokines (ILK) increases *S.* Enteritidis resistance levels in day-old chicks. This administration method reveals that resistance is associated with increasing circulating peripheral blood heterophils

(Kogut et al., 1993). Kogut and colleagues (1995) further investigated if ILK injections induce the production of heterophils and affect their phagocytic function. It was shown that immune lymphokine injection increases heterophil adherence. Heterophil chemotaxis assays showed a significantly greater random migration of heterophils in ILK-injected chickens. Furthermore, heterophils of ILK-injected chickens showed a significantly higher phagocytosis and bactericidal activity level than those of non-injected chickens (Kogut et al., 1995). Further studies to determine the differences in heterophil function between different poultry genetic lines would be beneficial for determining the genetic factors involved in disease resistance.

Summary

Together, the components of the avian immune system are efficient at eliminating foreign antigens from the host. When the immune system is compromised, the host is then susceptible to invading pathogens and other antigens. Certain bacteria, such as *Salmonella*, have resulted in severe economic impacts as well as loss of consumer confidence in the poultry industry. Short-term preventative methods such as probiotics have been researched in order to alleviate this issue; however, a long-term alternative is needed in order to reduce the occurrence of *Salmonella* contaminated consumer products. Current poultry production practices and unavoidable concentrations of *Salmonella* in poultry facilities ultimately reduce the likelihood of achieving zero tolerance for *Salmonella* in poultry. Furthermore, genetically selecting towards resistance to a particular type of *Salmonella* may result in the host becoming more susceptible to another harmful *Salmonella* serotype. Although complete elimination of *Salmonella* in poultry may not be practical, genetically selecting for *Salmonella* resistance could reduce the event of *Salmonella* contaminated poultry products. Several studies have shown that genetic traits are notably

involved in the resistance and susceptibility of poultry to *Salmonella* colonization. Furthermore, distinct phagocytic cells such as heterophils and PBMCs can provide a swift response to *Salmonella*. Although several studies have looked at these phenotypes in both chickens and turkeys, much less research has focused on turkey purebred lines. Subsequent chapters of this thesis will discuss phenotypic differences observed in six different turkey pedigree lines involving potential resistance and susceptibility to a *Salmonella* Heidelberg infection. The primary objective is to identify notable phenotypic differences and subsequently the genetic elements involved so that future genetic selection can lead to *Salmonella*-resistant poultry.

Chapter III

Materials and Methods

Experimental birds and housing

This project was approved and conducted under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee. In this study, 720 one day-old turkey poults of 6 different pedigree lines (represented as genetic lines A, B, C, D, E, F) (Table 1) were obtained from Aviagen Turkeys (Lewisburg, WV). Poults were separated by genetic line and gender at the hatchery. Each genetic line was wing-banded with a distinct color (blue, pink, purple, orange, green, and yellow respectively) for identification purposes. Birds were placed in floor pens (60 poults/gender/genetic line/pen) with female pens and male pens being in separate rooms of equal temperature and environment. All birds were placed in pens with fresh shavings, by genetic line and gender (1 pen/gender/genetic line), in the Virginia Tech Litton-Reaves BSL-2 animal research facility and provided water and non-medicated starter feed meeting primary breeder recommendations in mash form *ad libitum*. Prior to placement, random samples of fresh litter and feed were cultured to ensure *Salmonella*-free conditions. There were a total of 12 pens with 6 pens in each room; one room containing genetic lines A – F females and the other containing genetic lines A – F males. At three weeks of age, birds were divided into challenged and non-challenged groups. Challenge birds (30-40 birds/gender/genetic line) remained in floor pens and non-challenge birds (12 birds/gender/genetic line) were moved to cages (3 birds/gender/genetic line/cage) in a separate room for isolation.

Bacterial culture and bird inoculation

Prior to *Salmonella enterica* serovar Heidelberg (SH) challenge, cloacal swab tests were performed on 6-day old poults (6 poults/pen). Briefly, sterile swabs were inserted into the cloaca orifice approximately 1 cm, rotated against the cloaca lining, and placed directly into 5 mL of sterilized Brain Heart Infusion (BHI) broth filled tubes. Each of the tested birds was swabbed five times and all individual swabs were placed in one tube/bird. Tubes were then inverted and all five swabs/tube were removed. After a 24 hour (h) incubation at 37°C, prepared samples were spread onto XLT4 plates and incubated for another 24 h at 37°C. Colonies were analyzed using polymerase chain reaction (PCR) with an *S.* Heidelberg specific primer set and a *Salmonella* general primer set, followed by gel electrophoresis analysis. Blood collection, isolation of heterophils, and isolation of peripheral blood mononuclear cells were performed on non-challenged control birds located in cages in a separate room for isolation.

A field isolate of *S.* Heidelberg was used as the inoculum for this experiment. Culture enrichment was performed in tetrathionate broth base containing iodine-potassium iodide solution. Using XLT4 agar, a single colony of *S.* Heidelberg was isolated and used to prepare the inoculum by culturing in Luri-Bertani (LB) broth for 18-24 h. Serial dilutions were performed to obtain $1x10^7$ cfu/mL inoculum. Inoculum concentration was confirmed by plating onto XLT4, incubating at 37°C for 24 h, and performing colony counts. At 28 days of age, birds for challenge (30-40 birds/gender/genetic line) were orally gavaged with 1×10^7 *S.* Heidelberg (1 mL/bird; 10^7 cfu/mL).

Bacterial colonization of spleen, liver, and ceca

To determine possible differences in bacterial colonization levels among genetic lines and genders, spleen, liver, and cecum samples were collected from 4 non-challenged birds/gender/genetic line and 5-10 challenged birds/gender/genetic line at one and three days post-inoculation (dpi). Briefly, approximately 1 gram of spleen, liver, and cecum containing cecal contents were collected in individual sterile Whirl-Pak bags containing 9 mL of sterile water and stomached (BagMixer 400 P, Thermo Scientific, NJ) for 1 minute (min). One-milliliter of stomached sample was serially diluted in sterile 1% peptone water, and then 100 μL of diluted sample were spread onto XLT4 agar plates supplemented with 25 μg/mL Novobiocin and 20 μg/mL Ampicillin to prevent the growth of *E. coli* and other unwanted bacteria. Plates were then incubated at 37°C for approximately 24 h, followed by colony counting. The number of colonies was multiplied by the dilution factor and then divided by the total volume of culture plated to achieve the number of colony forming units (CFU) per mL of culture. Results were analyzed using the Fit Model platform in JMP 10.0 (SAS Institute Inc., Cary, NC). Colonization levels observed among genetic lines and genders were compared using Student's *t*-test following ANOVA with significance reported at $P \leq 0.05$.

Isolation of total DNA from ceca

For a more precise quantification of *S.* Heidelberg colonization levels in the ceca, total DNA was isolated from frozen cecal tissue and contents using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following the tissue and rodent tail manufactured spin-column protocol. Briefly, 40-50 mg of cecum tissue and contents were placed in individual 1.5-mL microcentrifuge tubes and mixed with 180 μL of Buffer ATL and 20 μL of proteinase K by

gentle vortexing. Samples were incubated at 56°C overnight (16-18 h) in a shaking mini incubator (VWR, Radnor, PA). Upon completion of lysis, 4 µL of RNase (Qiagen, Valencia, CA) were added to each sample to ensure pure gDNA. Samples were incubated at room temperature for 10 min and then mixed with 200 µL of Buffer AL by pipetting, followed by mixing with 200 µL of 100% ethanol by pipetting. The mixture was then transferred into a DNeasy Mini spin column (Qiagen, Valencia CA) situated in a 2-mL collection tube and centrifuged at 15, 970 x *g* for 1 min. The flow-through was discarded and the spin column was placed in a new 2-ml collection tube where 500 µL of Buffer AW1 were added to the spin column for the first washing step. The column was centrifuged for 1 min at 15, 970 x *g* and flow-through was discarded. The DNeasy Mini column was placed in a new 2-mL collection tube and 500 µL of Buffer AW2 were added for a second washing step. The DNeasy Mini column was placed in a new 2-mL collection tube and the column was centrifuged for 3 min at 16, 244 x *g* to dry the DNeasy membrane. Flow-through was discarded and the DNeasy Mini spin column was placed in a new 1.5-mL collection tube for the elution step. Isolated genomic DNA was eluted by adding 100 µL of Buffer AE directly onto the DNeasy membrane, incubating at room temperature for 10 min, and then centrifuging for 1 min at 15, 970 x *g*. The concentration of isolated genomic DNA was determined at OD 260 in a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, DE). The quality of genomic DNA was verified by evaluating the ratio of OD 260 to OD 280 readings and running on a 1% agarose gel electrophoresis.

Primer set design

In order to determine the presence of *S.* Heidelberg in the ceca, a qRT-PCR analysis was performed using genomic DNA (gDNA) extracted from cecal tissue and contents. A *S.* Heidelberg specific primer set was designed using a unique *Salmonella enterica* subsp. Enterica serovar Heidelberg strain, 07-4845, CRISPR1 repeat region (GenBank: JF724526) (Table 2). This region was located through multiple sequence alignment using Clustal Omega, NCBI BLAST, and Primer One. Primers were designed using the Primer Express 3.0 software (Applied Biosystems, Foster City, CA) and synthesized by MWG Operon (Huntsville, AL). The designed primer set was then tested through qRT-PCR analysis using gDNA from *S.* Heidelberg as a positive control and gDNA from *S.* Typhimurium, *S. kedougou, E. coli,* and sterile water as negative controls. Results were confirmed by running a 1% DNA gel. Results from qRT-PCR were analyzed using the 7500 Real-Time PCR (Life Technologies, Grand Island, NY).

Quantitative real-time PCR

Quantitative real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions in an ABI 7500 FAST Real-Time PCR System (Life Technologies, Grand Island, NY). Genomic DNA from ceca was diluted to a 25 ng/μL template in nuclease-free water, then 2 μL of the diluted gDNA were added to each well of a 96-well plate to obtain a 50 ng/μL template per well. Next, 8 μL of real time PCR master mix containing 5μL FAST SYBR Green Master Mix (Applied Biosystems), 0.5 μL each of 2 μM forward and reverse primers and 2 μL of sterile nuclease-free water per reaction were added to each well for a final volume of 10 μL per well. During the PCR reaction, samples were subjected to an initial denaturation phase at 95°C for 20 seconds (sec) followed by 40 cycles of denaturation at 95°C for 3 sec and annealing and extension at 60°C for 30 sec. Each

reaction was run in triplicate. Dissociation curves were generated to analyze individual PCR product after 40 cycles.

Heterophil and PBMC functional assays

In order to determine the phagocytic and bactericidal activity of turkey heterophils and PBMCs among genders and across genetic lines, blood was collected from 2 birds per gender, per genetic line (4 birds per genetic line) for use in heterophil and PBMC functional assays.

Isolation of heterophils and peripheral blood mononuclear cells (PBMCs)

Turkey heterophils and PBMCs were extracted from non-challenged males and females of each genetic line at 35 days of age using modified methods of Kogut and colleagues (1993; 1995). To induce the number of heterophils, 5 μg/kg of LPS were injected via brachial wing vein into 2 birds per gender in each genetic line (4 birds per genetic line and a total 24 birds). Five hours post-injection, the birds were euthanized by cervical dislocation, and 30 mL of blood per bird were collected through cardiac puncture (60 mL of blood per gender in each genetic line) immediately following euthanasia. The collected blood was stored on ice until cell isolation. Blood samples from the same gender and genetic line were combined (n = 1) into a 50-mL tube and then serum was centrifuged at 70 x *g* for 10 min at low deceleration (deceleration = 1). For density-based separation of cell populations, 15 mL of serum and buffy coat were collected and layered on top of approximately 6 mL of Histopaque-1077, which was over-laid on 9 mL of Histopaque-1119. The cells were centrifuged at 250 x *g* for 60 min at low deceleration (deceleration = 1). The interphase between samples and Histopaque-1077 was collected for PBMCs, while the interphase between Histopaque-1077 and Histopaque-1119 (Sigma-Aldrich, St. Louis, MO) was collected for heterophils. The collected cells were diluted in twice the

volume of incomplete DMEM media, followed by pelleting the cells at 250 x *g* for 10 min. The cells were re-suspended and washed with incomplete DMEM. Samples of heterophils were transferred to 15-mL tubes, while samples for PBMCs remained in 50-mL tubes. The supernatant was removed, and the cells were re-suspended with 2-3 mL of media and counted via Trypan blue exclusion. The total number of cells was calculated. The cell suspension was centrifuged as before, the supernatant was discarded, and the cells were re-suspended with proper volume of media to adjust the number of heterophils to 6 x 10^5 cells/well (0.5 mL) and that of PBMCs to 1 x 10^7 cells/mL in a 100-mm dish. The cells were seeded onto 24-well plates and 100-mm dishes and incubated overnight at 39°C.

Phagocytosis assay

Heterophils and PBMCs were isolated using density gradient separation described above. Approximately 5 x 10^5 cells/well heterophils or 6 x 10^5 cells/well PBMCs were incubated with a 1:10 (heterophil:SH) diluted number of *S*. Heidelberg (10^9 CFU/mL). The mixture of cells and *S*. Heidelberg was incubated at 39°C for 1 h, followed by addition of ice-cold PBS to stop phagocytosis. The cells were then stained using Diff-Quick staining solution (Thermo Fisher Scientific, Wilmington, DE) and the total number of heterophils showing phagocytosis out of the total number of heterophils was counted using a phase contrast microscope (40X – 100X). The number of phagocytic cells was calculated as percent of the total number of cells. Data were analyzed using the Fit Model platform in JMP Pro 10.0. Differences in genetic lines and genders were tested using Student's *t*-test following ANOVA with significance reported at $P \leq 0.05$.

Bactericidal assay

To examine resistance and susceptibility to *Salmonella* spp. in the six different genetic lines of turkeys, we measured survival ratio of *S*. Heidelberg incubated cultured with PBMCs.

Isolated PBMCs in 100-mm plates were washed and non-adherent cells were removed after the cells were incubated 24 h. The adherent PBMCs were harvested by trypsinization and the number of cells counted. Approximately 4 x 10^6 cells were transferred into a 1.5-mL microcentrifuge tube, followed by addition of either 1:10 or 1:100 diluted number of *S.* Heidelberg (10^9 CFU/mL). The bacteria were incubated either in medium alone or medium with antibiotics (50 μg/mL of Kanamycin and Gentamicin) as negative and positive controls, respectively. The mixture of PBMCs and *S.* Heidelberg was incubated at 39°C for 1 h. Bacteria were then pelleted by centrifugation for 2 min at 16, 244 x *g*. The bacterial pellets were re-suspended, diluted 1:5,000 with peptone water, and plated onto XLT4 agar plates. The number of colonies was counted 24 h post-incubation (39°C). The number of colonies was multiplied by the dilution factor and then divided by the total volume of culture plated to achieve the number of colony forming units (CFU) per mL of culture. Amount of SH remaining post-incubation with bactericidal PBMCs was analyzed. Results were analyzed using the Fit Model platform in JMP Pro 10.0 (SAS). Bactericidal differences observed among genetic lines and genders were tested using Student's *t*-test following ANOVA with significance reported at $P \leq 0.05$.

Microbial Profiling

Bacterial genomic DNA extraction

In order to test the specificity of designed primer sets and analyze differences in microbial profiling among genetic lines and genders, genomic DNA was extracted from pure cultures of *Salmonella*, *E. coli*, and *Enterococcus* species using the DNeasy Blood & Tissue Kit and a modified version of the Gram-negative and Gram-positive bacteria protocols. Briefly, 2-5 mL of pure culture were transferred to a 5-mL microcentrifuge tube, centrifuged, and pelleted.

For Gram-negative bacteria, pellets were re-suspended in 180 μL of Buffer ATL and 20 μL of proteinase K. Samples of Gram-positive bacterial species were re-suspended in 180 μL of enzymatic lysis buffer, incubated in a 37°C water bath for 30 min, and mixed with 180 μL of Buffer ATL and 20 μL of proteinase K. Samples were mixed thoroughly by pipetting and incubated at 56°C overnight (16-18 h) in a rotating incubator. Upon completion of cell lysis, 4 μL of RNase were added to each sample to ensure pure gDNA. Samples were incubated at room temperature for 10 min. After incubation, 200 μL of Buffer AL were added to each sample and mixed by pipetting to yield a homogeneous solution. Then, 200 μL of 100% ethanol were added and mixed by pipetting. The mixture was then transferred into a DNeasy Mini Spin column situated in a 2-mL collection tube and centrifuged at 15, 970 x *g* for 1 min. The flow-through was discarded and the spin column was placed in a new 2-mL collection tube where 500 μL of Buffer AW1 were added to the spin column. The column was centrifuged for 1 min at 15, 970 x *g* and flow-through was discarded. The DNeasy Mini column was placed in a new 2-mL collection tube, 500 μL of Buffer AW2 were added, and the column was centrifuged for 3 min at 16, 244 x *g* to dry the DNeasy membrane. Flow-through was discarded and the DNeasy Mini spin column was placed in a new 1.5-mL microcentrifuge tube for the elution step. To increase the final gDNA concentration in the eluate, elution was performed by adding 100 μL of Buffer AE directly onto the DNeasy membrane, incubating at room temperature for 10 min, and then centrifuging for 10 min at 15, 970 x *g*. The quality of gDNA was analyzed through NanoDrop and 1% TAE gel electrophoresis.

Bacterial-specific primer design

Specific qRT-PCR primer sets for *Salmonella* and *Enterococcus* species were designed using the common 16S rRNA region and multiple sequence alignment of at least 10 different

species of each bacterium. Primers were designed using Primer Express 3.0 software and synthesized by MWG Operon (Hunstville, AL). *E. coli* specific sequences were obtained from a previous study performed by Malinen and colleagues (2003). Specificity of each primer set was tested using isolated bacterial gDNA from each bacterial species. Bacterial gDNA from each of the six species was diluted to a 25 ng/μL template and each specific primer set was tested with each bacterial gDNA to confirm specificity according to design. Primer sequences and accession numbers are listed in Table 2.

Standard curves and quantification of cecal bacteria

qRT- PCR was performed on gDNA from cecal tissue and contents from control birds to examine abundance of total bacterial differences among genders and across genetic lines using an ABI 7500 FAST Real-Time PCR System ((Life Technologies, Grand Island, NY).). Standard curves were produced from isolated bacterial gDNA. Serial dilutions of the gDNA were made to create 10-fold serial dilutions from 100,000 to 10 pg/μL. Standard curves using real-time PCR amplification were prepared with bacterial specific primers (Table 2): 100,000; 10,000; 1,000; 100; 10 pg. Each 10 μL reaction contained 1 μL of gDNA template and 9 μL of real time PCR master mix containing 5 μL FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 0.5 μL each of 2 μM forward and reverse primers and 3μL of sterile nuclease-free water. Each concentration in each standard curve was performed in triplicate using three separate plates. Standard curves are illustrated in Figure 1.

Bacterial gDNA from cecal tissue and contents was diluted to a 25 ng/μL template in nuclease-free water, then 2 μL of the diluted gDNA were added to each well of a 96-well plate to obtain a 50 ng/μL template per well. Next, 8 μL of real time PCR master mix containing 5 μL FAST SYBR Green Master Mix (Applied Biosystems), 0.5 μL each of 2 μM forward and reverse

primers and 2 μL of sterile nuclease-free water per reaction were added to each well for a final volume of 10 μL per well. During the PCR reaction, samples were subjected to an initial denaturation phase at 95°C for 20 sec followed by 40 cycles of denaturation at 95°C for 3 sec and annealing and extension at 60°C for 30 sec. Each reaction was run in duplicate using bacterium specific primers. Data were compared to standard curves of *Salmonella*, *E. coli*, and *Enterococcus* species. Mean C_t values were calculated into the Y equation of each standard curve to determine the amount of *Salmonella, E. coli,* and *Enterococcus* present in the ceca. Due to the fact that the results did not fall within the mean C_t values of standard curves, only mean C_t values were referenced to compare effects of genetic line and gender on colonization levels. Data were analyzed using the Fit Model platform in JMP Pro 10.0. Differences in genetic lines and genders were tested using Student's *t*-test following ANOVA with significance reported at $P \leq 0.05$.

Chapter IV

Results

Mortality, cloacal swabs, and bacterial colonization of spleen, liver, and ceca

Although no mortality was observed post-SH inoculation, significant differences in mortality were found prior to inoculation. The genetic line X gender interaction revealed no significant statistical differences (Figure A- 1) while the main effect of genetic line on percent mortality resulted in lines A and F having significantly higher mortality than all other lines (P = 0.0024) (Figure 2). No significant differences were observed when looking at the effect of cumulative gender on pre-challenge mortality (Figure A- 2). Post-SH challenge, *Salmonella* colonization was detected in the non-challenged and challenged groups, qRT-PCR with gel analysis confirmed the absence of SH; therefore, another type of *Salmonella* was present in the ceca. SH used in the inoculation for this study was not a marked isolate, thus qRT-PCR was used to confirm that the inoculation culture was SH. Results demonstrated that the culture used for the inoculation in this study was SH. The "unknown" *Salmonella* serotype detected in both non-challenged and challenged birds was isolated and will be identified. No colonization was observed in either the spleen or liver of non-challenged and challenged birds at 1 and 3 dpi Interestingly, colonization of *Salmonella* in the ceca 1 dpi indicated a significant treatment X gender interaction with non-challenged females and challenged males having significantly higher bacterial counts than challenged females (P = 0.0048) (Figure 3). When genetic lines were viewed individually, line F showed the highest bacterial counts and was significantly different from lines A, B, and E (P = 0.0376) (Figure 4). Results 3 dpi indicated a significant 3-way interaction of treatment X gender X genetic line on colonization in the ceca, with non-challenged

males of line B and challenged males of line C having significantly higher bacterial counts than non-challenged and challenged males and females of all other genetic lines ($P = 0.0014$) (Figure 5).

Phagocytosis and bactericidal assays

Genetic line had no significant effect on phagocytosis of SH by heterophils (Figure A - 3). Cumulative gender-based phagocytosis of SH by heterophils illustrated that females had a significantly higher percentage phagocytosis than males ($P = 0.0053$) (Figure 6). Similarly, the main effect of genetic line had no significant impact on phagocytosis of SH by PBMCs (Figure A- 4). Cumulative gender-based phagocytosis of SH by PBMCs illustrated females having a significantly higher percentage phagocytosis than males ($P = 0.0270$) (Figure 7).

When incubated at a 1:10 PBMC to SH ratio, lines B and D conveyed the highest while line F exhibited the lowest bacterial counts when compared to all other genetic lines ($P = 0.0047$) (Figure 8). Furthermore, no significant differences were found when analyzing the effect of gender on bactericidal activity of PMBCs (Figure 9). Interestingly, cells incubated at a 1:100 ratio demonstrated genetic line (Figure 10) and gender (Figure 11) having no significant effect on bactericidal activity of PBMCs; however, bacterial counts across all lines were significantly higher than those of SH incubated in media containing antibiotics ($P = 0.0233$) (Figure 10). Similarly, bacterial counts of both males and females were significantly higher than those of SH incubated in media containing antibiotics ($P = 0.0002$) (Figure 11).

Microbial profiling

Microbial profiles of the ceca indicated that genetic line X gender had a significant effect on cecal colonization by *Enterococcus* with line E females showing the highest and line C males demonstrating the lowest amount of *Enterococcus* ($P = 0.0156$) (Figure 12). Interestingly, genetic line had a significant effect on *Salmonella* colonization as line E manifested the lowest while line A conveyed the highest colonization ($P = 0.0019$) (Figure 13). Results suggested genetic line E had the highest *Enterococcus* but lowest *Salmonella* colonization than all other lines, while line A birds displayed the highest *Salmonella* colonization with line C falling shortly behind. Furthermore, when analyzing the genetic line X gender interaction and the main effects of genetic line and gender on *E. coli* colonization, no significant differences were identified. Genetic lines B and C resulted in "undetermined" C_t values for both males and females. It is reasonable to suggest that the amounts of *E. coli* present were below detectable levels.

Table 1. Aviagen parental (purebred) genetic lines used in this study

Genetic line	Size	Gender	Selection
A		Male	Weight, FCR, and Livability
B	Very Heavy	Female	Weight, FCR, and Eggs*
C	Heavy	Female	Weight, FCR, and Eggs*
D	Heavy	Female	Weight, Eggs*, and Hatchability
E		Male	Weight, FCR, Leg Health, and Livability
F	Small	Female	Eggs* and Hatchability

* Genetic selection for reproductive traits (i.e. number of eggs set in 24 weeks of lay as well as hatch of settable eggs for the same 24 week period).

Table 2. Primers used for quantitative RT-PCR[1]

Target	Accession No.	Nucleotide sequence (5' → 3')
S. Heidelberg_F	JF724526	GCAAGGCCCGTATATGGATTT
S. Heidelberg_R		CGCCTTTAATCGCCTCTTATCG
*E. coli*_F	*	GTTAATACCTTTGCTCATTGA
*E. coli*_R		ACCAGGGTATCTAATCCTGTT
*Enterococcus*_F	AB015233	ACTGTTCATCCCTTGACGGTATC
*Enterococcus*_R		CGGCTGCTGGCACGTATT
*Salmonella*_F	FN356961	GGGAGGAAGGTGTTGTGGTTAA
*Salmonella*_R		TGCTTCTTCTGCGGGTAACG

[1]Primers designed by Primer Express software (Applied Biosystems, Foster City, CA).

*(Malinen et al., 2003).

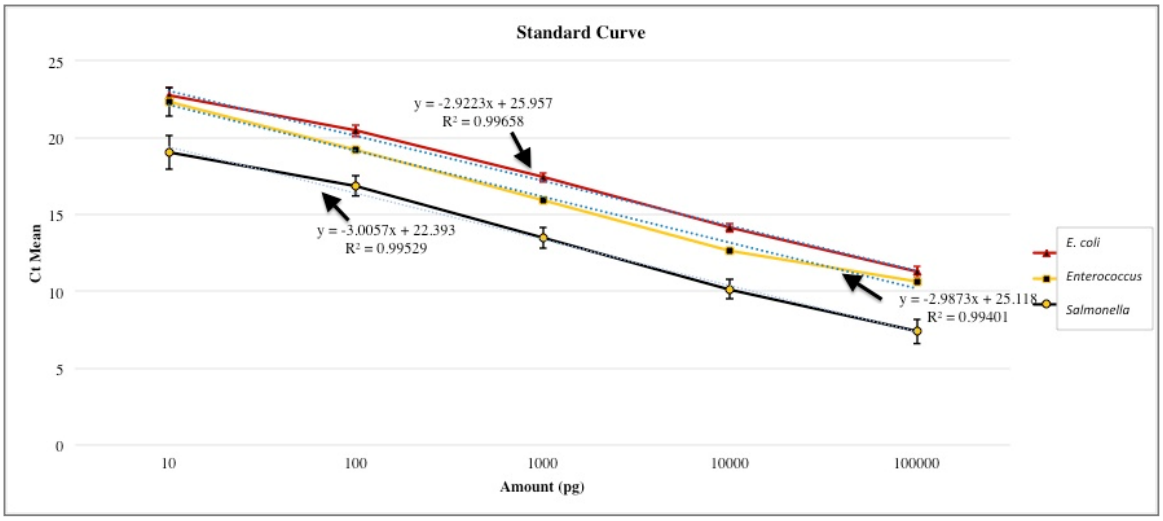

Figure 1: Standard curves of *E. coli*, *Enterococcus*, and *Salmonella* generated through qRT-PCR. Data are presented as mean threshold cycle (C_t); the linear equation and correlation coefficient (R^2) is provided for each linear standard curve. Amount of bacteria (0 - 100,000 picograms (pg)) is indicated on the x-axis while mean C_t is indicated on the Y-axis. Lower mean C_t values suggest higher bacteria amounts while higher mean C_t values demonstrate lower bacteria amounts.

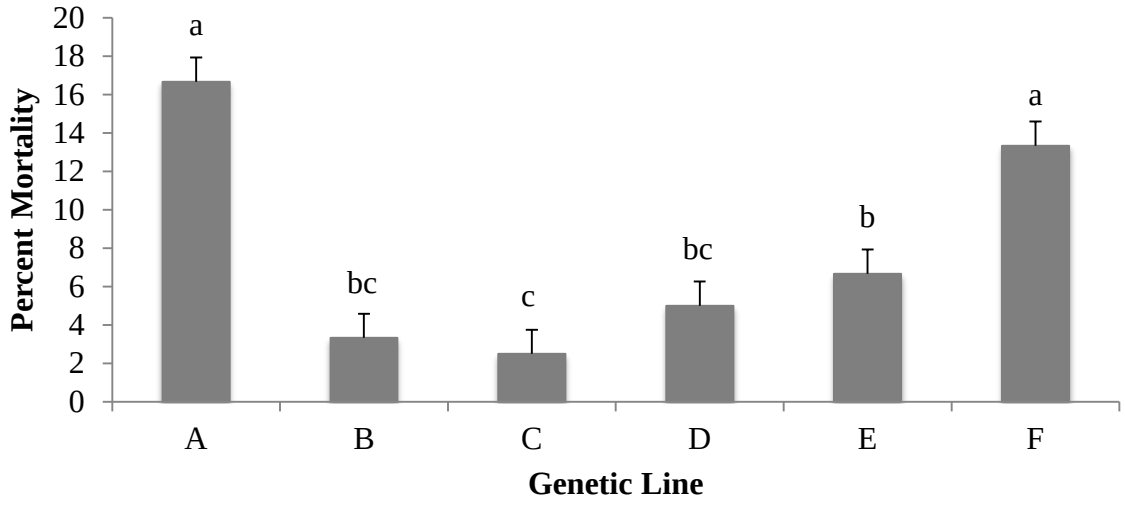

Figure 2. Effect of genetic line on mortality observed prior to an *S.* Heidelberg challenge in purebred turkeys. Data are presented as percent mortality (n = 2 pens/genetic line). Bars lacking a common letter (a-c) differ significantly. There was a significant effect of genetic line (A-F) on mortality ($P = 0.0024$).

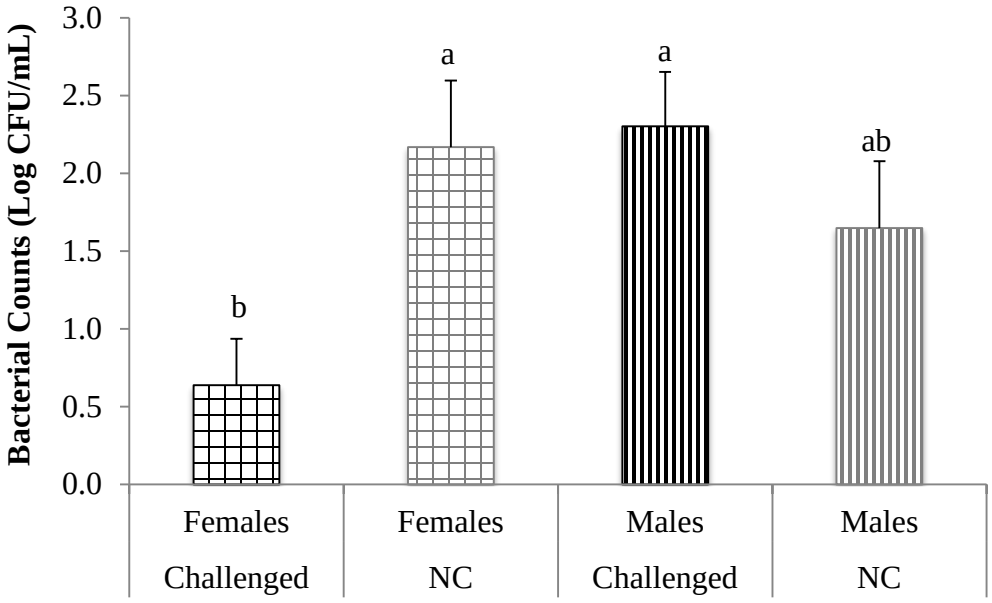

Figure 3. Effect of treatment X gender interaction on cecal colonization by *Salmonella* at one dpi. Data are presented as LS Means + SEM (n = 24 birds/gender (non-challenged (NC)); 36-60 birds/gender (challenged)). Bars lacking a common letter (a-b) differ significantly. Treatment (challenged; non-challenged) X gender interaction had a significant effect on cecal colonization by *Salmonella* at one dpi ($P = 0.0048$).

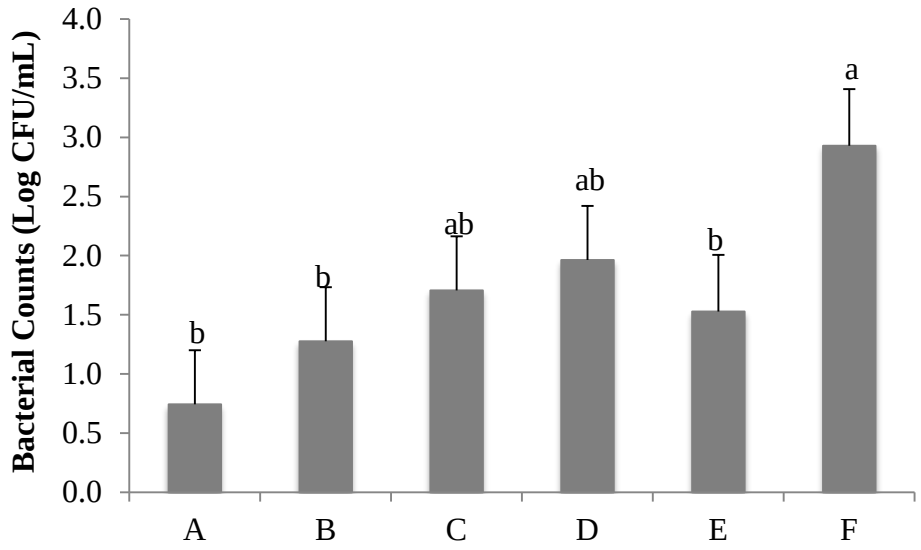

Figure 4. Effect of genetic line on cecal colonization by *Salmonella* at one dpi. Data are presented as LS Means + SEM (n = 28/genetic line). Bars lacking a common letter (a-b) differ significantly. There was a significant effect of genetic line (A-F) on cecal colonization by *Salmonella* at one dpi ($P = 0.0376$).

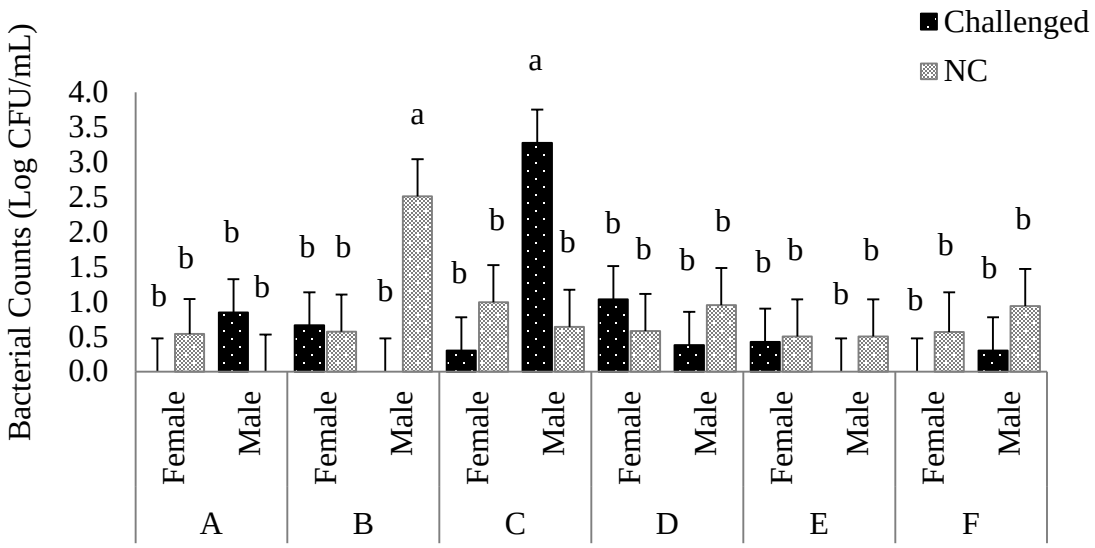

Figure 5. Effect of treatment X gender X genetic line interaction on colonization of ceca by *Salmonella* at three dpi. Data are presented as LS Means + SEM (n = 4/genetic line/gender (non-challenged (NC)) and 5/genetic line/gender (challenged)). Bars lacking a common letter (a-b) differ significantly. There was a significant effect of treatment X gender X genetic line (A-F) at three dpi ($P = 0.0014$).

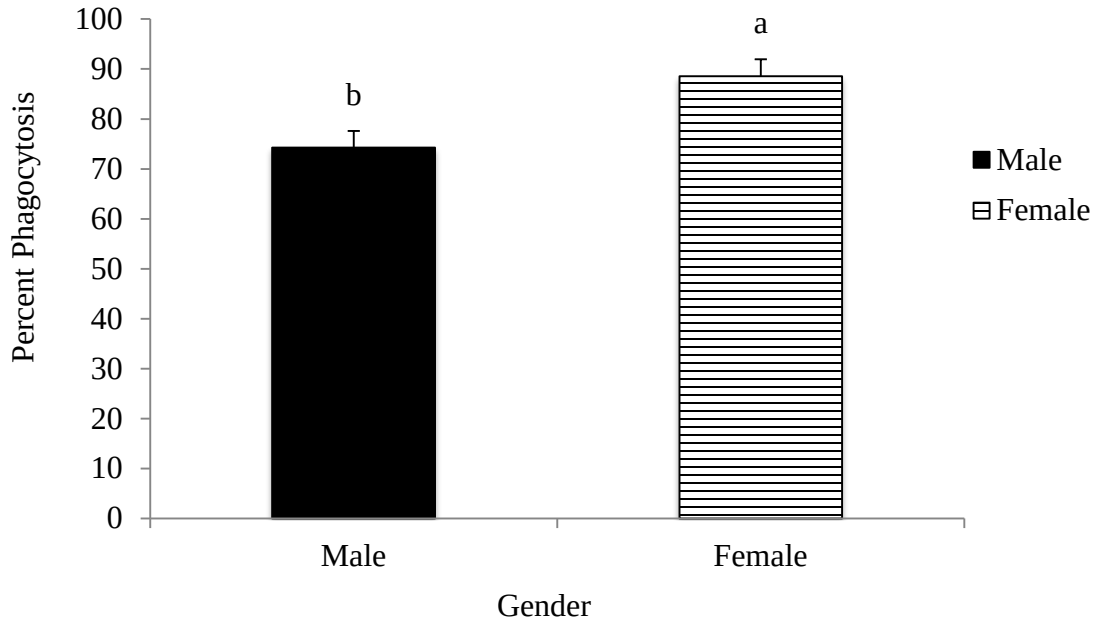

Figure 6. Effect of turkey cumulative gender on phagocytosis of *S.* Heidelberg by heterophils. Data were analyzed as LS Means + SEM (n = 6/gender). Bars lacking a common letter (a-b) differ significantly. There was a significant effect of gender on percent phagocytosis of *S.* Heidelberg by heterophils ($P = 0.0053$).

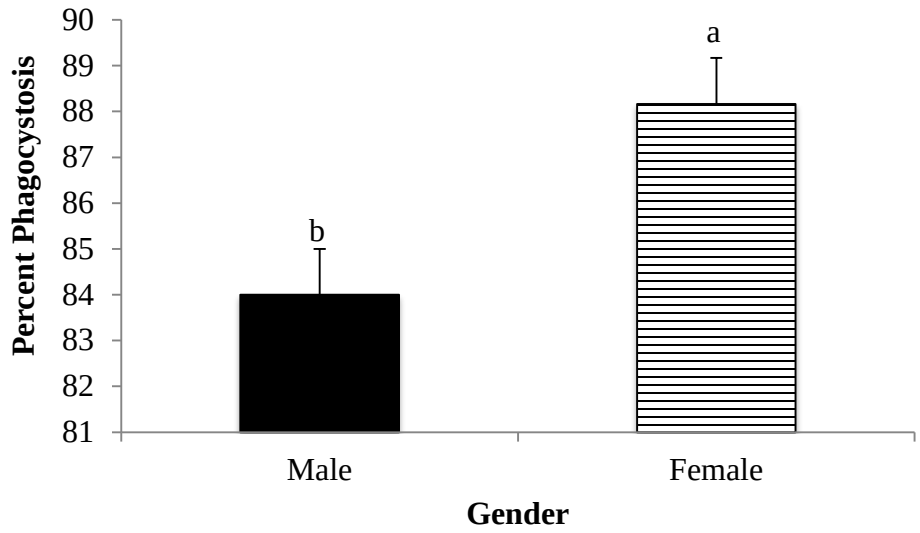

Figure 7. Effect of cumulative gender on phagocytosis of *S.* Heidelberg by turkey peripheral blood mononuclear cells (PBMCs). Data were analyzed as LS Means + SEM (n = 6/gender). Bars lacking a common letter (a-b) differ significantly. There was a significant effect of gender on phagocytosis of *S.* Heidelberg by PBMCs ($P = 0.0270$).

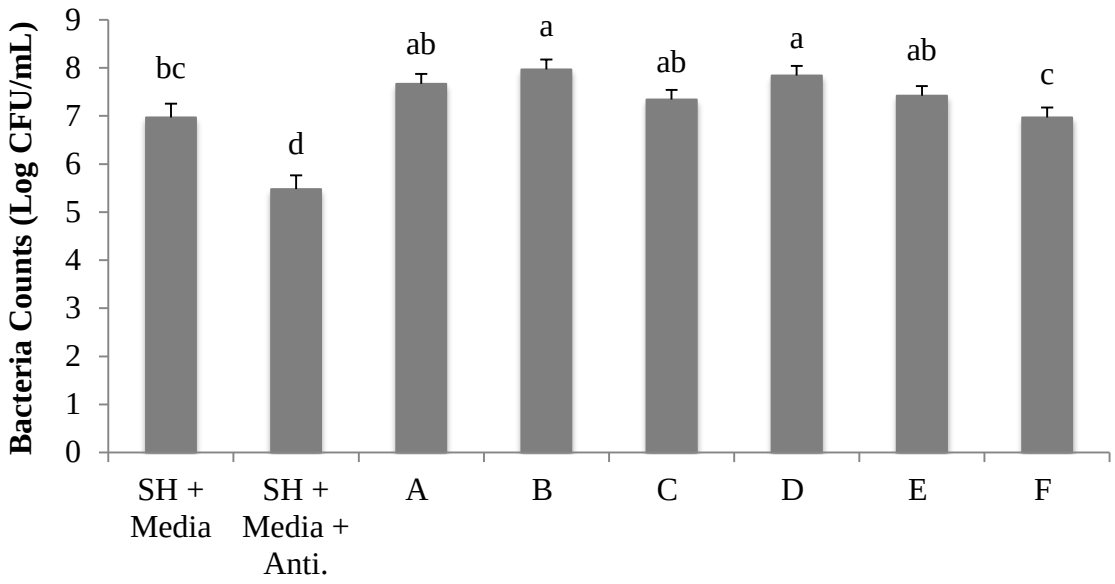

Figure 8. Effect of genetic line on bacterial killing of *S.* Heidelberg by turkey PBMCs incubated at a 1:10 PBMC to *S.* Heidelberg ratio. Data were analyzed as LS Means + SEM (n = 2); bars lacking a common letter (a-c) differ significantly. There was a significant effect of genetic line (A-F) on the killing of *S.* Heidelberg by turkey PBMCs ($P = 0.0047$). SH + Media = SH incubated in media alone; SH + Media + Anti. = SH incubated in media containing antibiotics (50 μg/mL of Kanamycin and Gentamicin).

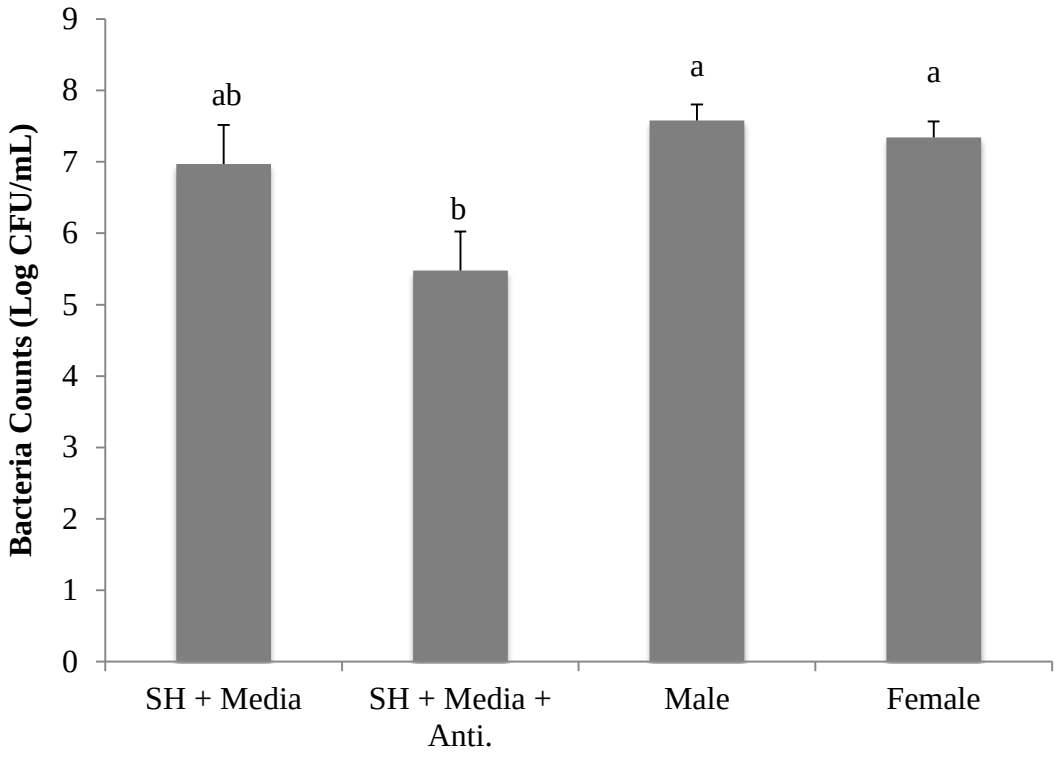

Figure 9. Effect of cumulative gender on bacterial killing of *S.* Heidelberg by turkey PBMCs incubated at a 1:10 PBMC to *S.* Heidelberg ratio. Data were analyzed as LS Means + SEM (n = 6/gender); bars lacking a common letter (a-b) differ significantly. There was a significant effect of gender on *S.* Heidelberg killing by PBMCs observed when comparing to *S.* Heidelberg incubated in media alone and *S.* Heidelberg incubated with antibiotics ($P = 0.0331$). SH + Media = *S.* Heidelberg incubated in media alone; SH + Media + Anti. = *S.* Heidelberg incubated in media containing antibiotics (50 μg/mL of Kanamycin and Gentamicin).

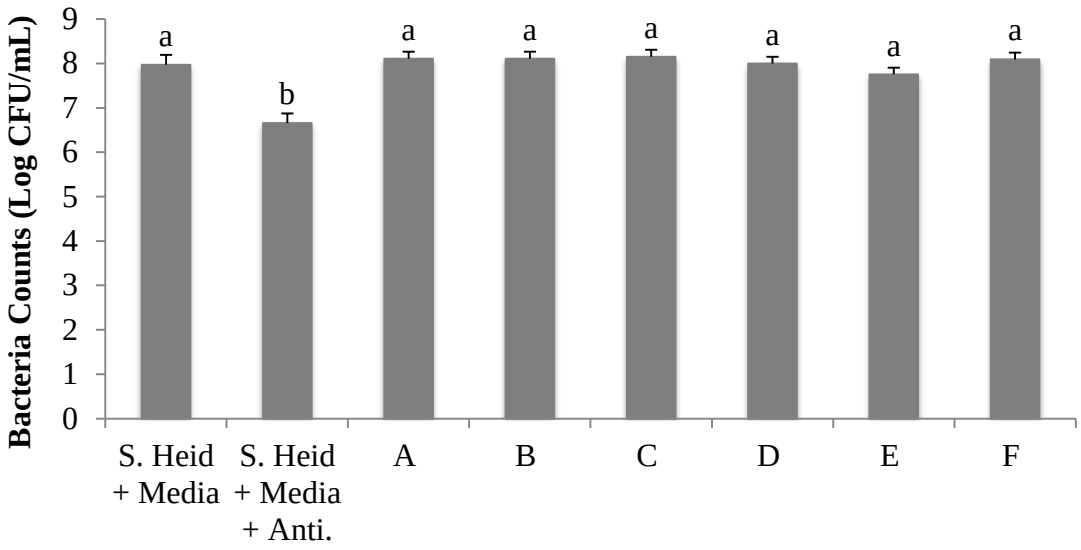

Figure 10. Effect of genetic line on bacterial killing of *S.* Heidelberg by turkey PBMCs incubated at a 1:100 PBMC to *S.* Heidelberg ratio. Data were analyzed as LS Means + SEM (n = 2 /genetic line); bars lacking a common letter (a-b) differ significantly. There was a significant effect of genetic line (A-F) on *S.* Heidelberg killing by PBMCs (*P* = 0.0233). SH + Media = *S.* Heidelberg incubated in media alone; SH + Media + Anti. = *S.* Heidelberg incubated in media containing antibiotics (50 μg/mL of Kanamycin and Gentamicin).

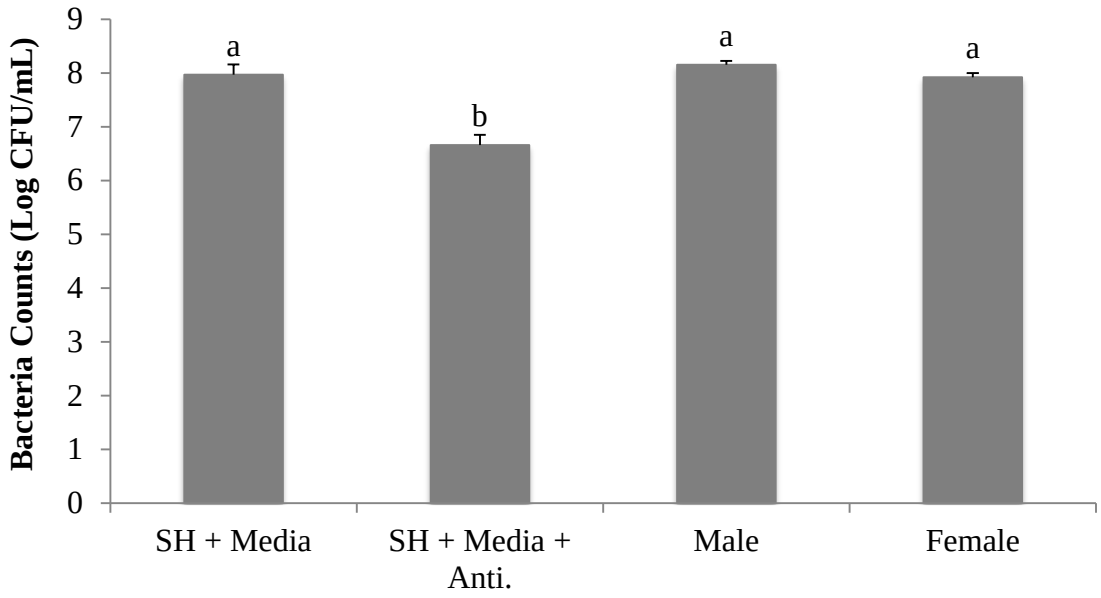

Figure 11. Effect of cumulative gender on bacterial killing of *S.* Heidelberg by turkey PBMCs incubated at a 1:100 PBMC to *S.* Heidelberg ratio. Data were analyzed as LS Means + SEM (n = 6/gender); bars lacking a common letter (a-b) differ significantly. There was a significant effect of gender on *S.* Heidelberg killing by PBMCs observed when comparing to *S.* Heidelberg incubated in media alone and *S.* Heidelberg incubated with antibiotics (P = 0.0002). SH + Media = *S.* Heidelberg incubated in media alone; SH + Media + Anti. = *S.* Heidelberg incubated in media containing antibiotics (50 μg/mL of Kanamycin and Gentamicin).

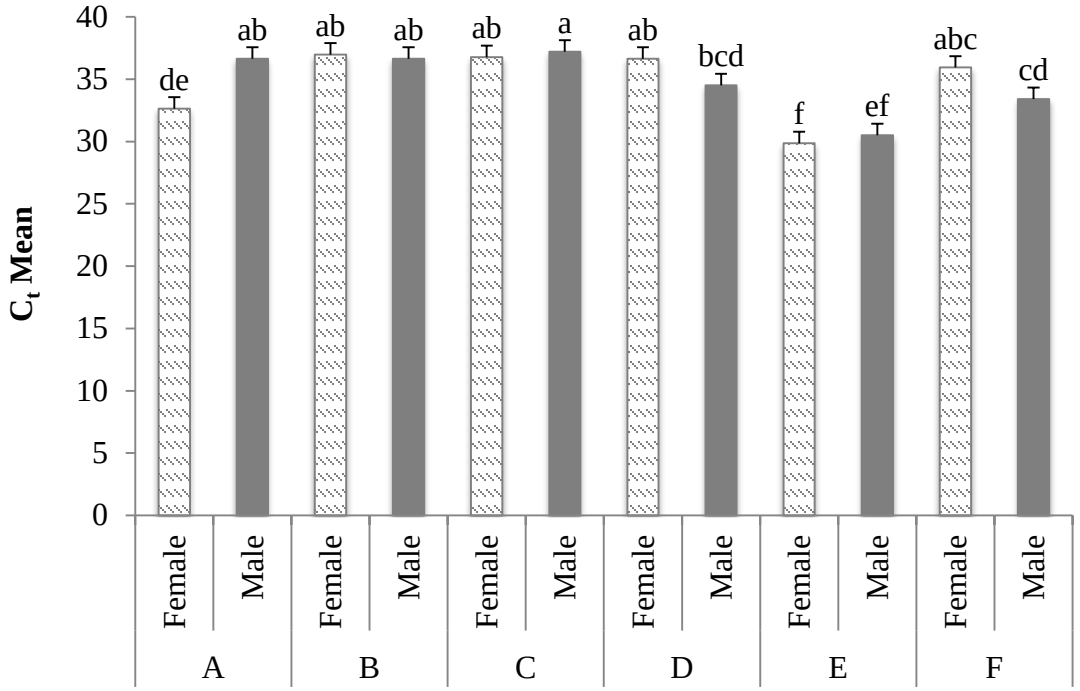

Figure 12. Effect of genetic line X gender on colonization of *Enterococcus* in the ceca of purebred turkeys. Data were analyzed as LS Means + SEM (n = 4 birds/gender/genetic line (non-challenged)); bars lacking common letters (a-f) differ significantly. There was a significant effect of genetic line (A-F) on load of *Enterococcus* in the ceca ($P = 0.0156$).

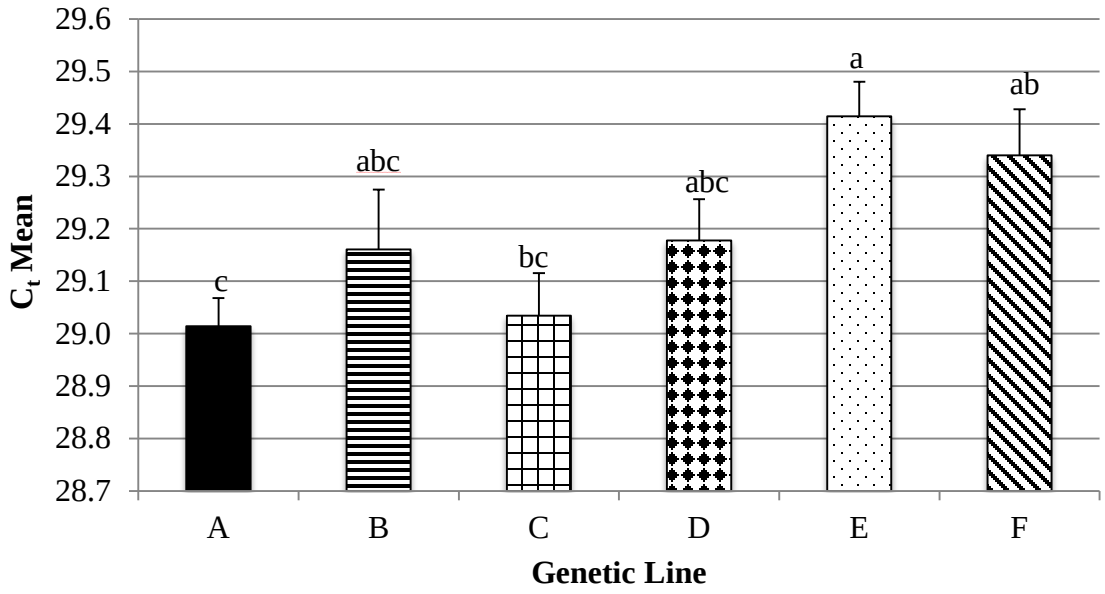

Figure 13. Effects of genetic line on colonization of an "unknown" *Salmonella* in the ceca of purebred turkeys. Data were analyzed as LS Means + SEM (n = 4 birds/gender/genetic line (non-challenged)); bars lacking a common letter (a-c) differ significantly. There was a significant effect of genetic line (A-F) on the load of *Salmonella* in the ceca ($P = 0.0019$).

Chapter V

Discussion

Although phenotypes of genetic resistance to *Salmonella* have been widely studied in chickens, less research has focused on determining these factors of *Salmonella* resistance in pedigree turkey lines. In this study, bacterial colonization, microbial profiles, and ex vivo effects of SH on leukocyte function were observed in six turkey purebred lines, differing broadly in disease susceptibility. According to Aviagen (personal communication), genetic lines emphasizing growth tend to be more sensitive to a stressful environment and have proven to be more likely to show respiratory illness or enteric illness if a bacterial challenge is present. Similarly, research of disease susceptibility in chicken lines has demonstrated that allocation of a substantial portion of resources towards a particular demand decreases the ability of the host to respond to other demands such as immunocompetence (Siegel and Honaker, 2009).

In this study, the main effect of genetic line had a significant impact on pre-challenge mortality with lines A and F demonstrating higher mortality than all other genetic lines. These results are interesting due to the fact that line F had the highest and line A showed the lowest bacterial counts at 1 dpi. These results conflicted with findings of microbial profiling, which conveyed that genetic line A had the highest colonization of *Salmonella* in the ceca. Significant differences in pre-challenge mortality could have been due to an increased susceptibility of genetic lines A and F to environmental stressors and an "unknown" *Salmonella* serotype that was later isolated from the ceca. Furthermore, pre-challenge mortality as a result of *Salmonella* exposure prior to SH challenge may have had a negative impact on the results of this study. As such, the birds were dually challenged, initially by an "unknown" *Salmonella* serotype and

subsequently by oral inoculation of SH, and potential competitive exclusion could have occurred between these two serotypes. Competitive exclusion among different types of *Salmonella* is feasible and often results in the serovar with the highest pathogenicity competitively excluding the others from the host population (Rabsch et al., 2000). Due to this likelihood, the effects of genetic line, treatment, and gender on the phenotypes of interest in this study, with the exception of phagocytic and bactericidal assays performed ex vivo, could not be directly correlated with SH.

Salmonella was detected in the ceca of both non-challenged and SH challenged groups; however, no colonization was noted to be in the spleen or liver. It is possible that *Salmonella* was actually present in the spleen, liver, and ceca and the loads were too small to detect through plating. There is a strong likelihood that these results would have been different had the samples been incubated for 24 h in an enrichment medium, such as tetrathionate broth, prior to plating. Interestingly, results of qRT-PCR with 1% TAE gel analysis confirmed that no SH was present in challenged or non-challenged groups; however, results indicated another type of *Salmonella* was present in the ceca of both challenged and non-challenged groups. Due to the opportunistic nature of *Salmonella*, it is suspected that this "unknown" *Salmonella* type was acquired from the environments which the birds had encountered. Furthermore, unexpected air-handling issues within bird facilities prior to SH inoculation could have resulted in birds being heat-stressed. Although air-handling issues occurred for an estimated two days approximately 1 week prior to SH inoculation, short-term stressors often result in decreased resistance to viral infections and surprisingly an increased resistance to bacterial and parasitic infections (Gross and Siegel, 1965; Gross, 1990). It is believed that the poults in this study encountered an "unknown" *Salmonella* serotype prior to heat stress; furthermore, exposure to heat stress rendered them more resistant to

the SH challenge or the presence of an "unknown" *Salmonella* serotype eliminated SH through competitive exclusion.

Interestingly, no *Salmonella* colonization was found in the spleen or liver among all genetic lines. However, significant differences in *Salmonella* colonization of the cecum was observed 1 dpi when analyzing treatment X gender interaction, with challenged males and non-challenged females demonstrating higher CFU/mL than challenged females. Furthermore, the main effect of genetic line was significant as line F exhibited the highest bacterial counts while line A showed the lowest bacterial counts with lines B and E falling shortly behind. Results suggested a significant effect of the three-way interaction of treatment X gender X genetic line on colonization in the ceca 3 dpi. Previous studies have found varying results debating the effects of a *Salmonella* challenge on bacterial colonization in the spleen, liver, and ceca. These data correspond with previous research where genetic line had a significant effect on *Salmonella* burden in ceca and cecal contents. Kaiser and Lamont (2001) found that genetic line had a significant effect on *Salmonella* Enteritidis pathogen load in the cecal content but not in the spleen of young layer chicks; however, there was no significant effect of sex on *Salmonella* Enteritidis numbers found in cecal contents. In a comparable study, intravenous inoculation of 10^6 *Salmonella* Enteritidis in 13-week-old chickens resulted in rapid contamination of liver, spleen, and lastly ceca. Furthermore, a significant effect of genetic line on levels of *Salmonella* Enteritidis was observed 3 dpi in the liver and ceca, 10 dpi in the ceca, and 15 dpi in the spleen (Girard-Santosuosso et al., 1998). Corroborating our findings, several studies have found that gender or genetic background correlate with *Salmonella* colonization in the ceca (Berthelot et al., 1998; Girard-Santosuosso et al., 1998; Beaumont et al, 1999; Kaiser and Lamont, 2001), suggesting that genetic background is potentially a key factor in determining *Salmonella*

resistance or susceptibility. Gender could potentially influence susceptibility to colonization due to resource allocation. It could be that the emphasis on egg laying in females requires less resources than the emphasis on growth in males. Furthermore, production traits are inversely proportional to disease resistance. The varying results found in these studies could have a variety of explanations such as differences in stress levels pre- and post-challenge, the age and condition in which the birds were challenged, and the presence of another *Salmonella* which may have competed with SH for attachment and colonization sites within the GI tract. Furthermore, males of a variety of species have been shown to be more susceptible than females to infections resulting from parasites, fungi, bacteria, and viruses (Klein, 2000). Studies have proven the effect of gene altering sex steroid hormones on susceptibility and resistance to infection. These studies suggest that genes located on the sex chromosomes are not the primary mediators of sex differences in host protection against infection. Rather, genes of the Ig superfamily are notably affected by sex steroids and are thought to modify the resistance and susceptibility to disease (Klein, 2000). Further research is required in order to pinpoint genes of resistance and susceptibility to *Salmonella* colonization in turkey purebred lines in order to achieve potential genetic selection against *Salmonella* colonization.

Heterophils are the avian equivalent to the mammalian neutrophil and are the first cells to defend the host against bacterial infection. Phagocytosis is an active and receptor-mediated internalization of foreign antigens by phagocytic cells such as heterophils and PBMCs. Heterophils have the rapid ability to kill bacterial pathogens through phagocytosis, degranulation, and generation of an oxidative burst (Swaggerty et al., 2003). Bactericidal assays permit the ability to test leukocytes in order to determine their ability to kill a culture of live bacteria. Ultimately, phagocytic cells ingest and destroy bacteria through cell lysis. In some

cases, bacteria are able to survive within the cell and multiply within macrophages. In this study, cumulative gender had a significant effect on phagocytosis while genetic line had a significant effect on bactericidal killing of SH by heterophils and PBMCs. These results were similar to findings of Redmond et al. (2011) where genetic crosses significantly affected the phagocytosis and bacterial killing, with heterophils from a broiler x Fayoumi advanced intercross line outperforming those from a broiler x Leghorn advanced intercross line. Results were also consistent with those of Swaggerty et al. (2003) suggesting that heterophil function and efficiency can be genetically transferred to progeny. Furthermore, heterophil function is sex-associated and can be genetically controlled by the rooster. These outcomes correspond with our findings of gender having a significant contribution to the process of phagocytosis of SH by heterophils and PBMCs. Curiously, results of these studies contradict findings of a previous study performed by Genovese et al. (2006) which proposed that heterophils isolated from commercial turkeys and Rio Grande turkeys on days 4, 7, and 14 post-hatch showed no significant differences in the percentage of heterophils phagocytizing *S.* Enteritidis. These differing results could be due to the age at which heterophils were isolated and the fact that only one commercial line was used for comparison.

Regarding the bactericidal assay in this study, an interesting result is that all genetic lines demonstrated an equivalent or higher bacterial count than SH incubated in media alone. Samples for this assay were plated to determine differences in bacterial counts post incubation with phagocytic PBMCs; however, samples were not viewed with a microscope prior to plating to determine whether or not the SH was killed post phagocytosis or remained live within the cell. These results suggest that SH survived within PBMCs for all genetic lines and potentially

multiplied within the cells of lines B and D resulting in a higher bacterial count than SH incubated in media alone.

Bacteria within the gut microbiota have shown the ability to attach to gastrointestinal epithelium with varying efficiencies; however, bacteria that do not attach to the epithelium have the potential to compete with others through rapid growth, substrate utilization, and production of bacteriocins that inhibit growth (Apajalahti et al., 2004). This principle, known as competitive exclusion, implies that one of two bacterial competitors will have an advantage over the other, ultimately resulting in the extinction of the inferior competitor. This study indicated turkey genetic line having a significant effect on the presence of *Salmonella* found in the ceca. In addition, the interaction of treatment X gender X genetic line had a significant impact on *Enterococcus* presence in the ceca. Although there were no significant differences observed when analyzing colonization of *E. coli* in the ceca, this could be due to the fact that the amount of *E. coli* was too minute to be detected through methods of qPCR. Genotype and gender are critical factors controlling the composition of the gut microbiota in chickens (Zhao et al., 2013). Kovacs et al. (2011) observed similar results in mice, demonstrating that genetic background significantly impacts the microbiota composition. Furthermore, their findings proposed that genetic background is a stronger determinant than gender when looking at effects of host susceptibility to diseases.

In conclusion, these data suggest that both genetic line and gender are key components involved in genetic resistance to *Salmonella* infection. Similarly, phagocytosis and bacterial killing of SH by heterophils and PBMCs suggested a relationship between efficiency of function and genetic line and gender. Genetic line also had a significant impact on *Salmonella* colonization in the ceca, while *Enterococcus* colonization was impacted by the interaction of

treatment X gender X genetic line. To our knowledge, this study is the first to evaluate differences of microbial profiles and leukocyte function in purebred turkey lines. More research is needed to determine the effects of a SH challenge on spleen, liver, and ceca bacterial colonization in turkey pedigree lines. Furthermore, additional research on microbial profiles, and heterophil and PBMC functions is needed in order to determine differences in the innate immune response of turkeys originating from different genetic lines and genders.

Chapter VI

Epilogue

A combination of the integrity of the small intestine and leukocyte function is essential for protection of the avian host against opportunistic bacteria such as *Salmonella*. With increasing recalls due to *Salmonella*-contaminated products, a long-term and cost-effective *Salmonella* preventative approach, such as genetic selection for *Salmonella* resistance, is needed in order to reduce detrimental economic impacts and maintain consumer confidence in the safety of poultry products. While numerous studies have focused on factors of *Salmonella* resistance in chickens, scarcely any research has emphasized on the components of *Salmonella* resistance and susceptibility in turkey pedigree lines. In order to achieve this strenuous goal, we must first identify differences in the innate immune response across genetic lines and subsequently pinpoint genetic markers that are responsible for *Salmonella* resistance and susceptibility. The results presented in this thesis indicate that both turkey genetic background and gender modulate factors that influence response to *Salmonella* resistance. These factors include bacterial colonization in the ceca, phagocytosis, and bacterial killing by heterophils and PBMCs. The evidence reported herein suggests that genetics and gender exhibit dissimilar effects on *Salmonella* colonization, phagocytosis, and bacterial killing depending on differences in innate immune response. Gender as an entirety and gender within genetic line significantly impacted *Salmonella* colonization in the ceca. Similar results occurred when analyzing the effects of genetic background and gender on colonization of *Enterococcus* found in the ceca, with the interaction of treatment X gender X genetic line showing a significant impact on *Enterococcus* colonization. This indicates that genetic selection against *Salmonella* carrier state in turkeys

could be feasible; furthermore, potential selection could be possible against other bacterial species such as *Enterococcus*. It would have been advantageous to look at the effects of genetic background and gender on other bacterial species such as *Bifidobacterium* and *Lactobacillus* to expand the knowledge of microbial profiles in turkeys.

Though the SH oral challenge in this study proved to be unsuccessful when analyzing SH colonization in the spleen, liver, and ceca, it is practical to conclude that a lack of response was due to either too low of an SH challenge dose, increased bacterial resistance due to pre-exposure heat stress, SH reduction through competitive exclusion, or a combination of these issues. Due to the fact that non-challenged groups were positive for an "unknown" *Salmonella* serotype, it could have been that challenged groups were positive for both the unknown serotype and SH. During the first week of this trial, there was an unintentional *Salmonella* infection presumed to have been contracted prior to SH inoculation. There was no detected SH in the flock; however, the unknown *Salmonella* identified as the cause of colonization in the ceca has not been yet serotyped. It is also important to note that, approximately one week prior to SH oral challenge, poults could have been inadvertently heat-stressed due to air-handling issues in animal facilities. Based on appearance of birds during this time frame, there were visual aspects such as the occurrence of split wings, flapping of wings, and panting that could suggest heat stress. Even so, birds were not tested for stress indicators such as corticosterone levels or heterophil/lymphocyte ratios; therefore, there is no way to confirm that heat stress actually occurred. The residual of pre-infection with an unknown *Salmonella* serotype, confounded with potential unintentional heat stress, could have obviously negatively impacted the results of the SH oral challenge in this study. It would be beneficial to repeat the SH challenge in this project to determine the carrier

state of SH in the pedigree lines observed; furthermore, providing potentially significant data on a bacterial species that is a well-known source of *Salmonella* related recalls.

Because heterophils are critical in the innate immune response, they are recognized as pivotal in the termination of *Salmonella* from the avian host; therefore, making them a key phenotype to consider when determining genetic factors involved in *Salmonella* resistance. Much work has been completed characterizing the many functions of heterophils and PBMCs in chickens and turkeys; however, little research has focused on turkey pedigree lines and the differences in heterophil and PBMC function across genetic lines and genders. This lack of knowledge makes it difficult to determine if there are differences in the heterophil and PBMC activities across genetic lines; therefore, resulting in certain genetic lines showing greater *Salmonella* resistance due to an increased ability to eradicate the bacterium. Our research indicated that gender as an entirety had a significant effect on phagocytosis of SH by heterophils. When analyzing phagocytosis of SH by PBMCs, the interaction of genetics and gender indicated females having a higher percentage phagocytosis than males. Bactericidal assays of PBMCs incubated with SH revealed that the interaction of genetics and gender, and gender alone had a significant effect on the bacterial killing of SH by PBMCs, with females often having results of higher bacterial killing than males. These results suggest a genetic effect on heterophil and PBMC function relative to SH capture and clearance. Once we are able to determine genetic markers and mechanisms involved, we may better employ these factors to our advantage in research of selection towards genetic resistance of *Salmonella* in poultry.

From this study, there is great promise for future research. The first effort should be to repeat the trial using poults known to be free from *Salmonella* and environmental stress to determine the true effects of a SH challenge on bacterial colonization in the spleen, liver, and

ceca post-challenge. In spite of the fact that the natural *Salmonella* infection and unintended potential heat stress did result in negative consequences for the results of this study, these challenges produced useful information on the potential occurrences in a real-world industry setting. Although producers strive to prevent disease and environmental stress, situations such as these often occur in commercial settings and it is useful to gain a better understanding of the impact of such things *Salmonella* colonization. Furthermore, infection with an unknown Salmonella sp. and various effects of genetics and gender on bacterial colonization in this study spike curiosity relative to further research in areas investigating competitive exclusion of *Salmonella* among genders and genetic lines. It would be interesting to learn how different *Salmonella* serotypes compete for resources in the host and if particular serotypes are capable of out-competing others, resulting in only one of the two serotypes having the ability to colonize in the gut. For example, could *Salmonella arizonae* eliminate *Salmonella* Heidelberg through competitive exclusion; therefore, resulting in only *Salmonella arizonae* being expressed in the ceca of infected birds and no effect from an SH challenge? Furthermore, when reflecting on the ex vivo effects of SH on heterophil and PBMC phagocytic and bactericidal activity, an interest in performing chemotaxis and adherence assays arises in order to obtain a better understanding of the differences in heterophil function among genders and across genetic lines.

There is great need for determining a long-term solution to *Salmonella* colonization and infection in poultry. Genetic selection towards resistance is a potential alternative to short-term preventatives and is well researched in chickens. However, few studies directly focus on the effects on genetics and gender relative to *Salmonella* resistance and susceptibility in turkey purebred lines. As has been demonstrated in chickens, genetic selection for *Salmonella* resistance could be achievable in turkey pedigree lines if the correct genetic markers of resistance are

identified. More research is needed in order to identify specific markers of genetic resistance to *Salmonella* in turkeys.

Chapter VII

References

Aley, S. B., M. Zimmerman, M. Hetsko, M. E. Selsted, and F. D. Gillin. 1994. Killing of *Giardia lamblia* by cryptdins and cationic neutrophil peptides. Infect. Immun. 62:5397-5403.

Angelis, M. D., S. Siragusa, M. Berloco, L. Caputo, L. Settanni, G. Alfonsi, M. Amerio, A. Grandi, A. Ragni, and M. Gobbetti. 2006. Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. Res. Microbiol. 157:792-801.

Apajalahti, J., A. Kettunen, and H. Graham. 2004. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. World Poult. Sci. J. 60:223-232.

Beal, R. K., C. Powers, T. F. Davison, and A. L. Smith. 2006. Immunological development of the avian gut. Pages 85-102 *in:* Avian Gut Function in Health and Disease. Iowa State Press, Ames, IA.

Beaumont, C., J. Protais, J. F. Guillot, P. Colin, K. Proux, N. Millet, and P. Pardon. 1999. Genetic resistance to mortality of day-old chicks and carrier-state of hens after inoculation with *Salmonella enteritidis*. Avian Pathol. 28:131-135.

Berthelot, F., C. Beaumont, F. Mompart, O. Girard-Santosuosso, P. Pardon, and M. Duchet-Suchaux. 1998. Estimated heritability of the resistance to cecal carrier state of *Salmonella enteritidis* in chickens. Poult. Sci. 77:797-801.

Blackwell, J. M., G. Tapasree, C. A. W. Evans, D. Sibthorpe, N. Papo, J. K. White, S. Searle, E. N. Miller, C. S. Peacock, H. Mohammed, and M. Ibrahim. 2001. SLC11A1 (formerly NRAMP1) and disease resistance. Cell Microbiol. 3:773-784.

Butcher, G. D., and R. D. Miles. 2003. The avian immune system. Veterinary medicine - large animal clinical sciences department, Florida Cooperative Extension Service. University of Florida, Gainesville, Florida. VM74.

Cheng, H.H., and S.J. Lamont. 2008. Genetics of disease resistance. Pages 59 -72 *in:* Diseases of Poultry, 12th ed. Blackwell Publ., Ames, Iowa.

Cheng, H. H., P. Kaiser, and S. J. Lamont. 2013. Integrated genomic approaches to enhance genetic resistance in chickens. Annu. Rev. Anim. Biosci. 1:239-260.

Dalloul, R. A., and H. S. Lillehoj. 2006. Poultry coccidiosis: recent advancements in control measures and vaccine development. Expert Rev. Vaccines 5:143-163.

Davison, F. 2014. The Importance of the Avian Immune System and its Unique Features. Pages 1-11 *in:* Avian Immunology, 2nd ed. Academic Press, San Diego, CA.

Davison, T. F., B. Kaspers, and K. A. Schat. 2008. Avian T cells: antigen recognition and lineages. Pages 91-102 *in:* Avian immunology, 1st ed. Academic Press, Amsterdam.

Desin, T. S., W. Köster, and A. A. Potter. 2013. *Salmonella* vaccines in poultry: past, present and future. Expert Rev. Vaccines 12:87-96.

Evans, E. W., G. G. Beach, J. Wunderlich, and B. J. Harmon. 1994. Isolation of antimicrobial peptides from avian heterophils. J. Leukoc. Biol. 56:661-665.

Farm Bureau, VA. 2012. Economic impact of Va. poultry industry tops $8 billion. https://vafarmbureau.org/NewsVideo/NewsHeadlines/tabid/347/articleType/ArticleView/articleId/1062/categoryId/53/Study_Economic_impact_of_Va_poultry_industry_tops_8_billion.aspx. Accessed July 2013.

Foley, S. L., R. Nayak, I. B. Hanning, T. J. Johnson, J. Han, and S. C. Ricke. 2011. Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. Appl. Environ. Microbiol. 77:4273-4279.

Gast, R.K. 2003. *Salmonella* infections. Pages 567-613 *in:* Diseases of Poultry, 11th ed. Iowa State Press, Ames, IA.

Genovese, K. J., H. He, V. K. Lowry, C. L. Swaggerty, and M. H. Kogut. 2006. Comparison of heterophil functions of modern commercial and wild-type Rio Grande turkeys. Avian Pathol. 35:217-223.

Girard-Santosuosso, O., P. Menanteau, M. Duchet-Suchaux, F. Berthelot, F. Mompart, J. protais, P. Colin, J. F. Guillot, C. Beaumont, and F. Lantier. 1998. Variability in the resistance of four chicken lines to experimental intravenous infection with Salmonella enteritidis phage type 4. Avian Dis. 42:462-469.

Goldsby, R. A., T. J. Kindt, B. A. Osborne, and J. Kuby. 2000. Antigens. Pages 57-75 *in:* Kuby Immunology, 4th ed. W. H. , Freeman & Co., NY.

Gordon, H. A., and L. Pesti. 1971. The gnotobiotic animal as a tool in the study of host microbial relationships. Bacteriol. Rev. 35:390-429.

Grimes, J. L., S. Rahimi, E. Oviedo, B. W. Sheldon, and F. B. Santos. 2008. Effects of a direct-fed microbial (primalac) on turkey poult performance and susceptibility to oral *Salmonella* challenge. Poult. Sci. 87:1464-1470.

Gross, W. B., and H. S. Siegel. 1965. The effect of social stress on resistance to infection with *Escherichia coli* or *Mycoplasma gallisepticum*. Poult. Sci. 44:998-1001.

Gross, W. B., and H. S. Siegel. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. Avian Dis. 27:972-979.

Gross, W. B..1990. Effect of exposure to a short-duration sound on the stress response of chickens. Avian Dis. 34:759-761.

Gross, W. B., and P.B. Siegel. 1997. Why Some Get Sick. J. Appl. Poult. Res. 6:453-460.

Harwig, S. S., K. M. Swiderek, V. N. Kokryakov, L. Tan, T. D. Lee, E. A. Panyutich, G. M. Aleshina, O. V. Shamova, and R. I. Lehrer. 1994. Gallinacins: cysteine-rich antimicrobial peptides of chicken leukocytes. FEBS Lett. 342:281-285.

Juul-Madsen, H. R., B. Viertlböeck, S. Härtle, A. L. Smith, and T. W. Göbel. 2014. Innate immune responses. Pages 121-147 *in:* Avian Immunology, 2nd Ed. Academic Press, San Diego, CA.

Kaiser, M. G., N. Lakshmanan, T. Wing, and S. J. Lamont. 2002. Salmonella enterica Serovar enteritidis Burden in Broiler Breeder Chicks Genetically Associated with Vaccine Antibody Response. Avian Dis. 46:25-31.

Kaiser, M. G., and S. J. Lamont. 2001. Genetic line differences in survival and pathogen load in young layer chicks after *Salmonella enterica* serovar enteritidis exposure. Poult. Sci. 80:1105-1108.

Kaspers, B., and P. Kaiser. 2014. Avian antigen-presenting cells. Pages 169-188 *in:* Avian Immunology, 2nd Ed. Academic Press, San Diego, CA.

Kaufman, J. 2014. The avian MHC. Pages 149-167 *in:* Avian Immunology, 2nd Ed. Academic Press, San Diego, CA.

Kennedy, M., R. Villar, D. J. Vugia, T. Rabatsky-Ehr, M. M. Farley, M. Pass, K. Smith, P. Smith, P. R. Cieslak, B. Imhoff, and P. M. Griffin. 2004. Hospitalizations and deaths due to *Salmonella* infections, FoodNet, 1996-1999. Clin. Infect. Dis. 38 (Suppl. 3):S142-148.

Kimura, A. C., V. Reddy, R. Marcus, P. R. Cieslak, J. C. Mohle-Boetani, H. D. Kassenborg, S. D. Segler, F. P. Hardnett, T. Barrett, and D. L. Swerdlow. 2004. Chicken consumption is a newly identified risk factor for sporadic *Salmonella enterica* serotype Enteritidis infections in the United States: a case-control study in FoodNet sites. Clin. Infect. Dis. 38 (Suppl. 3):S244-252.

Klein, S. L. 2000. The effects of hormones on sex differences in infection: from genes to behavior. Neurosci. Biobehav. Rev. 24:627-638.

Kogut, M. H., E. D. McGruder, B. M. Hargis, D. E. Corrier, and J. R. DeLoach. 1995. In vivo activation of heterophil function in chickens following injection with *Salmonella enteritidis*-immune lymphokines. J. Leukoc. Biol. 57:56-62.

Kogut, M. H., G. Tellez, B. M. Hargis, D. E. Corrier, and J. R. DeLoach. 1993. The effect of 5-fluorouracil treatment of chicks: a cell depletion model for the study of avian polymorphonuclear leukocytes and natural host defenses. Poult. Sci. 72:1873-1880.

Lahellec, C., and P. Colin. 1985. Relationship between serotypes of salmonellae from hatcheries and rearing farms and those from processed poultry carcasses. Br. Poult. Sci. 26:179-186.

Lamont, S. J. 2008. Variation in chicken gene structure and expression associated with food-safety pathogen resistance: integrated approaches to *Salmonella* resistance. Pages 57-66 *in:* Genomics of Disease. Stadler genetics symposia series. Springer, NY.

Lamont, S. J. 2010. *Salmonella* in chickens. Pages 213-231 *in:* Breeding for Disease Resistance in Farm Animals. CABI, Cambridge, MA.

Lee, M. D. 2007. Gene transfer and antibiotic resistance – what are those bacteria doing when we are not looking? in Chapter ARPAS Continuing Education Conference Proceedings 2007. Department of Population Health, College of Veterinary Medicine, and The University of Georgia.

Lehrer, R. I., A. K. Lichtenstein, and T. Ganz. 1993. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. Annu. Rev. Immunol. 11:105-128.

Liu, W., M. M. Miller, and S. J. Lamont. 2002. Association of MHC class I and class II gene polymorphisms with vaccine or challenge response to *Salmonella enteritidis* in young chicks. Immunogenetics. 54:582-590.

Logue, C. M., and C. W. Nde. 2007. *Salmonella* contamination of turkey from processing to final product-a process to product perspective. Foodborne Pathog. Dis. 4:491-504.

Malinen, E., A. Kassinen, T. Rinttila, and A. Palva. 2003. Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. Microbiology 149: 269-277.

Mariani, P., P. A. Barrow, H. H. Cheng, M. M. Groenen, R. Negrini, and N. Bumstead. 2001. Localization to chicken chromosome 5 of a novel locus determining salmonellosis resistance. Immunogenetics 53:786-791.

McCarthy, N., F. J. Reen, J. F. Buckley, J. G. Frye, E. F. Boyd, and D. Gilroy. 2009. Sensitive and rapid molecular detection assays for *Salmonella enterica* serovars Typhimurium and Heidelberg. J. Food Prot. 72:2350-2357.

Menconi, A., A. D. Wolfenden, S. Shivaramaiah, J. C. Terraes, T. Urbano, J. Kuttel, C. Kremer, B. M. Hargis, and G. Tellez. 2011. Effect of lactic acid bacteria probiotic culture for the treatment of *Salmonella* enterica serovar Heidelberg in neonatal broiler chickens and turkey poults. Poult. Sci. 90:561-565.

Muir, W. I., W. L. Bryden, and A. J. Husband. 2000. Immunity, vaccination and the avian intestinal tract. Dev. Comp. Immunol. 24:325-342.

O'Brien, A. D., D. L. Rosenstreich, I. Scher, G. H. Campbell, R. P. MacDermott, and S. B. Formal. 1980. Genetic control of susceptibility to *Salmonella* typhimurium in mice: role of the LPS gene. J. Immunol. 124:20-24.

Oláh, I., N. Nagy, and L. Vervelde. 2014. Structure of the avian lymphoid system. Pages 11-44 *in:* Avian Immunology, 2nd ed. Academic Press, San Diego, CA.

Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282:2085-2088.

Rabsch, W., B. M. Hargis, R. M. Tsolis, R. A. Kingsley, K.H. Hinz, H. Tschape, and A. J. Baumlert. 2000. Competitive exclusion of *Salmonella* Enteritidis by *Salmonella* Gallinarum in poultry. Emerg. Infect. Dis. 6:443-448.

Rathgeber, B.M, K. L. Budgell, and P. McCarron. 2013. *Salmonella* penetration through eggshells of chickens of different genetic backgrounds. Poult. Sci. 92:2457-2462.

Scallon, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States - major pathogens. Emerg. Infect. Dis. 17:7-15.

Sharma, J. M. 2003. The avian immune system. Pages 5-13 *in:* Diseases of Poultry, 11th ed. Iowa State Press, Aimes, IA.

Siegel, P. B., and C. F. Honaker. 2009. Impact of genetic selection for growth and immunity on resource allocations. J. Appl. Poult. Res. 18:125-130.

Smith, A. L., C. Powers, and R. K. Beal. 2014. The avian enteric immune system in health and disease. Pages 227-250 *in:* Avian Immunology, 2nd ed. Academic Press, San Diego, CA.

Strugnell, R. A., T. A. Scott, N. Wang, C. Yang, N. Peres, S. Bedoui, and A. Kupz. 2014. *Salmonella* vaccines: lessons from the mouse model or bad teaching?. Curr. Opin. Microbiol. 17:99-105.

Swaggerty, C. L., I. Y. Prevzner, V. K. Lowry, M. B. Farnell, and M. H. Kogut. 2003. Functional comparison of heterophils isolated from commercial broiler chickens. Avian Pathol. 32:95-102.

Swaggerty, C. L., P. J. Ferro, I. Y. Pevzner, and M. H. Kogut. 2005. Heterophils are associated with resistance to systemic *Salmonella* enteritidis infections in genetically distinct chicken lines. FEMS Immunol. Med. Microbiol. 43:149-154.

Tarr, A. 2011. Arkansas firm recalls ground turkey products due to possible *Salmonella* contamination. http://www.in.gov/isdh/files/Cargill_Meat_Solutions_Corporation_Expanded_Recall.pdf. Accessed October 2014.

Tarr, A. 2013. FSIS releases comprehensive strategy to reduce *Salmonella*. http://www.fsis.usda.gov/wps/portal/fsis/newsroom/news-releases-statements-

transcripts/news-release-archives-by-year/archive/2013/nr-12042013-01. Accessed October 2014.

Tizard, I. R. 2009. Veterinary immunology: an introduction. 8th ed. Saunders Elsevier, St. Louis, MO.

Torok, V. A., K. Ophel-Keller, M. Loo, and R. J. Hughes. 2008. Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. Appl. Environ. Microbiol. 74:783 -791.

U. S. Food and Drug Administration, U. S. FDA, 2010. The outbreak of *Salmonella* in egg. http://www.fda.gov/NewsEvents/Testimony/ucm226554.htm. Accessed November 2010.

Virginia Poultry Federation, VPF, 2013. Virginia Poultry Industry Facts and Figures 2012. http://www.vapoultry.com/assets/Virginia Poultry Industry Facts and Figures 12.pdf. Accessed October 2014.

Wigley, P. 2004. Genetic resistance to *Salmonella* infection in domestic animals. Res. Vet. Sci. 76:165-169.

Yun, C.H, H. S. Lillehoj, and E. P. Lillehoj. 2000. Intestinal immune responses to coccidiosis. Dev. Comp. Immunol. 24:303-324.

Zhao, S., D. G. White, S. L. Friedman, A. Glenn, K. Blickenstaff, S. L. Ayers, J. W. Abbott, E. Hall-Robinson, and P. F. McDermott. 2008. Antimicrobial resistance in *Salmonella enterica* serovar Heidelberg isolates from retail meats, including poultry, from 2002 to 2006. Appl. Environ. Microbiol. 74:6656-6662.

Zhao, L., G. Wang, P. Siegel, C. He, H. Wang, W. Zhao, Z. Zhai, F. Tian, J. Zhao, H. Zhang, Z. Sun, W. Chen, Y. Zhang, and H. Meng. 2013. Quantitative genetic background of the host influences gut microbiomes in chickens. Sci. Rep. 3:1163.

Appendix

Non-Significant Results

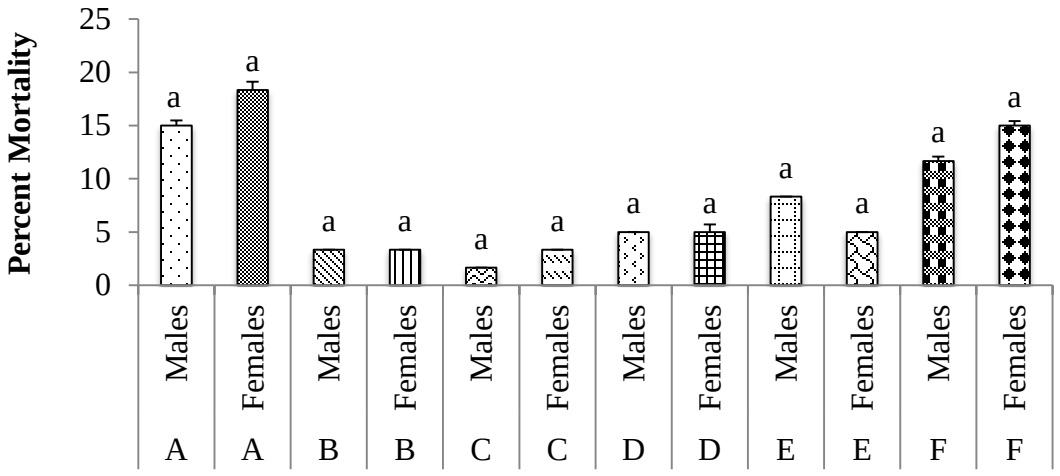

Figure A – 1. Effect of genetic line X gender on mortality observed prior to an *S.* Heidelberg challenge in purebred turkey lines. Data are presented as percent mortality (n = 1 pen/gender/genetic line); bars lacking common letters (a) differ significantly. There was no significant effect of genetic line (A-F) X gender on mortality.

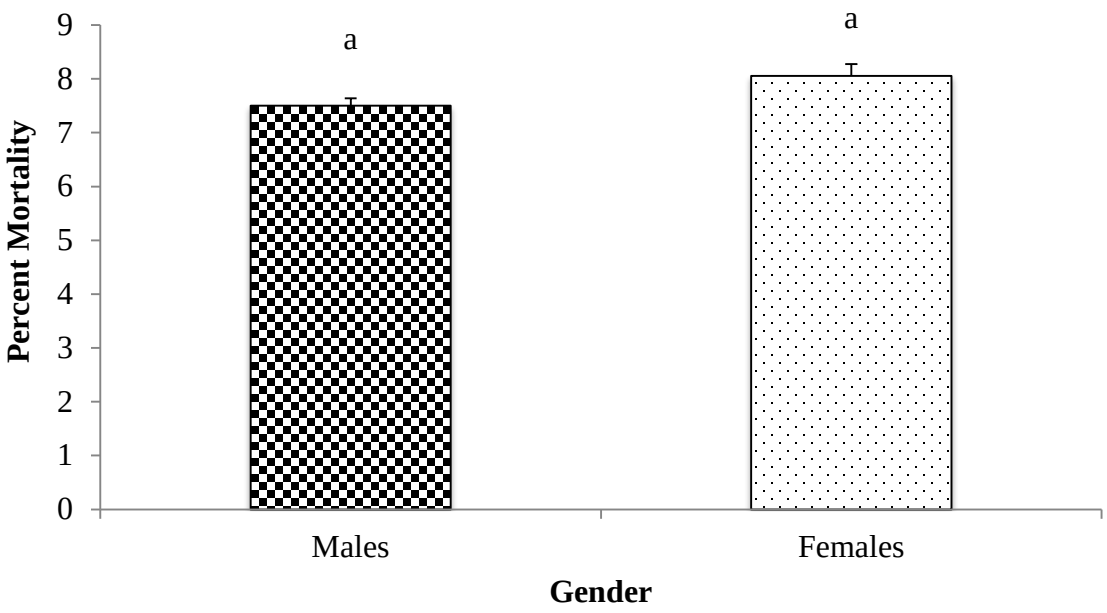

Figure A – 2. Effect of cumulative gender on mortality observed prior to an *S.* Heidelberg challenge in purebred turkey lines. Data are presented as percent mortality (n = 6 pens/gender); bars lacking common letters (a) differ significantly. There was no significant effect of cumulative gender on mortality.

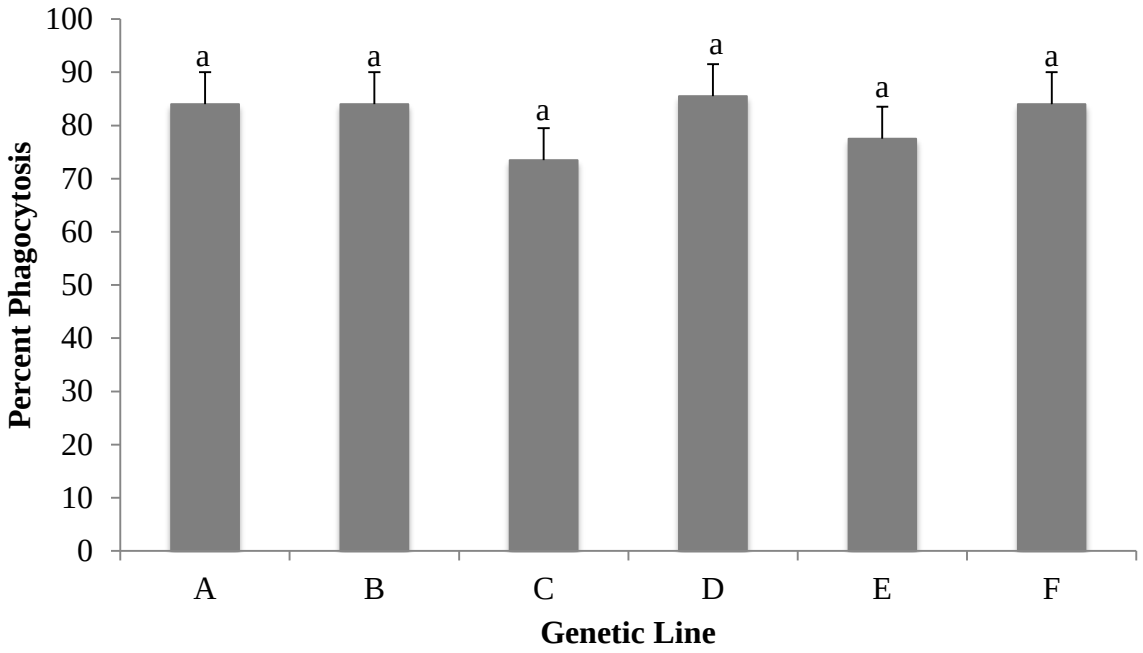

Figure A – 3. Effect of genetic line on phagocytosis of *S.* Heidelberg by turkey heterophils. Data were analyzed as LS Means + SEM (n = 2); bars lacking common letters (a) differ significantly. Genetic line (A-F) had no significant effect on phagocytosis of *S.* Heidelberg by turkey heterophils.

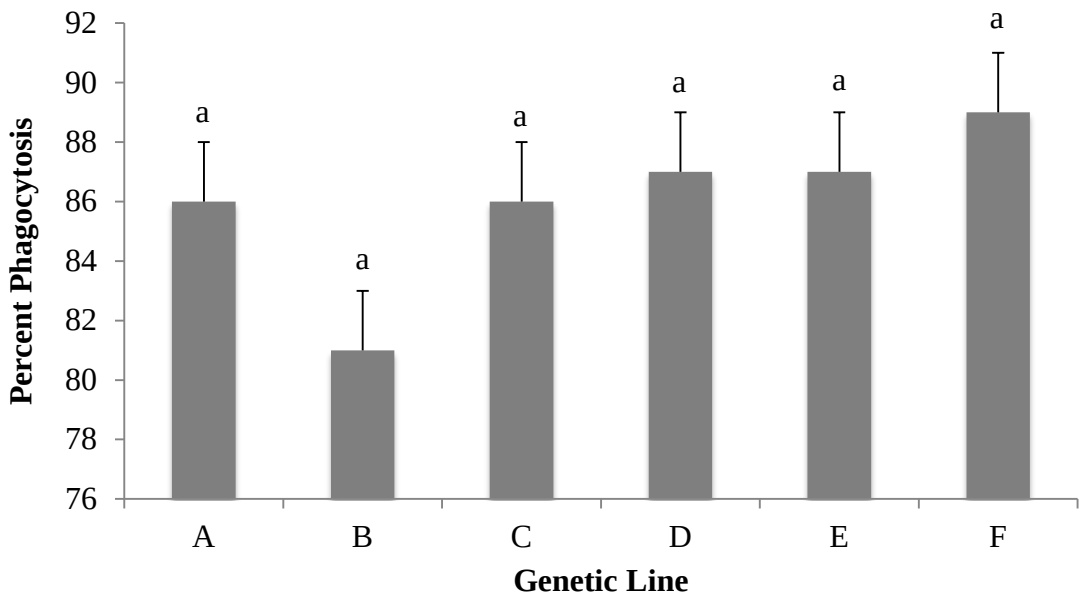

Figure A – 4. Effect of genetic line on phagocytosis of *S.* Heidelberg by turkey peripheral blood mononuclear cells (PBMCs). Data were analyzed as LS Means + SEM (n = 2/genetic line); bars lacking a common letter (a) differ significantly. Genetic line (A-F) had no significant effect on phagocytosis of *S.* Heidelberg by PBMCs.