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# Effects of genetic background and dietary immunomodulators on chicken heterophil function and *Salmonella* resistance

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# Effects of genetic background and dietary immunomodulators on chicken heterophil function and *Salmonella* resistance

by

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# LIST OF ABBREVIATIONS

Antimicrobial Peptide (AMP)

Ascorbic Acid (AA)

Bactericidal/Permeability Increasing Protein (BPI)

CC Chemokine Ligand (CCL)

Chemokine Ligand (CXCL)

Chronic Granulomatous Disease (CGD)

Complement Receptor (CR)

Dehydroascorbic Acid (DHA)

Deoxyribonuclease (DNase)

Diphenylene Iodonium (DPI)

Extracellular Traps (ETs)

Glucocorticoid Receptor (GR)

Glucocorticoid Response Element (GRE)

Glucocorticoids (GCs)

Glucose Oxidase (GO)

Heat Shock Protein (hsp)

Heterophil Extracellular Trap (HET)

Heterophil/Lymphocyte Ratio (H/L ratio)

Immunolymphokine (ILK)

Immunoreceptor Tyrosine Activation Motif (ITAM)

Inducible Nitric Oxide Synthase (iNOS)

Intercellular Adhesion Molecule (ICAM)

Mast Cell Extracellular Traps (MCETs)

Mitogen-Activated Protein Kinase (MAPK)

Monocyte Chemotactic Protein-1 (MCP-1)

Myeloperoxidase (MPO)

Natural Resistance-Associated Macrophage Protein (NRAMP)

Neutrophil Elastase (NE)

Neutrophil Extracellular Traps (NETs)

Pathogen-associated molecular pattern (PAMP)

Pattern Recognition Receptor (PRR)

Phagosome-Lysosome (P/L)

Protein Kinase C (PKC)

Proteinase 3 (PR3)

Reactive Oxygen Species (ROS)

Salmonella Containing Vacuoles (SCV)

*Salmonella enterica* serovar Enteritidis (SE)

*Salmonella enterica* serovar Typhimurium (ST)

*Salmonella* Pathogenicity Island (SPI)

Secretory Leukocyte Proteinase Inhibitor (SLPI)

Toll-like Receptor (TLR)

Type III Secretory System (TTSS)

# ABSTRACT

Immune responses in poultry can be influenced by genetic background, nutrition, environment and management, or any combination of the above. Chicken heterophils are the first line of defense that can launch a series of intra- and extracellular antimicrobial mechanisms. *Salmonella enterica* serovar Enteritidis (SE) is a causative agent of a bacterial foodborne illness, commonly occurring after consumption of contaminated eggs and meat. In 2008, SE affected 3 out of 100,000 U.S. inhabitants on average and it is now receiving increased public awareness. The limited scope of current knowledge about basis for the difference in heterophil responses during and after exposure to immunomodulators prompted the study of heterophil defense mechanisms against *Salmonella enterica* serovar Enteritidis (SE) infection in three genetically distinct chicken lines (Leghorn, broiler and Fayoumi) supplemented with dietary immunomodulators (β-glucan, ascorbic acid and corticosterone).

We hypothesize that heterophil function in three genetic chicken lines during exposure to dietary immunomodulators and challenge with SE will be significantly different. To test the hypothesis, we have isolated blood heterophils from three diverse chicken genetic lines; [outbred broiler and two highly inbred layer lines (Leghorn and Fayoumi)] that were fed diet supplemented with immunomodulators (β-glucan, ascorbic acid and cortisol) and challenged with SE. We used heterophil extracellular trap release (HETs, phagocytosis, bacterial killing and oxidative burst to determine heterophil function differences from different treatment groups.

The emphasis of this dissertation was on the role of genetic background and dietary immunomodulators on chicken heterophil function in order to prevent and control SE

colonization and shedding, using selected breeds of chickens. First, the baseline heterophil function was monitored *in vitro* using oxidative burst, phagocytosis and bacterial killing to study intracellular killing mechanisms. Additionally, HETs-DNA release has been for the first time described in avian species and used to explore a potential role of extracellular heterophil killing. We compared the responses of different chicken lines with dietary immunomodulatory effects when exposed to SE challenge. *In vivo* heterophil function and bacterial clearance by bacterial cultures were evaluated in both challenged and non-challenged chickens.

The responses of chicken heterophils to dietary immunomodulators and SE challenge were significantly influenced by genetic background, and dietary immunomodulators have significant effect on heterophil function in both non-challenged and challenged chickens. Increased SE clearance in challenged birds that were fed with supplemented diet was also observed. This dissertation provides evidence of the significant role of the genetic background of chicken innate immune system and it's responses to dietary immunomodulation and disease challenge. The results of the studies presented in this dissertation support the use of dietary immunomodulators to manipulate heterophil function in chickens from the same genetic background. The modification of the immune system of chickens with targeted genetic selection and diet supplementation in production settings can lead to improved SE (disease) resistance and reduced risk of food borne illnesses.

# CHAPTER 1. GENERAL INTRODUCTION

## INTRODUCTION

The innate immune system plays a fundamental role in the generation and maintenance of immunological balance. Mammalian neutrophils and avian heterophils, a granulocyte analogous to mammalian neutrophils, are able to cope with the great diversity of potential microbial challenges. As the first line of defense in the innate immune system, these sentinels detect and react to microbial conserved surface molecules called "Pathogen-associated molecular patterns, or PAMPs" (Medzhitov and Janeway, 2000). Additional major functions are to initiate the rapid killing and trapping of bacteria, fungi, protozoa, as well as viruses before they locally multiply and expand. Historically, studies on avian species have contributed greatly to the development of immunological understanding both in avian and mammalian species. Among avian species, chickens have provided an invaluable model for the exploration of basic immunological mechanisms. During their evolution, they have lost some of the abilities, such as lacking myeloperoxidase to mediate efficient oxidative killing. Interestingly, they achieve the same goal through the use of remaining mechanisms (Brune et al., 1972).

Chicken heterophils have ability to perform phagocytosis, bacterial killing, limited oxidative burst, and degranulation, and recently a novel extracellular function (heterophil extracellular traps; HETs) has been characterized and identified (Chuammitri et al., 2009). Together, these functional characteristics can contribute to bactericidal mechanisms in avian heterophils. Availability of the entire chicken genome sequence provides us with a more precise understanding of many key features of the avian immune system (Hillier et al., 2004).

Well-defined inbred chicken lines (*Gallus gallus domesticus*) have expanded research opportunities and understanding of chicken immunology (Deeb and Lamont, 2002; Zhou and Lamont, 1999).

Chickens provide the most inexpensive and important source of protein, meat and eggs worldwide including the North America. Research in chicken immunology plays a key role in increasing productivity of poultry as well as safeguarding public health. The benefits of immunomodulators, such as β-glucan, ascorbic acid (AA) and corticosteroid, on innate immune systems have been described in several studies. The ability of β-glucans to directly enhance of phagocytic and bactericidal capability of PMNs and phagocytes has been documented (Brown and Gordon, 2005). The immunomodulatory effect of ascorbic acid helps prevent tissue damage from free radicals and ameliorates disease during stress (Andreasen and Frank, 1999; Gross, 1992; Wang et al., 1997). Dietary corticosteroids regulate the productions of cytokines, chemokines, and inflammatory mediators and thus counterbalance immune systems that are overactive (Leung and Bloom, 2003; Rhen and Cidlowski, 2005).

Salmonellosis constitutes a major public health concern. The association between increased frequency of antimicrobial-resistant *Salmonella* and increased frequency of hospitalization are now increasing public awareness (CDC, 2009). A better understanding of the role of genetic background  and immunomodulatory effects of β-glucan and ascorbic acid on heterophil functions could lead to future applications and efficacious treatments for minimizing bacterial colonization and fecal shedding in poultry and other related species.

## OBJECTIVES AND SPECIFIC AIMS

*Our overall goal* was to identify immunomodulators and genetic profiles in chickens to enhance heterophil function and thereby improve resistance to *Salmonella enterica* serovar Enteritidis in commercial poultry, enhance animal health, production efficiency, and preharvest food safety.

*Our central hypothesis* is that the differences in chicken heterophil function in response to dietary immunomodulators and challenge with *Salmonella* Enteritidis will be observed in diverse chicken genetic lines using heterophil cellular function assays. We propose three specific research objectives to characterize the correlation of heterophil cellular function in three established chicken genetic lines.

*Objective 1*: Determine response of heterophils from different chicken lines to stimulation *in vitro* with *Salmonella* Enteritidis using heterophil function assays.

*Objective 2*: Determine response of heterophils from different chicken lines exposed to stress (corticosteroids) and immunomodulators (β-glucan, ascorbic acid) *in vivo* using heterophil function assays.

*Objective 3:* Determine response of heterophils from different chicken lines exposed to immunomodulator supplemented diet and challenge with Salmonella Enteritidis using heterophil function assays.

## DISSERTATION ORGANIZATION

This dissertation is composed of general introduction (Chapter 1) and review of the literature (Chapter 2) followed by one published paper (Chapter 3), submitted manuscript

(Chapter 4), and manuscript to be submitted to peer reviewed journal (Chapter 5). A general

conclusion is given in Chapter 6. References are cited at the end of each chapter.

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# CHAPTER 2. LITERATURE REVIEW

## HETEROPHIL BIOLOGY

Heterophils and neutrophils are essential as the first line of host defense against microbial infection. The heterophil of birds and reptiles is the equivalent of the neutrophil in mammals and fish (Macrae and Spitznagel, 1975). The term "Heterophil" was suggested by Kyes in 1929 and applies to granules of granulocytes that have variable sizes and staining characteristics (Lucas and Jamroz, 1961; Macrae and Spitznagel, 1975). Heterophils and neutrophils perform host defense through a variety of mechanisms. Bacterial adherence to phagocytes is accompanied by receptor activation and signal transduction, which triggers phagocytosis, degranulation and killing mechanisms (Brooks et al., 1996).

### Avian Hematology

The blood of all avian species contains a series of cells: erythrocytes, leukocytes and thrombocytes (Phillip et al., 2009). Five types of leukocytes are found in the blood of chickens: heterophils, eosinophils, basophils, lymphocytes and monocytes. Each of these lines retains their nucleus throughout the life of the cell in contrast to mammals (Phillip et al., 2009).

According to Lucas and Jamroz (1961), chicken heterophils are round cells and have a mean diameter of 8.7 μm (Lucas and Jamroz, 1961). The nucleus of circulating heterophils commonly has two to three lobes, with the average number of lobes reported to be 2.44 for chickens (Maxwell and Robertson, 1998). Characteristically, the nucleus of heterophils is less basophilic than the nucleus of eosinophils when stained with Romanowsky stain (Maxwell and Robertson, 1998). Electron microscopy showed prominent structures located

within the granular matrix that is referred to as "central granular bodies" in some chicken heterophils (Lucas and Jamroz, 1961; Maxwell and Robertson, 1998). Similar to the mammalian neutrophil counterparts, avian heterophil cytoplasm contains robust granules that can be characterized by shape and color. Unlike mammalian neutrophils, avian heterophils cannot be discretely defined as azurophil, specific, and gelatinase (tertiary) granules (Macrae and Spitznagel, 1975). The primary ellipsoid or fusiform granules of heterophils with Giemsa stain appear brick-red in color.

The second type of granules are rare with rod or dumb-bell shape and are more basophilic, less dense and smaller than the primary granule. This granule can also be seen in most avian species (Maxwell and Robertson, 1998). The identification of healthy heterophil nuclei can be obscured by granules. In response to inflammation, the granules may change in shape, color and number. Toxic changes of heterophils are found in severe inflammation as cell swelling with foamy cytoplasm or vacuolation, basophilic cytoplasm, and the appearance of toxic granules (Latimer et al., 1988; Phillip et al., 2009). The granules of avian heterophils have no myeloperoxidase (MPO) activity as found in azurophilic granules in neutrophils (Brune et al., 1972; Maxwell and Robertson, 1998).

**Granulopoiesis**

The mean proportions of erythroid cells, myeloid cells, and thrombocytes are 39.9%, 49.4%, and 6.0%, respectively, in bone marrow of clinically healthy birds (Phillip et al., 2009). Neutrophils and heterophils are short-lived cells, which are continuously generated from hematopoietic stem cells (HSCs) in the bone marrow by a process called granulopoiesis (Phillip et al., 2009; Theilgaard-Mönch et al., 2005). In neutrophils, granule proteins are not

synthesized *de novo* at sites of infection but pre-stored granules are synthesized during neutrophil differentiation. The granules are stored in specialized vesicles, known as azurophil, specific, and gelatinase granules (Theilgaard-Mönch et al., 2006).

Hematopoiesis in chickens occurs early in embryonic development then HSCs migrate to bone marrow (Fellah et al., 2008). The transcription signal of hematopoietic stem cells in bone marrow initiates differentiation of hematopoietic stem cells into myeloid progenitor cells, and then to granulocyte-macrophage progenitors (Theilgaard-Mönch et al., 2006). Terminal granulopoiesis that gives rise to a series of morphologically distinct stages of neutrophils and heterophils may differ slightly in their nomenclatures. While the neutrophil series can be named; *myeloblast, promyelocyte, myelocyte, metamyelocyte*, and *band/segmented neutrophils (PMN)*, chicken heterophil-granulocyte series are called *granuloblast, megagranuloblast, promyelocyte*, *mesomyelocyte, metamyelocyte*, and *mature heterophil*, respectively (Lucas and Jamroz, 1961; Maxwell and Robertson, 1998; Theilgaard-Mönch et al., 2005).

Neutrophil granules are sequentially synthesized during differentiation (Faurschou and Borregaard, 2003). Azurophilic granules are readily formed at the promyelocyte stage, but specific and genatinase granules are produced successively at the metamyelocyte stage (Theilgaard-Mönch et al., 2005). Maxell and Robertson (1998) reviewed several studies regarding hematopoiesis in chickens and showed that only a small area in bone marrow is reserved for mature granulocytes; whereas, the predominant area is used for production and maturation of erythrocytes (Maxwell and Robertson, 1998). In the majority of avian species, lymphocytes are the most numerous leukocytes in the circulation with some notable exceptions. Heterophils appear to be the predominant granulocytes in several species of

parrots (Psittacine order), waterfowl (Anseriformes order; ducks, geese, swans), ostrich, ring-necked pheasant, pigeon, and rosy flamingo (Lucas and Jamroz, 1961; Maxwell and Robertson, 1998). In small chicks, significant numbers of heterophils (up to 50%) are released from the spleen into the circulation at the time of hatching, which may indicate bone marrow is not as well developed in hematopoiesis as in the adults (Maxwell and Robertson, 1998). Heterophil ultrastructure examined by Maxwell and Robertson (1998) showed the presence of heterophilic myelocytes as principal granulocytes in several lymphoid organs in embryos. This form of cellular development is another example of extramedullary granulopoiesis that is also reported by Bar-Shira and Friedman (2006) at small intestine, cecum and colon (Bar-Shira and Friedman, 2006).

**Avian Hematology and Pathophysiological Relevance**

Heterophils play a role in acute inflammatory reactions and physiological stress. The Heterophil/Lymphocyte ratio (H/L ratio) can be used as an indicator of physiological stress, (Maxwell and Robertson, 1998). Heterophil recruitment or mobilization arises from bone marrow interactions with the hypothalamic-pituitary-adrenal cortical axis (HPA) as a consequence of stress. The reference values for the H/L ratios are about 0.20, 0.50 and 0.80 for low, optimal and high degrees of stress, respectively (Maxwell and Robertson, 1998).

**Heterophil Granules**

There are at least two predominant types of granules in neutrophils, the azurophilic and the specific. They are produced during neutrophil differentiation as mentioned above. Their granular contents are determined by the proteins synthesized during granulopoiesis (Segal, 2005; Theilgaard-Mönch et al., 2005). There are limited studies as to how many types or

categories of avian (*Gallus gallus*) heterophil granules exist. According to the cytochemical studies by Macrae and Spitznagel (1975), granules from chicken heterophils and rabbit neutrophils are categorized into type A and type B. The third type of granules that are smaller and denser than the former two types may be present. The granules contain a variety of acid hydrolases including lysozyme, β-glucuronidase, acid phosphatase, β-galactosidase etc. (Macrae and Spitznagel, 1975). Although chicken heterophils lack peroxidase in the primary granule or type A granule, they contain lysozyme that makes this granule distinct. Table 1. shows granules currently identified in either neutrophils or chicken heterophils.

## HETEROPHIL FUNCTION

Heterophils/neutrophils are activated during infection and non-infectious stresses such as burns and injuries. Heterophils constitutively express Toll-like receptors (TLRs), which are directly induced by both an oxidative burst and a degranulation response (Kogut et al., 2005). According to the review by Papayannopoulos and Zychlinsky (2009), two major intracellular antimicrobial strategies have been described as "phagocytosis" and "intraphagosomal degranulation" (Papayannopoulos and Zychlinsky, 2009).

The recently discovered release of Neutrophil Extracellular Traps (NETs) as a means of extracellular antimicrobial killing has explored the neutrophils role in innate immunity (Brinkmann et al., 2004). In this dissertation, functional activities of neutrophils and heterophils are reviewed together since they are closely related not only in their development but also in their function.

**Phagocytosis and Bacterial Killing**

Pathogens have been evolving various mechanisms of subverting the processes of phagocytosis and intracellular killing. Some examples are prevention of receptor recognition, opsonization, cell signaling, and actin polymerization. *Salmonella* inhibits actin polymerization during phagocytosis with its virulent factors (Finlay and Brumell, 2000). The process of phagocytosis is generally defined as the internalization of the large particles, such as bacteria, immune complexes, or apoptotic cells; otherwise, it is referred to as "endocytosis" if the ingested particles are less than 0.5 μm (Ernst and Stendahl, 2006). The phagocytosis is solely performed by professional phagocytes; macrophages, neutrophils, and dendritic cells, which express specific receptors, e.g. IgG Fc receptors (FcγR) and complement receptors (CR). The phagocytic process is initiated by binding of target particles with relevant receptors on the surface of phagocytic cells. The interaction of the clustered receptors with cytosolic molecules results in phagosome-lysosome (P/L) fusion, oxidative burst, bacterial killing and degranulation of antimicrobial peptides (AMPs) and enzymes. An extended pseudopod encapsulates the target particles together with the host plasma membrane and forms phagosomes (Ernst and Stendahl, 2006).

Comparative studies among chicken heterophils, human, and canine neutrophils by flow cytometry, revealed significantly fewer phagocytosed latex spheres by chicken heterophils than by human or canine neutrophils (Brooks et al., 1996). No significant differences were found in phagocytosis, but a significant increase in bacterial killing was found in heterophils treated with ascorbic acid (Andreasen and Frank, 1999). It was also reported that avian heterophils were less phagocytic and produced less oxidant in response to zymosan than canine and human neutrophils (Brooks et al., 1996). *In vitro* studies demonstrated an

enhancement of functional activities of the heterophils including chemotaxis, adherence, phagocytosis, and bacterial killing induced by immunolymphokine (ILK) (Kogut et al., 1998).

**Oxidative Burst**

The generation of oxygen free radicals and reaction products are collectively referred to as reactive oxygen species (ROS). ROS are produced as a consequence of NADPH oxidase activity, which pumps superoxide ($O_2^-$) into the phagocytic vacuole (Segal, 2005). NADPH oxidase is located in neutrophils, eosinophils, and mononuclear phagocytes (Babior et al., 2002). ROS production in PMNs is mediated by a multi component enzyme complex and NADPH oxidase, which are composed of flavocytochrome $b_{558}$ and other subunits (Lambeth, 2004; Segal, 2005). The <u>N</u>ADPH <u>OX</u>IDASE (NOX) families are composed of membrane-associated flavocytochrome $b_{588}$ (gp91$^{phox}$ and p22$^{phox}$) and cytosolic components (p47$^{phox}$, p67$^{phox}$, small GTPase; Rac , Rac 2, and Rap1A) (Roos et al., 2003). Failure to assemble into a functional complex results in chronic granulomatous disease (CGD) (Babior et al., 2002; Roos et al., 2003; Segal, 2005). NADPH oxidase plays a pivotal role in microbial killing via the electron transport chain through phagocytic membrane. This multi component enzyme catalyzes the generation of superoxide ($O_2^-$) from oxygen and NADPH in the following reaction:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^-$$

The superoxide is first produced and then converted to hydrogen peroxide ($H_2O_2$). Hydrogen peroxide together with metal ions such as iron ($Fe^{3+}$) or copper ($Cu^{2+}$), generates an extremely reactive and toxic hydroxyl radical (OH$^•$) that can attach double bonds in

organic substances. In neutrophils, hypochlorous acid (HOCl) is produced as a potent antimicrobial and cytotoxic agent. HOCl accounts for most bacterial killing of the ROS. In addition, singlet oxygen ($\Delta^1 O_2$) and ozone ($O_3$) can alternatively be produced by MPO (Babior et al., 2002; He et al., 2003; Lambeth, 2004; Roos et al., 2003). Heterophils have limited ability to produce ROS presumably because these cells do not generate enough HOCl from $H_2O_2$ for bactericidal activity (Maxwell and Robertson, 1998; Penniall and Spitznagel, 1975). Many studies have shown that chicken heterophils in tissues do lack MPO, which is very important for oxygen dependent antimicrobial mechanisms (Brune et al., 1972; Maxwell and Robertson, 1998; Montali, 1988; Penniall and Spitznagel, 1975).

**Degranulation**

Oxidase pumps electrons into the phagocytic vacuole, inducing release of cytoplasmic granules into the vacuole (Lambeth, 2004; Segal, 2005). In neutrophils, both azurophilic and specific granules fuse with the phagocytic vacuole soon after phagocytosis. At a resting state, granule contents are maintained at pH 5.0 by a buffering system. The consumption of protons by superoxide in the vacuole of activated cells elevates vacuolar pH (Segal, 2005). Potassium ion ($K^+$) is the major cation in the cytoplasm and $K^+$ transport and is markedly diminished when the pH rises above 8.0 (Reeves et al., 2002). Chloride ion ($Cl^-$) is also critical for microbial killing by halogenation by NADPH oxidase and MPO. The activation of granular enzymes and oxidase during degranulation is directly related to the coupling of chloride ion influx when bacteria are phagocytosed (Segal, 2005).

In chicken heterophils, direct evidence of degranulation was reported using phase contrast microscopy or measuring β-glucuronidase (Farnell et al., 2006; Trowell and Brewer,

1976). Administration of unmethylated CpG oligonucleotides enhanced the degranulation (He et al., 2005). Degranulation of heterophils was significantly inhibited by an inhibitor of p38 MAPK (Mitogen-Activated Protein Kinase), selective inhibitor of c-Jun N-terminal kinase (JNK), and by CR-mediation, an inhibitor of Phosphoinositol-3 Kinase (PI-3K). Similar results were not obtained when degranulation was investigated using an inhibitor of extracellular signal-regulated kinase (ERK), whereas inhibitors of phospholipase C (PLC) could decrease heterophil degranulation (Kogut et al., 2002; Kogut et al., 2003a).

## EXTRACELLULAR FUNCTIONAL ACTIVITY

Neutrophil Extracellular Traps (NETs) were first reported by Brinkmann and co-workers in 2004 (Brinkmann et al., 2004). This structure was produced by activated neutrophils in response to either biological or chemical stimulators. Since then, there have been many studies that show similar structures in human, rabbit, mice, horse, bovine, feline, chinchilla, and fish neutrophils (Alghamdi and Foster, 2005; Beiter et al., 2006; Brinkmann et al., 2004; Clark et al., 2007; Ermert et al., 2009; Hong et al., 2009; Lippolis et al., 2006; Palić et al., 2007b; Wardini et al., 2009). A growing body of knowledge regarding extracellular traps (ETs) can be found in the PubMed database. NETs are formed by mammalian neutrophils and they contain proteins secreted from azurophilic (primary) granules such as neutrophil elastase (NE) and MPO, and some other cytosolic granules as listed in Table 1.

In the recent review by Papayannopoulos and Zychlinsky (2009), NETs are a novel existing mechanism of extracellular antimicrobial killing (Papayannopoulos and Zychlinsky, 2009). In chicken heterophils, this mechanism is employed in response to potent stimulants (PMA and $H_2O_2$) (Chuammitri et al., 2009). Neutrophils control *Aspergillus* infection

primarily by NETs rather than by phagocytosis and degranulation (Papayannopoulos and Zychlinsky, 2009). Killing bacteria by neutrophils with compromised intracellular mechanisms can be achieved by NET-mediated killing. The phenomenon of phagocytic cell death following NETs formation has been recently named as either NETosis or ETosis (Steinberg and Grinstein, 2007; Wartha et al., 2008).

As mentioned earlier in this chapter, two major intracellular antimicrobial strategies have been described, i.e. phagocytosis and degranulation. Recent discoveries of NETs in mammalian neutrophils (human, rabbit, mouse, bovine, horse, and cat neutrophils as listed in Table 2) have changed our understanding of the neutrophils' extracellular killing ability. Relatively recently, ETs have been discovered in fish neutrophils and chicken heterophils (Chuammitri et al., 2009; Palić et al., 2007a; Palić et al., 2007b). It has become apparent that NET structures initiate killing against both Gram-positive and Gram-negative bacteria. The NETs are also formed in response to other pathogenic organisms, particularly; fungi, and protozoa (Table 3). As the body of knowledge expands, NETs and their products could be used to study some disease conditions, progressions and interventions, such as, preeclampsia, CGD, and autoimmune vasculitis (Bianchi et al., 2009; Gupta et al., 2006; Kessenbrock et al., 2009). We have shown evidence in chickens that heterophils produce extracellular traps (HETs) after stimulation with PMA or $H_2O_2$ in vitro (Chuammitri et al., 2009).

**Structures and Compositions of NETs and Extracellular Traps (ETs)**

The structures of NETs in most species are predominated by nuclear materials, histones and DNA. Wide varieties of cytoplasmic granules, charged peptides, proteins and free radicals are associated with NETs as well. Compositions of effector molecules currently

known to be present in NETs and other extracellular traps are noted in Table 4. Neutrophils

utilize cytoplasmic granules to disrupt or chelate the essential nutrients required by some

pathogens. For example, neutrophils release lactoferrin to chelate iron and to interfere with

its uptake by bacteria (Martinelli et al., 2004). In addition, histones ensnare bacteria and

prevent invasion and multiplication using positively charged ions (Hirsch, 1958).

Apart from the generation of microbicidal action of ROS, neutrophils employ a variety of

non-oxidative factors to combat invading pathogens as effector molecules. These factors

disrupt the microbial cell wall and membrane or inhibit the growth of microbes. Those

effector molecules contain highly charged peptides and proteins such as defensins,

cathelicidins (e.g. LL-37), MPO, Bactericidal/Permeability Increasing Protein (BPI), and

cationic serine proteases known as serocidins such as NE, cathepsin G (CTSG), proteinase 3

(PR3), and azurocidin (AZU). Histones are able to promote bacterial lysis but the exact

microbicidal mechanism has not been clearly understood.

**Processes of NET Formation**

NETs contain proteins from all granular compartments, histones and DNA (Brinkmann et

al., 2004; Lögters et al., 2009a; Papayannopoulos and Zychlinsky, 2009). Upon activation by

potent neutrophil stimulants listed in Table 2, such as IL-8 and PMA, neutrophils release

NETs to the surrounding area. The receptors on neutrophils and other phagocytes (FcγR,

TLR, or CR on neutrophils, FcεR on mast cells, and FcεR on eosinophils) can be involved in

the initiation of NET generation (Brinkmann and Zychlinsky, 2007; von Kockritz-Blickwede

et al., 2008; Yousefi et al., 2008). The binding with receptors activates protein kinase C

(PKC), which initiates a signal transduction cascade that induces the assembly and activation

of the NADPH oxidase complex (Brinkmann and Zychlinsky, 2007; Fuchs et al., 2007).

CGD patients or NOX deficient mice have a disruption or absence of NET release (Bianchi

et al., 2009; Ermert et al., 2009; Fuchs et al., 2007; Yousefi et al., 2009). Sufficient evidence

supports NET formation by ROS. The conversion of $O_2^-$ to $H_2O_2$ inside the phagosome

induces NET because $H_2O_2$ can penetrate membranes and has relatively long half-life

(Brinkmann and Zychlinsky, 2007; Fuchs et al., 2007). Treating neutrophils isolated from

CGD patients with Glucose Oxidase (GO) could stimulate downstream of ROS pathways,

resulting in the production of exogenous-$H_2O_2$ and generation of NET (Brinkmann and

Zychlinsky, 2007; Fuchs et al., 2007; Yousefi et al., 2009).

**Blocking, Inhibition and Degradation of NET Formation**

Many pathogenic bacteria and fungi have DNases bound to their cell wall that are capable

of NET degradation (Brinkmann and Zychlinsky, 2007). The NET structural backbone is

composed of chromatin (histones and DNA) which is inevitably destroyed by DNases. Many

pathogenic bacteria possess DNases or similar enzymes (Beiter et al., 2006; Buchanan et al.,

2006; Sumby et al., 2005; Walker et al., 2007; Wartha et al., 2007). The granular protease

activity on NETs can be blocked by the action of secretory leukocyte proteinase inhibitor

(SLPI) (Brinkmann et al., 2004). In the presence of cytochalasin D, an inhibitor of actin

polymerization, NETs persisted but phagocytosis ceased. NET is also dismantled with

DNase, which results in the negligible killing of bacteria. Application of the potent oxidase

inhibitor, diphenylene iodonium (DPI), at the minimal concentration not only blocked the

oxidative burst, but it also disrupted the formation of NETs (Brinkmann and Zychlinsky,

2007; Fuchs et al., 2007). Exogenous catalase, which degrades $H_2O_2$ to oxygen and water,

completely blocked NET formation, whereas an inhibitor of catalase, 3-amino-1,2,4-triazole (AT), increased NET formation (Table 2) (Fuchs et al., 2007).

**Novel Cell Death Program**

Live neutrophils actively release NETs, initiating cell death (Fuchs et al., 2007). This form of cell death was distinctly different from apoptosis or necrosis, and was termed "NETosis" in a recent review by (Steinberg and Grinstein, 2007).The term "ETosis" is later used to describe the similar process since extracellular traps can be formed by other cells (Wartha et al., 2008), such as heterophil extracellular traps (HETs) in chicken heterophil; mast cell extracellular traps (MCETs); and eosinophil-derived extracellular traps (Chuammitri et al., 2009; von Kockritz-Blickwede et al., 2008; Yousefi et al., 2008). Nuclear morphology from TEM and SEM microscopy revealed that cells undergoing death after NET generation were morphologically dissimilar to apoptotic or necrotic nuclei caused by infections, chemicals or stimuli. In contrast to apoptotic or necrotic nuclei, activated neutrophils making NET successively dissolve their nuclear alignment (membranes) and the cytoplasmic granules, therefore, permitting the mixture of nuclear materials and granule components to be submerged inside the cytosol and finally combine them to generate NET components (Fuchs et al., 2007).

**Proposed Functions of NETs as Modulators of Infection, Inflammation, and Pathological Conditions**

NETs may functionally modulate the inflammatory response by versatile means (Papayannopoulos and Zychlinsky, 2009). NETs may serve as a physical containment that prevents further spreading of bacteria, fungi, mycobacteria and protozoa (Brinkmann et al.,

2004; Guimarães-Costa et al., 2009; Papayannopoulos and Zychlinsky, 2009; Ramos-Kichik et al., 2009; Urban et al., 2006). From a resource perspective, it is plausible that cationic AMPs in NETs trap negatively charged microbes and restrain them by charge-mediated mechanism (Wartha et al., 2008). Furthermore, long stretches of netting may also capture and sequestrate noxious proteases from granules that could potentially be inducing the damage to adjacent sites of infection or inflammation (Brinkmann et al., 2004). In accordance with antimicrobial properties, NETs may be essential for effectively increasing local concentration by minimizing diffusion and facilitating synergy among antimicrobial agents on NETs (Brinkmann and Zychlinsky, 2007; Papayannopoulos and Zychlinsky, 2009). On a down side, NETs might also have a detrimental effect on host tissues due to excessive accumulation of cell free DNA (cf-DNA) upon degradation of NETs by DNases. This phenomenon could be harmful if cf-DNA is deposited at small capillaries of lungs or kidneys plays a role in the development of autoimmune disease and other clinical diseases, such as lupus erythematosus, small-vessel vasculitis and preeclampsia (Brinkmann et al., 2004; Gupta et al., 2006; Kessenbrock et al., 2009; Lögters et al., 2009b). Some NETs are not sufficiently degraded by DNAses within circulation and thus may occlude capillaries, impair microcirculation, cause enzymatic damage of tissues, and promote inflammation (Lögters et al., 2009a). Evidence from the interaction between platelets and neutrophils is also emphasized in the pathophysiology of organ failures by accumulated proteases that are injured during the formation of NETs in severe sepsis that resulted from the impairment of microcirculation of liver sinusoids and liver damage (Clark et al., 2007; Lögters et al., 2009a).

# GENUS *SALMONELLA*

The genus *Salmonella* is a member of the family Enterobacteriaceae. The *Salmonellae* are Gram-negative bacilli that cause enteric (typhoid) fever and gastroenteritis in humans and animals (Scherer and Miller, 2001). Traditional identification of *Salmonellae* based on the Kauffman-White scheme has identified over 2,463 different *Salmonella* serovars. *Salmonella* serovars are categorized based on three major antigenic determinants: the flagellar (*H*) antigen, the somatic (*O*) antigen, and the capsular (*Vi*) antigen (Libby et al., 2004; Scherer and Miller, 2001). Currently, two species of *Salmonella, Salmonella enterica* and *Salmonella bongori* can be separated and identified systematically *Salmonella enterica* and *Salmonella bongori*. Using 16S rRNA sequence analysis separated and identified systematically. The latter species lacks SPI-2, which is crucial for systemic infection (Libby et al., 2004). *Salmonella enterica* subspecies I differ from *S. bongori* (formerly *S. enterica* subspecies V) and *S. enterica* subspecies II, IIIa, IIIb, IV, VI and VII serovars in their ability to circulate within populations of warm-blooded animals (Wray and Wray, 2000). The species *S. enterica* is further divided into six subspecies namely; *enterica, salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* (Libby et al., 2004). The nomenclatures of *Salmonella* have been established by Kaufmann and White Kauffman in 1920s and 1930s and new serotypes are listed in annual updates of the Kauffmann-White scheme. *Salmonella enterica* serovar Typhimurium is used in place of the first citation, and then *Salmonella* Typhimurium is used in the subsequent references.

Agglutination of specific O antigens is used to group *Salmonellae* into 6 serogroups: A, B, C1, C2, D, and E respectively (Libby et al., 2004; Scherer and Miller, 2001). The O-antigen of *S. enterica* serovar Enteritidis and serovar Gallinarum consists of the O12-antigen

and the O9-antigen, the latter is the immunodominant determinant (Luk and Lindberg, 1991).

For epidemiological purposes, phage typing has been developed to differentiate *Salmonella*

isolates from outbreaks, and has been applied especially for *Salmonella enterica* serovar

Typhimurium (ST) and *Salmonella enterica* serovar Enteritidis (SE) isolates (Libby et al.,

2004; Scherer and Miller, 2001). *Salmonella* can be host-adapted, and cause severe systemic

disease in animal species. The vast majority of serovars of *Salmonella enterica* show no host

adaptation. *Salmonella* such as, ST and SE are frequently isolated from a wide variety of

vertebrates. These two serovars may be considered the least host adapted serovars (Libby et

al., 2004). Serovars that are included in the host-adapted category are; *Salmonella* Typhi and

*S.* Paratyphi (human), *S.* Gallinarum and *S.* Pullorum ( chickens)*, S.* Dublin (cattle), *S.*

Choleraesuis (pigs) and *S.* Typhimurium and *S.* Enteritidis in mice, human and poultry

(Libby et al., 2004; Scherer and Miller, 2001). *S.* Enteritidis is a zoonotic pathogen that

persists in the chicken cecum or ovaries without triggering clinical signs in the host (Libby et

al., 2004).

### *Salmonella* **Pathogenicity Island (SPI)**, *Salmonella* **Virulence Plasmid (***Spv***), and Virulence Factors**

Molecular phylogenetic study of *Salmonella* infections has shown that approximately

20% of the *Salmonella* genome consists of genetic material (bacterial plasmids or other

mobile genetic elements) from plasmid-mediated horizontal transfer (Wray and Wray, 2000).

Based chiefly on ST genome, several SPIs and *Spv* have been identified by its virulence

factors. There are more than five SPIs and numerous smaller pathogenicity "islets" in the

genome sequence of ST (Finlay and Brumell, 2000; McClelland et al., 2001) as well as for *S.*

Enteritidis phage type 4, SE PT4 (Thomson et al., 2008). The SPIs, as obtained by horizontal gene transfer (HGT), can be defined by the GC contents of these islands, which are much lower than those of the remaining chromosomal DNA sequence. SPI-1 and SPI-2 are major virulence factors of *Salmonella* (Dieye et al., 2009). **SPI-1** encodes a type III secretory system (TTSS), which is required for the uptake of *Salmonella* by intestinal epithelial cells. Invasion factor (*InvF*) regulates the expression of secreted SPI-1 effector proteins, *AvrA, SipABCD, SopE, SopE2*, *SopB*, and *SopD* that encode within SPI-1.Effector proteins are translocated into the host cytoplasm through the TTSS (Libby et al., 2004). Genes encoded on SPI-1 allow *Salmonella* serovars to trigger the process of endocytosis called "macropinocytosis", that is accompanied by membrane ruffling (Wray and Wray, 2000). **SPI-2** is essential for encoding and producing proteins that are required for intramacrophage or host phagocyte survival.  For example, *SpiC* is required for preventing fusion of phagosome and lysosome. SPI-2 may also interfere with the movement of the NADPH oxidase to *Salmonella* -containing vacuoles (SCV) thereby preventing phagocyte- dependent oxidative killing (Finlay and Brumell, 2000; Libby et al., 2004). **SPI-3** and/or **SPI-4** encode the *PhoP/PhoQ* that regulates $Mg^{+2}$ and $Ca^{+2}$ transportation required for both intracellular growth and survival (Libby et al., 2004; Scherer and Miller, 2001). **SPI-5** is required for enteric but not systemic Salmonellosis. It encodes *SopB* required for fluid secretion and neutrophil recruitment.  However, the *SopB* protein is translocated by the SPI-1. *Salmonella* possess *Salmonella* Virulence Plasmid or *spv* that encodes genes (*spvR* and *spvABCD*) that affect bacterial growth during the systemic phase of infection inside macrophages. The plasmid encodes two fimbrial operons, plasmid encoded fimbriae (*pef*) and long polar fimbriae (*lpf*), for adherence to Peyer's patches (PP) and intestinal villi. For resistance to

complement factors and for cell invasion, the *rck* gene is also encoded within the virulence

plasmid, but is found only in *S.* Typhimurium  (Scherer and Miller, 2001). *RpoS*, encoded

within *spv*, is required for growth during stationary phase and the acid tolerance response.

*Salmonella* could potentially induce diarrhea by secreting cholera-like toxins, putative

*Salmonella* enterotoxin ( *Stn*), and toxin of unknown compositions (Libby et al., 2004;

Scherer and Miller, 2001; Wray and Wray, 2000). *Salmonella* colonize, interfere, and induce

fluid secretion causing diarrhea by their virulence factors.

## *Salmonella* Enteropathogenicity

### Localized Phase of Infection

After ingestion of *Salmonella*-contaminated food or water, SE survived the low gastric

pH (pH = 3) in a study with mice (Libby et al., 2004; Wray and Wray, 2000). Two types of

acid-tolerance systems have been described: one that is activated on exposure to acid or

during the growth (log) phase via *fur* gene and another gene (*RpoS*) that is developed in the

stationary phase (Libby et al., 2004). The M cells located in gut-associated lymphoid tissue

(GALT) of intestinal wall are by far the preferred niche that *Salmonella* utilize for attaching

and entering the host cells. M cells may facilitate the uptake of invading *Salmonella* because

of the absence of goblet cells, polymeric immunoglobulin receptors (pIgR), and low

concentrations of secretory IgA (sIgA). They also have a thin adhesive matrix (glycocalyx)

and irregular brush border that is easily attached to by fimbriae of *Salmonella* (Libby et al.,

2004). After uptake, *Salmonella* induce the formation of membrane protrusions called

"membrane ruffling" by rearrangement of cell cytoskeleton (Brumell et al., 1999). Disruption

of cellular frameworks leads to cell death, sloughing of epithelium, and opening of new

opportunities for *Salmonella* to invade the submucosa. The increased production of IL-8 (chemoattractant) from ruptured cells causes the migration of neutrophils and macrophages to the infected site.

### Systemic Phase of Infection

Many genes encoded within SPI-2 are induced following invasion of host cells and play a vital role in replicating bacteria within the SCV of phagocytes (Finlay and Brumell, 2000). *Salmonella* can be detected in reticuloendothelial organs (RES), such as spleen and liver, within 1 day post infection (p.i.)(Dunlap et al., 1992; Richter-Dahlfors et al., 1997). *Salmonella* survive in the macrophage and upregulate at least 35 proteins, including one of the stress response proteins, *GroEL* (Wray and Wray, 2000). A plasmid-encoded protein, *Rck,* interrupts the host complement system (Wray and Wray, 2000). Essentially, the *Salmonella* virulence plasmid (*spv*) and SPI-2 are required for systemic infection in mice. The *spv* genes increase the rate of intracellular replication (Wray and Wray, 2000). SPI-2 encodes a TTSS required for survival within SCV in macrophages for prevention of P/L fusion. SPI-2 is present in all lineages commonly associated with extraintestinal or systemic infection (Wray and Wray, 2000).

### *Salmonella* and Immunity

A key feature that distinguishes pathogenic from nonpathogenic micro-organisms is their ability to overcome innate immune defenses (Murphy et al., 2008). PMNs, macrophages, and DCs are involved in *Salmonella* infections at the sites of invasion of the intestinal tracts. Besides macrophages that serve as means of dissemination, neutrophils can be the mobile elements for systemic infection because Salmonella can survive intracellularly and can

multiply within phagolysosomes (Dunlap et al., 1992; Libby et al., 2004). Shortly after oral exposure, *Salmonella* can be cleared by peristalsis, villous brush border movements, and competitive exclusion by secretory IgA (Libby et al., 2004). The development of adaptive immunity either by the generation of systemic antibodies (IgM or IgG) or mucosal antibodies (sIgA) from infection is considered to be particularly important, since these antibodies can bind to the bacteria and prevent their uptake by M cells and epithelial cells (Libby et al., 2004; Scherer and Miller, 2001). Innate immune cells are absolutely necessary in controlling *Salmonella.* Macrophages produce IL-12 to activate NK cells to release IFN-γ, which in turn upregulates inducible nitric oxide synthase (iNOS) in macrophages, subsequently it leads to the production of nitric oxide that can kill ingested bacteria (Libby et al., 2004). Cationic antimicrobial peptides (CAMPs), as a component of innate immunity, are contained in neutrophil granules, phagosomes, mucosal surface, and integument. These peptides can interact, in part, with negative charges of *Salmonella* LPS, thus leading to disruption of bacterial cells (Scherer and Miller, 2001).

**Chicken Salmonellosis**

Salmonellosis is zoonotic and causes economic damage in the chicken industry due to its high mortality and morbidity. Outbreaks of SE in humans have been linked to the consumption of contaminated meat and eggs. Persistently infected and carrier chickens are a food safety threat. *Salmonella* could cause detrimental effects on egg production. Chicken heterophils play a key role in protecting chickens from the development of systemic disease following SE infections by confining the bacteria to the intestine. Chicken heterophils are the first line of defense against *Salmonella* infections. The pathogenesis of chicken

Salmonellosis is different in some aspects from that of human and mice. In chickens, SPI-1 and SPI-2 genes are the two most important PIs required for the colonization of the intestinal tract, liver, and spleen (Rychlik et al., 2009). The deletion mutant of SPI-1 was the only SPI that induced significantly less heterophil infiltration to the site of infection. Zhang-Barber *et al.* (1999) showed that the clearance of ST in challenged chickens was mainly due to specific antibodies. They also showed that using components from defective mutants of ST as a vaccine has potential to prevent chickens from Salmonellosis (Zhang-Barber et al., 1999).

Among *Salmonella enterica* serovars that infect chickens, serovar Enteritidis has shown the highest invasiveness of gut lamina propria tissue (Berndt et al., 2007). SE can colonize the intestinal tracts and invade the livers, spleens, ovaries, and oviducts of inoculated hens (Gast et al., 2007; Keller et al., 1995). A relatively small incidence of egg contamination has been a common feature of most experimental infection studies, even when hens received large oral doses of SE, and the animals were still at the risk of illnesses (Gast et al., 2007). The deposition of SE within the eggs (both yolk and albumen) seems to result from the colonization of different regions of reproductive tracts in laying hens. Warm temperatures may account for *Salmonella* penetration to reach the yolk contents in infected hens (Gast et al., 2007).

The ability of SE to colonize organs showed no difference among hen strains (Lindell et al., 1994). Results from the same study showed that SE fecal shedding and the production of SE-contaminated eggs were significantly different among chicken strains by 7-14 days post-inoculation. A long-term SE contamination of environment derives from the fecal shedding from carrier chickens. This is a common way of horizontal transmission among the flock,

which affects other non-infected chickens as well as the safety of workers in the poultry house (Lindell et al., 1994).

## CHICKEN GENETICS AND DISEASE RESISTANCE

### General Information of Chickens

The genetic information of chicken species exist at two fundamental levels; genetic variation between individuals within a population, and genetic differences between populations (Muir and Aggrey, 2003). The selection for faster growth rate in broiler chicken has adversely affected immune competence against viral and bacterial diseases in those chickens (Zekarias et al., 2002). In most cases, chicken strains with strong genetic resistances are poor producers (Zekarias et al., 2002). In order to achieve the optimal condition in both production and health, chickens of divergent strains with known disease resistance have been crossbred. However, this attempt showed undesirable outcomes in maintenance and development of disease resistant genes under non-challenged environments (Zekarias et al., 2002).

### Chicken Genome

Chickens have been evolutionarily separated from mammalian genomes for approximately 310 million years (Hillier et al., 2004). According to International Chicken Genome Sequencing Consortium of the chicken genome project, a draft genome sequence of the red jungle fowl, *Gallus gallus* is estimated to have 20,000–23,000 genes in its one billion base pairs of sequence (Hillier et al., 2004). Genetic variations with polymorphism in the chicken genome map contain approximately 2.8 million single-nucleotide polymorphisms (SNPs) (Wong et al., 2004). DNA from females of inbred lines was used to minimize

heterozygosity and provide sequence for both the Z and W sex chromosomes. The chicken karyotype (2n = 78) is made up of 38 autosomes and one pair of sex chromosomes, with the female as the heterogametic sex (ZW female, ZZ male). Within the chicken genome there are substantial reductions in interspersed repeat content, pseudogenes, segmental duplications, and intrachromosomal rearrangements (such as inversions) (Wong et al., 2004).

**Chicken Breeds and Fundamental Basis of Chicken Genetics**

Inbred chickens have been utilized for research by many investigators (Abasht et al., 2009a; Abasht et al., 2009b; Cheeseman et al., 2007; Cheeseman et al., 2004; Cheeseman et al., 2008; Malek et al., 2004; Redmond et al., 2009; Zhou and Lamont, 1999). The intensity and duration of genetic selection can result in the loss of genetic variation (loss of alleles) (Muir and Aggrey, 2003). Studies of inbred and outbred chickens revealed that layers in general had less overall genetic variations than broilers. In addition, between-line variation is higher than within-line variation, which indicated that maintaining more lines generate more total diversity (Muir and Aggrey, 2003). Another strategy proposed to conserve the alleles is through the development of a series of highly inbred chicken stocks, which could be maintained in small numbers.

**Common Breeds of Chickens**

Meat producing chickens, broilers, have been intensively selected for faster growth, efficient feed conversion, and high white-meat yield in order to meet consumer demands. Broilers are crossbreds from a three-way or four-way cross of specific closed pure breeding lines (Muir and Aggrey, 2003). These birds are grown in large-scale production farms.  In order to satisfy both producers and consumers, broilers have been highly selected in both

production efficiency and disease resistance which will be an ongoing process. Outbred broilers have been studied for their disease resistance and antibody level after vaccinations against *S.* Enteritidis and *E.coli* (Cheeseman et al., 2007; Huff et al., 2006; Kaiser et al., 2002; Kaiser et al., 1998; Redmond et al., 2009).

In layers, the egg production trait is continued as the ultimate goal under selection. However, the emphasis has been shifting lately toward persistency of laying eggs rather than peak production (Muir and Aggrey, 2003). It has been reviewed in Muir and Aggrey (2003) that an average number of eggs per hen per year was 309 as of 1998 (Muir and Aggrey, 2003). Among commercial layer strains, white Leghorns are by far the most popular layer chicken in large-scale commercial egg industry, as well as, in biological research. Leghorns, especially ones that are highly inbred, have been used in disease resistance and MHC-restricted immune response studies (Bacon et al., 2000; Zhou and Lamont, 1999; Zhou and Lamont, 2003). Highly inbred leghorns are also selected and studied for their resistance to SE (Abasht et al., 2009b; Cheeseman et al., 2008; Kaiser et al., 1998; Malek et al., 2004; Redmond et al., 2009).

In the 1954, Fayoumi chicken eggs were brought from Egypt to the United States for poultry genetics programs. Because of their traits, they are highly disease resistant. This breed has been manipulated in order to isolate good genes for biotechnology techniques by Iowa State University poultry geneticists. Originally from Fayoumi breed, the M5.1 and M15.2 lines are highly inbred with an inbreeding coefficient of 0.99. They share an identical genetic background and differ only in the chromosome bearing MHC genes. Furthermore, they are genetically distant from either broiler or Leghorn lines (Zhou and Lamont, 1999). Many studies have confirmed that the Egyptian birds are much more resistant to some viral

diseases, such as avian leukosis, and coccidiosis (Kim et al., 2009; Kim et al., 2008). Fayoumis are under ongoing investigation for their resistance to SE (Abasht et al., 2009a; Redmond et al., 2009).

**Genetic Implications in Disease Resistance**

In studies of the immune system, inbred strains are often used to limit the potential effects of genetic variation. In particular, several inbred lines that differ in disease susceptibility were established in order to identify genes by linkage analysis (Abasht et al., 2009b; Bacon et al., 2000; Muir and Aggrey, 2003; Zhou and Lamont, 1999; Zhou et al., 2001). Genetic resistance to diseases is a multigenic trait governed mainly by the immune system and its interactions with many physiologic and environmental factors (Zekarias et al., 2002). Genetic resistance to pathogens in chickens has been identified, most notably in the functional diversity of MHC, especially B locus that is involved exclusively in antigen presentation (Lamont, 2000; Muir and Aggrey, 2003).  Moreover, individual variation in the immune response is sometimes linked to TCR, immunoglobulins, cytokines and certain proteins (Zekarias et al., 2002).

**General Features of Chicken Immune System**

Chickens lack equivalent structures of lymph nodes, the primary site for antigen presentation in mammals, but they have lymphoid nodules along the lymphatics (Cheng and Lamont, 2008; Kaiser et al., 2005). Chickens have primary lymphoid organs and secondary lymphoid organs. The thymus and the bursa of Fabricius are two examples of PLO discovered in chickens. Examples of SLO include spleen, bone marrow, conjunctival-associated (CALT), bronchial-associated (BALT), and gut-associated lymphoid tissues

(GALT). The bursa of Fabricius is the site where the immunoglobulin repertoire is developed by a process of gene conversion. In chickens, a significant numbers of circulating γδ T cells are also present (Kaiser et al., 2005).

### Chicken Innate Immunity

Birds have well-developed innate defense mechanisms. The functions of cells in chicken innate immunity are to detect, fight, and destroy invading pathogens immediately after they enter the protective barrier of the body. It is now clear that this immunity initiates a cascade of events, including recruitment of various immune components necessary for interplay between innate and adaptive responses (Juul-Madsen et al., 2008; Medzhitov and Janeway, 2000). Pathogens possess "Pathogen-Associated Molecular Patterns or PAMPs". PAMPs are often shared by large groups of microorganisms, for example, LPS in Gram negative bacteria; lipoteichoic acid in Gram positive bacteria; and Zymosan in fungi (Medzhitov and Janeway, 2000). Cells of innate immunity could recognize the vast majority of target pathogens via germ line DNA-encoded elements named "Pattern Recognition Receptors or PRRs". PRRs are present in two distinct forms; soluble (LPS-binding protein, alternative complement pathway components), and cell-associated components including Toll-like receptors (TLRs), C-type lectins and various intracellular receptors (Juul-Madsen et al., 2008; Medzhitov and Janeway, 2000; Zekarias et al., 2002). Heterophils depend on the expression of various PRRs to detect the presence of pathogens. Recently, many chicken TLRs have been identified including chTLR 1/6/10-like, 2, 3, 4, 5, 7/8, and 9, and some of which are implicated in *S.* Enteritidis infection (Abasht et al., 2009b; Juul-Madsen et al., 2008; Kogut et al., 2005).

The non-cellular innate components include ciliary movement, skin fatty acids, peristaltic movement, the gastric pH, and AMPs. In chickens, many AMPs have been identified; chicken cathelicidins or fowlicidins (Goitsuka et al., 2007; van Dijk et al., 2009; Xiao et al., 2006) and β-defensins or gallinacins appear to exist only in chickens (Hasenstein et al., 2006; Juul-Madsen et al., 2008). Both avian fowlicidins and gallinacins may have a very crucial role in avian heterophils for killing SE because these cells lack oxidative mechanisms (Brune et al., 1972; Hasenstein et al., 2006). The cellular part of the chicken innate immunity includes cells of myeloid origin such as granulocytes (heterophils, eosinophils and basophils), macrophages, dendritic cells, and thrombocytes as well as NK cells (Juul-Madsen et al., 2008; Zekarias et al., 2002).

### Chicken Adaptive Immunity

The most important cells in chicken adaptive immunity are T cells, B cells, and macrophages as antigen presenting cells (APCs) (Cheng and Lamont, 2008). T cells, the principal cells of cell-mediated immunity (CMI), recognize foreign antigens by T cell receptors (TCRs) after the antigens have been processed and presented by APCs. As in mammals, avian T cells have two types of surface receptors that bind antigens: $\alpha\beta$ TCR and $\gamma\delta$ TCR. Chickens have a higher proportion of' $\gamma\delta$ TCR T cells than mice or humans and may reach 30-50% of circulating lymphocytes, but $\alpha\beta$ TCR-expressing T cells remain as the main type of effector cells in the chicken (Cheng and Lamont, 2008; Muir and Aggrey, 2003; Zekarias et al., 2002). Recent studies suggest that $\gamma\delta$ T cells recognize protein antigens directly without antigen processing (Muir and Aggrey, 2003). The auto-reactive T cells in chickens might have originated from this group of cells. The avian T cell repertoire is

generated exclusively through rearrangement of multiple TCR genes. Sub populations of T cells express three different lineages of T cell receptors: TCR1 ($\gamma\delta$ T cells), TCR2 ($\alpha\beta$ T cells expressing a family of V$\beta$1 genes) and TCR3 ($\alpha\beta$ T cells expressing a second family of V$\beta$2 genes) (Muir and Aggrey, 2003). Two important functional subsets of T cells, CD4^{+} and CD8^{+} T cells can be differentiated by accessory molecules (Cheng and Lamont, 2008; Muir and Aggrey, 2003; Zekarias et al., 2002).

The bursa of Fabricius is not only the site for B cell differentiation, but also an organ designed to permit gene conversion to occur (Muir and Aggrey, 2003; Zekarias et al., 2002). Progenitor B cells colonize at the bursa at 10-day embryonic age (E10) and undergo proliferation and differentiation into functional cells. In the chicken, immunoglobulin (Ig) diversity is created by a transfer of sequence from pseudo V genes to the single rearranged functional V gene (Zekarias et al., 2002). Between day 18 of embryogenesis (E18) and the 6th week of age, chicken B cells undergo the gene conversions within the bursa leading to Ig diversification. Immunoglobulins in chickens have only three main classes; IgM, IgG, and IgA (Muir and Aggrey, 2003). Because chicken IgG is larger than its mammalian counterpart, the chicken IgG is often called IgY (Cheng and Lamont, 2008).

**Genetic Resistance to *Salmonella***

### Chicken Genetic and Selections for *Salmonella* Resistance Traits

Many specific genes are associated with genetic control of response to *Salmonella* species (Cheng and Lamont, 2008). Specific genes (e.g., the MHC) are known to play a role in disease resistance including *Salmonella* (Lamont, 1998). In a recent report, several natural resistance-associated macrophage proteins (NRAMP1)-linked markers were identified to be

associated with splenic bacterial burden in *Salmonella* Enteritidis (Cheng and Lamont, 2008; Lamont, 1998). Patterns of resistance across inbred poultry lines were similar for different Salmonella serovars (Gast et al., 2007; Muir and Aggrey, 2003). It is believed that resistant birds are in some ways able to control early replication of the *Salmonella* within the phagocytic environment, until a secondary response develops.

**Significance of Inbred Chicken Lines in Molecular Genetics Research**

Congenic lines with 99% genetic identity (almost all the loci are homozygous) have proved to be useful for studying genes or groups of genes. Several genes were selected as candidates for immune response or transcription activation in Leghorn and Fayoumi origin (Zhou et al., 2001). Inbred and outbred chickens have been studied in great detail for their disease resistance. Using highly inbred chicken lines, MHC congenic Leghorn and Fayoumi, or advance intercross lines from broiler sire crosses with dams from Leghorn or Fayoumi, or outbred broilers in challenged experiments the genetic lines have proved to have a strong effect on *Salmonella* resistance (Cheeseman et al., 2007; Cheeseman et al., 2004; Chiang et al., 2008; Redmond et al., 2009). Differentially expressed genes were detected in the comparisons between the lines when they were infected with SE (Chiang et al., 2008).

There have been numerous chicken gene expression studies of certain cytokines, chemokines, or effector molecules that participate in response to SE infection. Genes that participate in the control and response to SE include *MHC Class I, MHC Class II, TRAIL, Caspase-1 (CASP1), IAP-1, Prosaposin, SAL1, NRAMP1 (SLC11A1), TNC, TGFβ2, TGFβ3, TGFβ4, GM-CSF, IgL, INOS, CD3, CD8,CD28, TLR2,TLR4, TLR5, MD-2, IL-6, IL-8(CXCLi2), IL-10, IL-18,IFN-γ,VIL1, CXCLi1(K60)* and *Gallinacins*. (Abasht et al., 2009a;

Cheeseman et al., 2004; Cheeseman et al., 2008; Cheng and Lamont, 2008; Hasenstein et al., 2006; Kogut et al., 2003b; Lamont et al., 2002; Malek et al., 2004; Malek and Lamont, 2003; Redmond et al., 2009; Swaggerty et al., 2004). Polymorphisms in some of these genes were correlated to bacterial loads in the cecum or spleen and vaccine antibody response. The effect of line on resistance to other pathogens, for example coccidiosis, was also evident in the studies from chicks challenged with *Eimeria tenella*. The Fayoumi line appeared clearly the most resistant line, while Leghorn line was the most susceptible (Pinard-Van Der Laan et al., 1998).

**The Chicken Major Histocompatibility Complex (MHC)**

The chicken MHC was originally described as blood group (B) system. The MHC molecules (class I, class II and class IV or B-F, B-L and B-G, respectively) belong to immunoglobulin superfamily (Cheng and Lamont, 2008; Muir and Aggrey, 2003; Zekarias et al., 2002). Antibody production against a variety of antigens (total serum IgG level) is associated with the antigen processing and presentation of the chicken MHC. Congenic chicken lines are laboratory generated by mating two inbred strains, and backcrossed with the descendants with one of the original strains (Lamont, 1998).

<p align="center">**IMMUNOMODULATORS**</p>

There are many studies and publications which used various kinds of immunomodulators to enhance the function of the mammalian neutrophils and chicken heterophils. The selected immunomodulators for my thesis study, β-glucan, ascorbic acid and corticosteroids, are reviewed as follows:

**β-glucan as an Immunomodulator**

Beta (β)-1,3 glucans are potent immunomodulators derived from the cell walls of yeast, fungi, and bacteria (Adams et al., 1997). β-glucans have a long life in vertebrate systems, which lack either the appropriate glucanases to degrade these molecules through oxidation in the liver or excretion through glomerular filtration (Brown and Gordon, 2005). β-1,3 glucans possess anti-inflammatory and anti-tumorigenic properties (Brown et al., 2003). One of immunomodulatory effects of β-glucans is to enhance clearance of apoptotic cells (Brown and Gordon, 2005). Particulate β-glucans with large molecular weight can activate leukocytes directly. Their effects include the stimulation of phagocytic, cytotoxic and microbicidal activities of PMNs and phagocytes. These molecules can stimulate the production of reactive oxygen intermediates (ROI), pro-inflammatory cytokines, and chemokines, such as interleukin (IL)-1β, IL-6, IL-8 and TNF-α (Brown and Gordon, 2005). Soluble β-glucans with lower molecular weight (< 5,000–10,000 daltons) do not generally stimulate leukocytes directly and they might act as β-glucan receptor antagonists (Brown and Gordon, 2005). In some instances, they induced cytokines, such as IL-6 and IL-8, and nuclear transcription factors, such as NF-κB and NF-IL-6 (Adams et al., 1997; Brown and Gordon, 2005).

The β-glucan receptor has been identified on both immune and non-immune cells, including monocytes, macrophages, neutrophils, eosinophils, Langerhans cells, NK cells, endothelial cells, alveolar epithelial cells and fibroblasts (Brown and Gordon, 2005; Herre et al., 2004; Taylor et al., 2002). A number of cellular receptors implicated in these activities comprise of CR3, also known as CD11b/CD18 or Mac-1, lactosylceramide, scavenger

receptors and Dectin-1 (Brown and Gordon, 2005; Brown, 2006; Brown et al., 2003; Herre et al., 2004; Taylor et al., 2002). Dectin-1 is composed of a carbohydrate-like binding domain, a stalk region, and a cytoplasmic tail containing ITAM-like motif (Brown and Gordon, 2005). Dectin-1 receptor was originally believed to be a dendritic cell specific receptor but it is currently known to be expressed on monocyte/macrophage and neutrophil lineages to mediate the recognition and immune response to β-glucans (Brown and Gordon, 2005; Taylor et al., 2002). More recently, Nerren and Kogut (2009) suggested that chickens possess a Dectin-1-like β-glucan receptor on heterophils as the primary PRR for β-glucan (Nerren and Kogut, 2009). The primary signaling of this receptor is independent of the TLRs (Brown and Gordon, 2005).

Binding of its receptors on neutrophils and macrophages, β-1,3 glucan can enhance phagocytosis, endocytosis and subsequent oxidative burst by activating the alternative complement pathway and stimulating inflammatory mediators via the lipoxygenase pathway (Ainsworth, 1994). It can also induce the production of pro-inflammatory cytokines and chemokines in collaboration with the TLRs (Brown and Gordon, 2005). Preliminary data suggested that particulate β-glucans and zymosan initiated signal transduction through protein kinase C (PKC), phospholipase $A_2$ ($PLA_2$), protein tyrosine kinase (PTK), MAPK, and pertussis toxin pathways (Adams et al., 1997). Other downstream components of the TLR signaling require MyD88 and NF-κB. Immunomodulatory mechanisms of β–glucans, such as zymosan and soluble glucans, might involve both activation and inhibition pathways (Brown, 2006).

**Relevant Studies with β-glucans**

The experimental studies of β-glucan and its immunomodulatory effects have been studied both *in vivo*, and *in vitro* using isolated neutrophils, heterophils, and cell lines. Studies with β-glucan in particulate and soluble forms of zymosan showed that the β-glucan component of zymosan was actually responsible for zymosan phagocytosis in channel catfish (*Ictalurus punctatus*) (Ainsworth, 1994). This led to the assumption that channel catfish neutrophils possessed Mac-l-like receptor (CR3) that facilitated phagocytosis of zymosan. In another study of sea bass (*Dicentrarchus labrax*) alternative complement pathway and lysozyme activity significantly enhanced under dietary β-glucan supplementation (Bagni et al., 2000). In murine monocytic cell line study (Adams et al., 1997), soluble β-glucan (PGG-glucan) activated NF-κB-like and NF-IL-6-like transcription factors thus enhanced leukocyte microbicidal activities without inducing inflammatory cytokines. Mouse neutrophils/ macrophages CR3 (Mac-1) acted as a potential receptor for β-glucans as seen in humans (Xia et al., 1999).

Brown et al (2003) demonstrated for the first time that the response of zymosan and live fungi required the recognition by Dectin-1, a specific receptor for fungal pathogens, in addition to the TLRs in mice peritoneal macrophages (Brown et al., 2003). The study by Engstad et al. (2002) showed increase in neutrophil degranulation in human leukocytes by β-glucan. Soluble β-glucan also helped priming blood leukocytes to secrete TNF-α and IL-6 when leukocytes were stimulated with LPS (Engstad et al., 2002). Another human study showed that soluble β-glucan (PGG) enhanced myeloid and megakaryocyte progenitor cell

proliferation, oxidative burst response, in addition to the activation of NF-κB-like nuclear factor (Wakshull et al., 1999).

Many studies were conducted using chickens to determine the actions of β-glucans aiming for improving chicken immunity and production. Study by Lowry et al. (2005) reported that β-glucan supplement in diet significantly enhanced phagocytosis, bacterial killing and oxidative burst of heterophils from day-old chicks.  In this study increased protection against SE organ invasion was also observed (Lowry et al., 2005). *In vitro* treatment of abdominal macrophages with 0.1% β-1,3–1,6-glucan significantly increased phagocytic and killing of *Salmonella* Enteritidis.  The 14-day *in vivo* treatment of β-glucan was sufficient to eliminate the SE from spleen and liver (Chen et al., 2008). Recently, Dectin-1-like β-glucan receptor was reported in chicken heterophils. This receptor facilitated the generation of ROS following the stimulation with Dectin-1 specific agonist (Nerren and Kogut, 2009). Addition of β-glucan also inhibited organ colonization by *Salmonella Typhimurium* in broiler chicks (Revolledo et al., 2009). Decrease in respiratory infection by *Escherichia coli* was also experimentally demonstrated in broiler chicks fed with β-glucan (Huff et al., 2006).

## Ascorbic Acid as an Immunomodulator

Ascorbic acid is synthesized in the liver (mammals and birds) or in the kidneys (amphibians, reptiles and birds) using glucose molecules by converting them to L-gulono-γ-Iactone and then to ascorbic acid (AA) (Padh, 1991). Like most terrestrial animals, domestic fowl adequately synthesize daily requirements of AA from the liver or kidney (Pardue and Thaxton, 1986). The tissue levels of AA in poultry vary drastically depending on species,

breed, sex, age, tissue source, environmental, nutritional and pathological conditions as collated by Pardue and Thaxton (1986). Mahmoud et al. (2003) reported that domestic chickens (*Gallus gallus domesticus*) can synthesize AA to meet their physiological needs, thus it is not recommended to supplement to poultry diets (Mahmoud et al., 2003). The lowest tissue concentrations of AA (4-6.5 mg/100 g) have been found in pulmonary and muscle tissues. The mean concentration of AA in the blood of all chicken breeds and sexes is 14 μg/ml. The adrenal glands contain a higher concentration of AA (178 mg/100 g). Other tissues, including the spleen, liver, intestines, and testes contain AA at concentrations several times greater than the concentration in plasma (Pardue and Thaxton, 1986). Physiologically, mammalian neutrophils consume considerable amounts of oxygen to generate $H_2O_2$ during oxygen dependent killing of ingested pathogens. As reviewed by Erickson (2000), AA could improve neutrophil motility and antimicrobial properties in patients with chronic granulomatous disease and bronchial asthma (Erickson et al., 2000).

### Roles of Ascorbic Acid in Immunity

Ascorbic acid has many functional properties, which include; biochemical function to provide electrons to keep metal ions in their reduced forms, biosynthesis of procollagen and collagen for the formation of extracellular matrix, and antioxidant and free radical scavenger to protect cells from oxidative damages (Padh, 1991). Ascorbic acid has been reported to decrease superoxide production by neutrophils. Moreover, AA inhibits the activation of the oxidant-sensitive transcription factor NF-κB, which mediates the production of proinflammatory cytokines such as IL-1 and TNF-α (Conner and Grisham, 1996; Erickson et

al., 2000). The theoretical role of AA to prevent autooxidation of chicken heterophil granules at the site of infection was also suggested (Andreasen and Frank, 1999).

One of the desirable functions of AA is to protect tissue deterioration from harmful oxidants generated by activated phagocytes. Tissue damages could be preventable by a process called "ascorbate recycling" (Wang et al., 1997). Ascorbate recycling, which is induced by gram-positive, gram-negative bacteria, and even pathogenic fungi, *Candida albicans*, occurs when extracellular ascorbate is oxidized, transported as dehydroascorbic acid (DHA), and reduced intracellularly to ascorbate (Wang et al., 1997). AA in human neutrophils is largely localized in cytosol and is not bound to proteins (Washko et al., 1989). Increases in AA accumulation could be explained by extracellular oxidation of AA to DHA, preferential uptake of DHA, and intracellular reduction back to AA (Washko et al., 1993). Ascorbate recycling does not occur in bacteria or fungi so it may represent a specific eukaryotic defense mechanism against detrimental oxidants. AA has been shown to protect against bacterial and viral diseases and to reduce the impact of detrimental stress in chickens (Andreasen and Frank, 1999; Gross, 1992). In humans, AA has been documented to upregulate neutrophil chemotaxis, phagocytosis, and bacterial killing. AA failed to increase phagocytosis, but significantly increased killing of *Staphylococcus aureus* by chicken heterophils. The addition of either AA or DHA to a neutrophil suspension caused a dramatic increase in the generation of ROS (DeChatelet et al., 1972). This phenomenon could be an alternative method to produce $H_2O_2$ without MPO and might explain antimicrobial activity of heterophils under MPO deprivation.

### Ascorbic Acid and Stress

Ascorbic acid is involved in the synthesis of cortisol hormones and has strong association with the depletion of adrenal AA with increased secretion of adrenocortical hormones (Pardue and Thaxton, 1986). The AA depletion is established as an indicator of increased cortisol release in mammals. A Similar event has been proposed as a classical stress response in chickens (Siegel, 1971). It was suggested by Gross (1992) that the anti-stress effects of AA occur in the adrenals (Gross, 1992). Maurice et al. (2007) reported that AA synthesis was not influenced by corticosterone-induced stress in chickens, but the alteration of tissue AA concentrations was caused by dietary corticosterone (Maurice et al., 2007). Dietary supplementation with AA increased chicken tolerance to heat stress and decreased heat associated mortality as well as improved growth rates and production parameters (Lohakare et al., 2005; Pardue et al., 1985). As reviewed by Pardue and Thaxton (1986), AA addition to the diet increased chicken resistance to coccidiosis, *Salmonella gallinarum* (fowl typhoid), Newcastle virus, and *Pasteurella multocida*.

### Relevant Studies with Ascorbic Acid

Many studies have been conducted in order to determine the effects of ascorbic acid in humans, animals, and phagocytes for many decades. The following research contributed as background for this dissertation research. AA improved innate immunity by increasing bactericidal and antiviral activities in broiler chicken heterophils (Andreasen and Frank, 1999; Gross, 1992; Lohakare et al., 2005). Ascorbic acid also alleviated the loss of chickens from heat strees (Aengwanich et al., 2003; Kutlu and Forbes, 1993; Mahmoud et al., 2003; Mahmoud et al., 2004; McKee et al., 1997; Pardue et al., 1985) and enhanced synthesis of

AA during the stress depending on the level of corticosteroids which was affected by a gender of chickens (Maurice and Lightsey, 2007).

**Corticosterone as an Immunomodulator**

### General Information of Corticosteroids

By definition, "stress" is the integration of an animal's defense mechanisms and a stimulus (stressor) in any circumstance (Siegel, 1971). Under the stress condition, there is an increase in corticosteroids (Siegel, 1995). In most vertebrates, 80-90% of circulating corticosteroids are specifically bound to globlulins (Rhen and Cidlowski, 2005; Siegel, 1995). The vast majority of unbound corticosteroids (biologically active form) can effectively pass through target tissues, bind to respective cytoplasmic receptors, and exert their actions (Rhen and Cidlowski, 2005; Siegel, 1995).

Glucocorticoid receptors have been found in virtually every nucleated cell type in the body (Munck et al., 1984). In chickens, the study of the expression of glucocorticoid receptors (GR) is very limited. Relatively recently, Kwok *et al*. (2007) have revealed that chicken GR (cGR) encodes amino acids that share 73% sequence homology to that of human and mouse (Kwok et al., 2007). Two isoforms of human GRs, hGR$\alpha$ and hGR$\beta$, are the products of alternative splicing of exon 9 of *gr* gene. GR$\beta$ is unable to bind GCs due to the difference at the carboxy terminus from GR$\alpha$ (Bamberger et al., 1995). Like humans, chicken *gr* genes have 9 exons and they are widely expressed in various tissues, such as pituitary, muscle, ovary and kidney. Studies have not shown the evidence of chicken GR $\beta$-isoform (Kwok et al., 2007).

Glucocorticoids (GCs) are extremely lipophilic so that they primarily enter cell cytoplasm by passive diffusion (Leung and Bloom, 2003). The GR is localized predominantly in cytoplasm.  Without binding with ligand, GR is anchored to a heteromeric complex of heat shock proteins (hsp), which includes a dimer of hsp 90 molecule (Bamberger et al., 1995; Leung and Bloom, 2003). Following the entry into cytosol, GCs subsequently bind to GRs and lead to a conformational change in the receptor by dissociation from the hsp 90 complex (Newton, 2000).  Free GRs then translocate to the nucleus where they bind to DNA sequences known as glucocorticoid response elements (GREs), which regulate transcription of target genes.

The ratio of GR$\alpha$ and GR$\beta$ (approximately 600:1) in a cell affects the cell's sensitivity to glucocorticoid, with higher levels of GR$\beta$ leading to glucocorticoid resistance (Rhen and Cidlowski, 2005). GC resistance is a common finding and can attribute to decreased expression of GR$\alpha$, increased expression of GR$\beta$, or phosphorylation of GRs. It is hypothesized that hGR$\beta$ competes with hGR$\alpha$ for GR binding sites since it has been shown that hGR$\beta$ can also bind to GREs (Rhen and Cidlowski, 2005).

**Molecular Features of Anti-Inflammatory Effects of Corticosteroids**

GRs interact directly with the p65 subunit of NF-$\kappa$B and inhibit the NF-$\kappa$B/AP-1 (*Jun* and *Fos*; Activator Protein 1) interaction, binding of DNA, and represses transcriptional activity of target genes (Leung and Bloom, 2003). NF-$\kappa$B is associated with the induction of multiple genes in immune responses. Inhibition of this transcription factor limits the expression of a broad spectrum of genes encoding cytokines, chemokines, adhesion molecules, effector proteins, such as IL-1$\beta$, IL-6, IL-11, IL-13, IL-16, GM-CSF, TNF-$\alpha$,

matrix metalloproteinase 9 (MMP9), CXCL8 (IL-8), CCL5 (RANTES), MCP-1, eotaxin, ICAM-1, VCAM-1, iNOS, etc. (Leung and Bloom, 2003; Rhen and Cidlowski, 2005). IκB plays a vital role in controlling NF-κB translocation to the nucleus since the activities of NF-κB in the nucleus are tightly regulated by this protein (Choi et al., 2003; Leung and Bloom, 2003; Rhen and Cidlowski, 2005). GRs also inhibit histone acetylation through direct inhibition of CREB binding protein (CBP)-associated histone acetyltransferase (HAT) activity and through the recruitment of the co-repressor for histone deacetylases (HDAC) to the NF-κB activation complex. It is also proposed that the increased expression of IκBα by GCs could inhibit translocation of NF-κB to the nucleus (Leung and Bloom, 2003).

**Immunosuppressive and Immunomodulatory Effects of Corticosteroids**

In order to understand the anti-inflammatory effects of GCs, the following mechanisms have been addressed. GCs inhibit transcription of cyclooxygenase 2 (Rhen and Cidlowski, 2005). Lipocortin synthesis, a specific inhibitor of $PLA_2$ and regulator in the production of prostaglandins, leukotrienes, and platelet activating factor (PAF), was increased by the action of GCs (Leung and Bloom, 2003; Newton, 2000). Expression of genes that are currently known for resolution of inflammation, such as the SLPI, annexin 1 increases by GCs. SLPI might block NF-κB activation in addition to its anti-inflammatory effects on neutrophils (Leung and Bloom, 2003; Rhen and Cidlowski, 2005). Phosphorylation of IκB leads to its ubiquitination and degradation by the proteasome, unmasking a nuclear localization of NF-κB. Apoptosis of granulocytes at sites of infection is recognized and internalized by macrophages. Another possible mechanism of anti-inflammatory function of GCs is to promote phagocytosis of PMNs which are undergoing apoptosis. After binding with GRs

GCs may re-program monocyte differentiation towards an anti-inflammatory phenotype (Heasman et al., 2003). NF-κB is involved in promoting and delaying PMN apoptosis. Evidence suggested the action of GCs which target NF-κB in neutrophils to promote apoptosis facilitate the resolution of inflammation (Choi et al., 2003). It is also likely that GCs engage NF-κB of neutrophils in synthesis of "survival proteins or anti-apoptosis" (Heasman et al., 2003).

**The Effect of Corticosterone on Chickens**

The effects of acute and chronic corticosterone can be observed by changes in physiological condition and productivity of chickens. The corticosterone-induced stress in the chicken farms might occur due to densely populated housing conditions and feed restriction. These factors cause reduced weight gain and egg production as well as abnormal behavior such as feather pecking and cannibalism in chickens (El-lethey et al., 2001).

The dietary corticosterone supplementation increased the serum corticosterone level and heterophil to lymphocyte (H/L) ration due to increased circulating heterophils. The dietary corticosterone reduced heterophil diapedesis to tissue because of the decreased expression of adhesion molecules on the surface of both heterophils (ICAM-1) and the endothelial cells (E and P-selectin) (Puvadolpirod and Thaxton, 2000; Shini et al., 2009). The H/L ratio has been used as a sensitive indicator of stress response in chicken as it increases following an increase in plasma corticosterone levels (Post et al., 2003a). Other blood biochemical parameters, such as concentration of corticosterone, glucose, cholesterol, and nitrate, are also accepted as indicators of adaptation to a stressor (Thaxton et al., 2006). Corticosterone also causes the changes in heterophil size, shape, granularity and lymphocyte cytoplasmic

characteristics as visualized by electron microscopy. The corticosterone also affects the release of immature heterophils from bone marrow to a peripheral blood circulation and stimulates lymphocyte redistribution to blood circulation, which results in increased H/L ratio (Singh et al., 2009). Shini et al. (2008) found changes in heterophil morphology, granulation, longevity, H/L ratios and immune response after exposure to dietary corticosteroids (Shini et al., 2008).

Corticosterone was also reported to elevate blood glucose, cholesterol, and triglyceride (TG) in chickens (Puvadolpirod and Thaxton, 2000; Singh et al., 2009). The increase in cholesterol and TG may result in increased liver weight and hepatic lipid due to the increased gluconeogenesis by corticosterone, which leads to increased weight gain in broiler chickens (Puvadolpirod and Thaxton, 2000). The effect of corticosterone supplementation in egg production has been reported to delay and decrease egg production in mature hens. The increased level of corticosterone in yolk and albumin may also indicate the adaptation of hens to environmental stress (Singh et al., 2009).

The immunosuppressive effects of corticosterone on some of lymphoid organs in chickens were demonstrated by the decrease in relative weight of spleen, thymus and bursa of Fabricius in experiment chickens (Puvadolpirod and Thaxton, 2000). It was also reported that rapidly elevated concentration of corticosterone in bloodstream caused the reduction in antibodies against sheep red blood cells in chickens and increase in heterophil counts (Post et al., 2003b). Administration of corticosterone in chicken diet showed higher H/L ratios and serum corticosterone level but reduced body weight and egg production. Regarding the immune response, dietary corticosterone reduced the antibody response to tetanus toxoid, sheep RBC, inflammatory responses to *Mycobacterium* antigen and phytohemagglutinin

(PHA). These results suggested that corticosterone impaired both humoral and cell-mediated immunity in chickens (El-Lethey et al., 2003). Al-Muranni et al. (2002) conducted studies in order to use H/L ratio as a criterion for *Salmonella* resistance and found that the H/L ratio, antibody titer, phagocytic activity of colloidal carbon, and plasma corticosterone concentration were significantly higher in *S*. Typhimurium resistant chickens (Al-Murrani et al., 2002).

**Summary**

Heterophils are short-lived cells that have the pivotal roles in chicken innate immunity. Host defense mechanisms are performed through a variety of killing mechanisms, both intracellularly (phagocytosis, intraphagosomal degranulation, and oxidative burst) and extracellularly (HETs). As with mammalian neutrophil counterparts, proteolytic granules are securely stored in vesicles within their cytoplasm until heterophils are activated. Heterophils constitutively express various types of PRRs, for example, FcR and TLRs, for immediate detection of microbes and initiate the immune response. The biological (microbes) and/or physicochemical (oxidants and chemical) stimulation can directly or indirectly induce both intracellular and extracellular killing mechanisms from these cells.

Food-borne disease caused by chicken salmonellosis (SE) provokes economic damage to the chicken industry and public health. Persistent infection and the shedding of SE by chickens could be minimized by improving the disease resistance. This mission can be achieved by genetic selection or by dietary supplementation. Chicken lines of either meat- or layer types with known genetic resistance to *Salmonella* infection would be the first priority to be preferentially selected and developed to meet high meat yield and disease resistance.

After genetic screening, supplementation of biological (e.g. β-glucans from fungal cell walls) or synthetic products (e.g. ascorbic acid and corticosterone), as promising immunomodulatory effects, can be evaluated *in vitro* (cellular function) and *in vivo* (bacterial clearance) to determine any beneficial outcomes to SE resistance. This kind of research will be of significance because what is learned can contribute to broader understanding of heterophil function as well as SE resistance.

**Table 1.** Cytoplasmic granule contents in neutrophils and chicken heterophils.

| Species | Cytoplasmic Granules | References |
|---|---|---|
| **Chicken heterophil** | α-Glucosidases | Maxwell and Robertson, 1998 |
| | β-Glucuronidase | Harmon, 1998 |
| | Acid phosphatase | |
| | Acid trimetaphosphatase | |
| | Alkaline phosphatase | |
| | Arylsulphatase | |
| | Cathepsin | |
| | Elastase | |
| | Glucose-glycogen glucosyltransferase | |
| | Lysozyme | |
| | Phosphorylase | |
| | Uridine diphosphate | Evans et al., 1995 |
| | Chicken Heterophil Peptides 1 and 2 (CHP1, 2) | Brockus et al., 1998 |
| | Gallinacin (avian β-Defensin) | Harmon, 1998 |
| | | Hasenstein et al., 2006 |
| | | Hahn-Dantona et al., 2000 |
| | Matrix metalloproteinase 9 (MMP-9) | |
| | or Gelatinase B-like | Xiao et al., 2006 |
| | Fowlicidin-1, 3 (Chicken Cathelicidin) | Goitsuka et al., 2007 |
| **Human neutrophil** | **Azurophil (primary) granules:** | Segal, 2005 |
| | Myeloperoxidase (MPO) | Faurschou and Borregaard, 2003 |
| | Cathepsin G | |
| | Elastase | |
| | Proteinase 3 | |
| | Bactericidal/Permeability Increasing Protein (BPI) | |
| | α, β Defensins | |
| | **Specific (Secondary) granules:** | |
| | Lactoferrin | |
| | Ttranscobalamin II | |
| | Neutrophil gelatinase-associated lipocalin | |
| | Flavocytochrome $b_{558}$ | |
| | **Gelatinase (Tertiary) Granules:** | |
| | Gelatinase | |
| | Lysosomes: | |
| | Acid hydrolases | |
| | Elastase (ELA2) | Urban et al., 2009 |
| | Defensin 1, 3 | |
| | Lactotransferrin (LTF) | |
| | Azurocidin (AZU1) | |
| | Cathepsin G (CTSG) | |
| | Myeloperoxidase (MPO) | |
| | Proteinase 3 (PR3) | |
| | Lysozyme C (LYZ) | |

**Table 2.** Summary of known potent stimulators, inhibitors or DNA degradators of NETs.

| Hosts | Stimulators | Inhibitors/Degradators | References |
|---|---|---|---|
| **Human neutrophil** | Antineutrophil Cytoplasm Autoantibodies (ANCAs) Complement factor 5a (C5a) f-Met-Leu-Phe (f-MLP) GM-CSF Glucose Oxidase (GO) IL-1β IL-8 Ionomycine LPS Platelet Activation Factor (PAF) PMA Syncytiotrophoblast microparticles (STBM) TLR2, 5, 7/8 agonist TNF-α Zymosan | Amino Triazole (AT) Catalase Cytochalasin D Diphenylene Iodonium (DPI) DNase FBS Group A Streptococcus *sda1* *Pneumococcal* Endonuclease | Bartneck et al., 2009 Beiter et al., 2006 Bianchi et al., 2009 Brinkmann et al., 2004 Buchanan et al., 2006 Clark et al., 2007 Fuchs et al., 2007 Guimarães-Costa et al., 2009 Gupta et al., 2005 Jaillon et al., 2007 Kessenbrock et al., 2009 Munafo et al., 2009 Ramos-Kichik et al., 2009 Urban et al., 2009 Urban et al., 2006 Wang et al., 2009 Wartha et al., 2007 Yost et al., 2009 Yousefi et al., 2009 |
| **Murine neutrophil** | PMA | Cytochalasin D DNase | Ermert et al., 2009 Jann et al., 2009 Urban et al., 2009 |
| **Bovine neutrophil** | Ionomycin PMA | β-hydroxybutyrate Cytochalasin D DNase | Behrendt et al., 2010 Grinberg et al., 2008 Lippolis et al., 2006 |
| **Equine neutrophil** | Spermatozoa *Escherichia coli* | Seminal plasma DNase | Alghamdi and Foster, 2005 |
| **Feline neutrophil** | *Leishmania* promastigotes | Restriction enzymes (*Eco*R1 and *Hin*dIII) | Wardini et al., 2009 |
| **Fish neutrophil** | β-glucan Calcium Ionophore (CaI) LPS PMA | Cytochalasin B | Palić et al., 2007a Palić et al., 2007b |
| **Mast cells (BM-derived) Murine BMMCs Human MC line** | GO PMA | Catalase Cytochalasin D DNase DPI GO | von Kockritz-Blickwede et al., 2008 |
| **Human eosinophil** | C5a Eotaxin IL-5 LPS | Cytochalasin D DNase | Yousefi et al., 2008 |
| **Chicken heterophil** | H₂O₂ PMA | | Chuammitri et al., 2009 |

**Table 3.** Summary of pathogenic bacteria, fungi, and protozoa known to activate NETs and ETs.

| Organisms | References |
|---|---|
| **Bacteria** | |
| *Aeromonas salmonicida* | Alghamdi and Foster, 2005 |
| *Enterococcus faecalis* | Beiter et al., 2006 |
| *Escherichia coli* | Brinkmann et al., 2004 |
| *Listeria monocytogenes* | Buchanan et al., 2006 |
| *Mycobacterium tuberculosis* | Clark et al., 2007 |
| *Salmonella* Typhimurium | Ermert et al., 2009 |
| *Shigella flexneri* | Fuchs et al., 2007 |
| *Staphylococcus aureus* | Grinberg et al., 2008 |
| *Streptococcus dysgalactiae* | Gupta et al., 2006 |
| *Streptococcus pneumoniae* | Gupta et al., 2005 |
| *Streptococcus pyogenes* | Jaillon et al., 2007 |
| | Katzenback and Belosevic, 2009 |
| | Lippolis et al., 2006 |
| | Munafo et al., 2009 |
| | Ramos-Kichik et al., 2009 |
| | von Kockritz-Blickwede et al., 2008 |
| | Wartha et al., 2007 |
| | Yost et al., 2009 |
| **Fungi** | |
| *Aspergillus fumigatus* | Bianchi et al., 2009 |
| *Aspergillus nidulans* | Ermert et al., 2009 |
| *Candida albicans* | Jaillon et al., 2007 |
| *Cryptococcus neoformans* | Urban et al., 2006 |
| | Urban et al., 2009 |
| **Protozoa** | |
| *Leishmania amazonensis* | Baker et al., 2008 |
| *Plasmodium falciparum* | Behrendt et al., 2010 |
| *Eimeria bovis* | Guimarães-Costa et al., 2009 |
| | Wardini et al., 2009 |

**Table 4.** Co-localized effector molecules on NETs or ETs.

| Structure/Granules | References |
| --- | --- |
| *Nuclear Materials* | Beiter et al., 2006 |
| DNA | Brinkmann et al., 2004 |
| Histones (H1, H2A, H2B, H3, H4) | Chuammitri et al., 2009 |
| | Ermert et al., 2009 |
| *Primary Granules* | Fuchs et al., 2007 |
| Neutrophil elastase (NE) | Guimarães-Costa et al., 2009 |
| Myeloperoxidase (MPO) | Gupta et al., 2006 |
| Proteinase 3 (PR3) | Gupta et al., 2005 |
| Bacterial Permeability Increasing Protein (BPI) | Jaillon et al., 2007 |
| Cathepsin G | Kessenbrock et al., 2009 |
| | Munafo et al., 2009 |
| *Specific Granules* | Palić et al., 2007a |
| Gelatinase | Palić et al., 2007b |
| Lactoferin | Ramos-Kichik et al., 2009 |
| | Urban et al., 2006 |
| *Miscellaneous* | Urban et al., 2009 |
| p22$^{phox}$ | von Kockritz-Blickwede et al., 2008 |
| p67$^{phox}$ | Wang et al., 2009 |
| CD11b | Wardini et al., 2009 |
| Calprotectin | Wartha et al., 2007 |
| Tryptase | Yousefi et al., 2009 |
| Mitochondria | |
| Peptidylarginine deiminase 4 (PAD4) | |
| LL37 (AMP) | |
| Pentraxin 3 ( PTX3) | |

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# CHAPTER 3. CHICKEN HETEROPHIL EXTRACELLULAR TRAPS (HETS): NOVEL DEFENSE MECHANISM OF CHICKEN HETEROPHILS

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## Abstract

Recent findings in mammals and fish have revealed that neutrophil nuclear material associated with cytoplasmic granular content is released in the form of neutrophil extracellular traps (NETs) that can trap and kill invading microorganisms *in vitro* and *in vivo*. To determine if a similar mechanism is present in chicken heterophils, hydrogen peroxide ($H_2O_2$) and phorbol myristate acetate (PMA) were used for stimulation of blood-derived heterophils. Stimulated heterophils produced structures that were characterized using immunocytochemistry and confocal microscopy as heterophil extracellular traps (HETs). Released HETs contained DNA, histone-DNA complex and elastase from heterophil cytoplasmic granules. HETs released from chicken heterophils are structurally similar to

NETs found in mammalian and fish neutrophils. Extracellular DNA released from heterophils was quantified by Picogreen assay. Stimulation with PMA or $H_2O_2$ significantly increased the HET-DNA release index *in vitro* compared to non-stimulated heterophils ($1.11 \pm 0.04$ and $1.55 \pm 0.10$, respectively), and $H_2O_2$ stimulation induced significantly higher HET-DNA release than PMA ($P < 0.001$). Thus, HETs are now characterized as an important heterophil-mediated defense mechanism in chickens.

## 1. Introduction

Granulocytic phagocytes are an essential part of innate immune defense, with a primary role to attack and kill microorganisms. Granulocytes in different species utilize similar mechanisms (oxidative burst, degranulation, phagocytosis, and neutrophil extracellular trap release) to efficiently fulfill this role. An important interspecies difference between chicken heterophils and mammalian, as well as some fish, neutrophils is the lack of myeloperoxidase (MPO) in heterophil granules (Brune *et al*., 1972; Segal, 2005). Because MPO is crucial in utilizing superoxide product and halide ions to produce hypochloric acid, the lack of this enzyme is likely related to reduced potential of the heterophil to display a strong oxidative burst. The measurable oxidative response in heterophils is minimal when compared to the activity of mammalian neutrophils (Harmon, 1998; Stabler *et al*., 1994). Heterophils and neutrophils also can degranulate and release proteolytic enzymes and various antimicrobial peptides contained in cytoplasmic granules to aid the killing of microorganisms intracellularly or extracellularly (Farnell *et al*., 2003). It appears that chicken heterophils have maintained the ability to phagocytize and kill microorganisms without the activity of

MPO by utilizing an array of specific proteins such as avian beta-defensins and other granular enzymes, elastase and, potentially, the release of heterophil extracellular traps.

Despite observed physiological differences, chicken heterophils, as part of innate immunity, also have physical, biochemical and functional properties similar to polymorphonuclear granulocytes (neutrophils) in mammals (Harmon, 1998). A new mammalian and fish neutrophil killing mechanism has recently been described as Neutrophil Extracellular Traps (NETs) (Brinkmann *et al*., 2004; Fuchs *et al*., 2007; Gupta *et al*., 2006; Lippolis *et al*., 2006; Palić *et al*., 2007a; Palić *et al*., 2007b). NETs are composed of chromatin and are associated with the content of neutrophil cytoplasmic granules (Fuchs *et al*., 2007; Brinkmann *et al*., 2004). Neutrophils can be stimulated to produce NETs *in vitro* by inhibitor of Protein Kinase C, and phorbol myristate acetate (PMA), as well as by pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), lipoteichoic acid (LTA) and β-glucans (Brinkmann *et al*., 2004; Fuchs *et al*., 2007; Wartha *et al*., 2007a; Palić *et al*., 2007b; Jaillon *et al*., 2007; Palić *et al*., 2007a). The NETs consist of nuclear material (histones and DNA), granular enzymes, and lysozyme (Cho *et al*., 2005) as confirmed by immunocytochemistry and specific DNA staining in human (Brinkmann *et al*., 2004; Fuchs *et al*., 2007), mouse (Beiter *et al*., 2006; Wartha *et al*., 2007a; Buchanan *et al*., 2006; Katharina *et al*., 2006), rabbit (Brinkmann *et al*., 2004), equine (Alghamdi and Foster, 2005), bovine (Lippolis *et al*., 2006), fathead minnow *(Pimephales promelas)* (Palić *et al*., 2007b), and zebrafish (*Danio rerio*) neutrophils (Palić *et al*., 2007a). The released NETs can induce extracellular killing of different bacterial pathogens and fungi (Brinkmann *et al*., 2004; Urban *et al*., 2006; Wartha *et al*., 2007b; Beiter *et al*., 2006; Wartha *et al*., 2007a).

Based on functional similarity of heterophils and neutrophils, and presence of NETs in phylogenetically diverse animal species, we hypothesized that the release of DNA associated with granular enzymes is conserved and present in chicken heterophils. Using cytochemistry, confocal microscopy, and extracellular DNA quantification assay, we demonstrated for the first time that chicken heterophils stimulated with phorbol myristate acetate and hydrogen peroxide *in vitro* have the ability to produce structures that are morphologically similar to NETs in mammals and fishes, and suggest that those structures be named Heterophil Extracellular Traps.

## 2. Materials and Methods

### 2.1. Chicken Heterophil Isolation

Whole blood from several healthy adult, male and female Leghorn chickens of the highly inbred Ghs-6 Leghorn line (>95% inbred) (Zhou and Lamont, 1999) was collected, and chicken heterophils were isolated by a slight modification of a previously described technique (Andreasen and Frank, 1999). One volume of chicken blood was gently mixed with one volume of 1% methylcellulose (Sigma-Aldrich, USA), centrifuged (15 x $g$, 15 min), and the plasma and buffy coat layer above the erythrocytes then removed to another tube. The remaining erythrocyte interface was gently washed with Hank's Balanced Salt Solution without calcium & magnesium and phenol red (HBSS, 1X, Cellgro, USA) supplemented with 0.1% fetal bovine serum (FBS, Altanta Biologicals, USA) and the wash added to the plasma layer. The plasma samples were centrifuged (370 x $g$, 10 min), supernatant discarded and the cell pellets resuspended in HBSS w/FBS. Cell suspension was placed on discontinuous density gradient (specific gravity of 1.077 over 1.119 g/ml; Sigma-Aldrich,

USA), centrifuged (500 x $g$, 30 min), 1.077/1.119 interfaces and 1.119 bands were collected, resuspended using HBSS w/FBS, and washed (370 x $g$, 10 min). The cell pellets were resuspended in red blood cell (RBC) lysis buffer (1 g/L Potassium Bicarbonate, $KHCO_3$; Fisher Scientific, USA; and 0.87 g/L Ammonium Chloride, $NH_4Cl$; Sigma-Aldrich, USA; in deionized water) and RBCs lysed for 3 min. After lysing, cells were topped with HBSS and washed (370 x $g$, 5 min). The supernatant was discarded and the cell pellet resuspended with HBSS. Heterophil viability was determined with 0.1% trypan blue solution. Cells were counted by Coulter Particle Counter Model Z1 (Beckman Coulter, USA) and cell suspension adjusted to 5 x $10^6$ cells/ml. The ratio of heterophil to non-heterophil cells was determined using Diff-Quik (Dade Behring, USA) stained cytospin preparations of cell suspensions.

## 2.2. Heterophil Extracellular Trap (HET) release

Circular coverslips (12 mm diameter) (Fisher Scientific, USA) were placed into 24 well tissue culture cluster plates (Corning, USA). Cells were placed on coverslips in the wells ($2.5x10^5$ cells/well), followed by HBSS with calcium and magnesium and without phenol red ($HBSS^+$). $HBSS^+$ (unstimulated control), Phorbol myristate acetate (PMA, Sigma-Aldrich, USA;1 µg/ml), and hydrogen peroxide ($H_2O_2$, Fisher Scientific, USA; 0.15 mM) were added, plates were centrifuged(250 x $g$, 5 min,  4°C), and incubated at 41°C with 5% $CO_2$ for 3 hours (optimal concentration and incubation time was determined in preliminary experiments).

## 2.3. Immunocytochemistry

Plates with control or activated cells were centrifuged (250 x $g$, 5 min), supernatant removed, and cells fixed with 4% paraformaldehyde (Polyscience, USA) for 25 min at room

temperature. After washing with phosphate buffered saline (PBS), Image-iT FX signal enhancer solution (Molecular Probes, USA) was applied for 30 min, cells were washed, incubated for 90 min in blocking solution (2% normal donkey serum; 2% normal goat serum; 1% bovine serum albumin; 0.4% Triton X-100), and double-labeled with a primary antibody cocktail containing rabbit anti-Elastase (Abcam, USA) and mouse anti-DNA/Histone 1 (Chemicon International,USA), antibodies diluted to 1:150, and 1:1500, respectively. Primary antibodies were incubated on slides overnight at room temperature in a sealed humidified container. After incubation, slides were washed, incubated in the secondary antibody cocktail containing goat anti-mouse Cy5 (Molecular Probes, USA) and goat anti-rabbit Cy3 (Jackson Immunoresearch Lab, USA) antibodies diluted to 1:200 and 1:500, respectively. Slides were washed and mounted with ProLong Gold with DAPI (Molecular Probes, USA). Negative controls were carried out in parallel during all processing by omitting the primary antibodies.

## 2.4. Confocal microscopy and light microscopy

Cells and associated structures labeled with DAPI and antibodies were visualized and images captured using a Leica confocal scanning laser microscope (TCS-NT; Leica Microsystems, USA). Heterophils stained with Diff-Quik Stain Set (Dade Behring, USA) according to the company's protocol were examined with a Zeiss Axioplan 2 upright microscope (Carl Zeiss MicroImaging, USA) using bright field and differential interference contrast (DIC). Images were captured with an AxioCam MRc color camera (Carl Zeiss MicroImaging,USA). All figures were prepared using Photoshop (version 10.0, Adobe, USA), Freehand (version 10.0, Macromedia, USA) and Image J (version 1.38, NIH, USA).

## 2.5. Quantification of DNA release from stimulated heterophils

Heterophils ($2.5 \times 10^5$ cells in HBSS) were seeded into 96-well plates and stimulated with 0.1 µg/ml PMA or 1 mM $H_2O_2$ and incubated at 41°C with 5% $CO_2$ for 2 hours (optimal stimulant concentration and incubation time was determined in preliminary experiments). Non-stimulated heterophils were used as controls. HETs generated by stimulated heterophils were digested with 500 mU/ml Micrococcal Nuclease (MNase;Worthington Biochemical, USA) for 20 min at 37°C. The nuclease activity was inactivated with 5 mM EDTA and the supernatants were collected for DNA quantification using Picogreen dsDNA kit (Molecular Probes, USA) according to manufacturer's instructions. Plates were read in fluorescence plate reader (SpectraMAX Gemini XS, Molecular Devices, USA; excitation 492 nm, emission 520 nm; with SOFTMax PRO software 4.0). The HET-DNA release was analyzed using GraphPad Prism version 5.0 (GraphPad Software, USA). The HET-DNA release index was calculated using the following formula:

$$\text{HET-DNA release Index} = DNA_{stim} / DNA_{non\text{-}stim}$$

Where DNA: average amount of DNA content released from heterophils. The data were presented as mean ± standard error of the mean (SEM). The differences in HET-DNA release from PMA or $H_2O_2$ were determined by Student's *t*-test. $P < 0.05$ was considered as statistically significant.


## 3. Results and Discussion

In this report, the presence of heterophil extracellular traps is described for the first time in an avian species. The release of extracellular fibers, consisting of DNA, histones and granular enzymes, appears to be conserved in the innate immune system of phylogenetically

diverse species (fish, birds, and mammals). It was determined that phorbol myristate acetate and hydrogen peroxide induce HETs formation in chicken heterophils, indicating that products of oxidative metabolism can be involved in HET release mechanisms. The structure of HETs was characterized with immunocytochemistry and confocal microscopy (Figs. 1, 2) and DNA release induced by application of PMA or $H_2O_2$ was quantified (Fig. 1G).

### 3.1. Chicken heterophils released structures similar to extracellular traps upon activation

Chicken heterophils released NET-like structures after activation with stimulants (PMA or $H_2O_2$) at 41°C for 3 hours. Extracellular fibers attached to or connecting multiple heterophils were observed with bright field microscopy in stimulated cells, while similar structures were not observed in non-stimulated control cells on Diff-Quik stained slides (Fig. 1, A-C). Stimulation with PMA and $H_2O_2$ induced release of long fibers that were associated with chromatin material, but also projected beyond cell membranes to adjacent cells or surrounding space (Fig. 1, A-C).

The release of HETs from stimulated heterophils was correlated with stimulant concentration and incubation time (data not shown), but not with the type of surface used for attachment of cells. Cell incubation on uncoated glass circular coverslips for 3 hours in 24-well plate format provided optimal conditions for HETs production (optimization data not shown). To further investigate structure and morphology of the observed fibrous structures, immunocytochemistry was performed on stimulated heterophils.
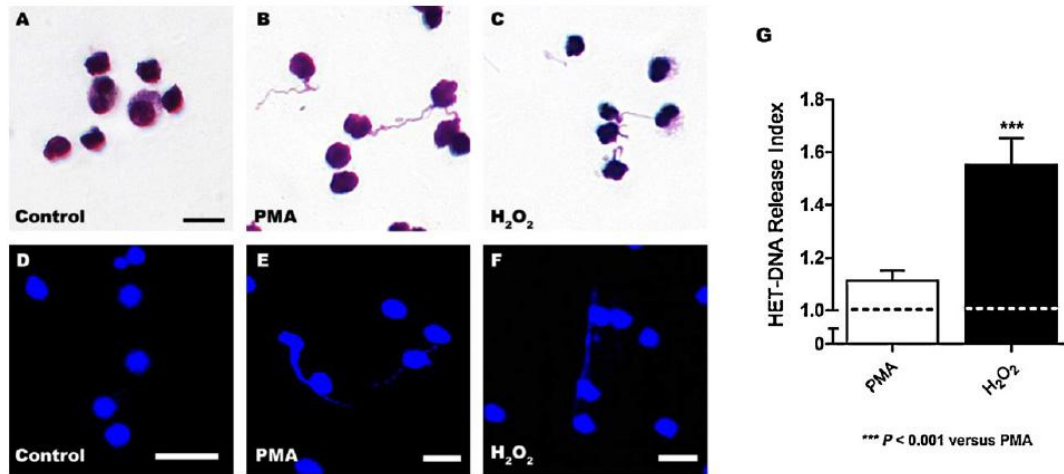
**Fig. 1.** Chicken heterophils release HETs upon stimulation with PMA or Hydrogen peroxide. (A–C) Fibrous structures originating in nuclear region and protruding extracellularly are released by stimulated chicken heterophils and observed using differential interference contrast (DIC) on Diff-Quik stained slides. (D–F) Heterophil cell nuclei and released HETs were labeled with DNA specific stain (DAPI) and co-localization was observed using confocal microscopy. Scale bars: 10 μm. (G) Quantification of extracellular HET-DNA formation after stimulation with PMA or $H_2O_2$ is presented as increase of extracellular DNA release compared to non-stimulated cells (dotted lines). HET-DNA release index in $H_2O_2$ stimulated is significantly higher compared to PMA stimulated heterophils (mean ± S.E.M., $n = 19$, ***$P < 0.001$).

### 3.2. DNA, Histones and Elastase are constituents of HETs

DNA was found to be a major constituent of fibrous structures released by chicken heterophils upon stimulation. Stimulated heterophils and associated structures were stained with DNA-specific binding dyes (DAPI) and the presence of DNA on the extracellular fibers was confirmed by fluorescent confocal microscopy (Fig. 1, D-F). Structures observed in bright field and confirmed with fluorescent confocal microscopy were morphologically

consistent with neutrophil extracellular traps described in mammalian and fish neutrophils and are therefore referred to as Heterophil Extracellular Traps or HETs. Specific DNA staining and immunocytochemistry with anti-DNA/histone H1 and anti-elastase antibodies revealed that DNA, histones and cytoplasmic granule contents (elastase) are present on HETs (Fig. 2). HETs structures observed with confocal microscopy were similar to NETs components as first reported by Brinkmann *et al.* (2004). Chicken HETs structures show co-localized immunoreactivity of DNA-histone H1 complex (red) and elastase (green) (Fig. 2, D and H). The observed co-localization of DNA, histones and granular content suggests that morphology of chicken HETs appears similar to NETs found in human beings, rabbits, fathead minnows and zebrafish (Brinkmann *et al*., 2004; Fuchs et al., 2007; Gupta *et al*., 2005; Palić *et al*., 2007b; Palić *et al*., 2007a).



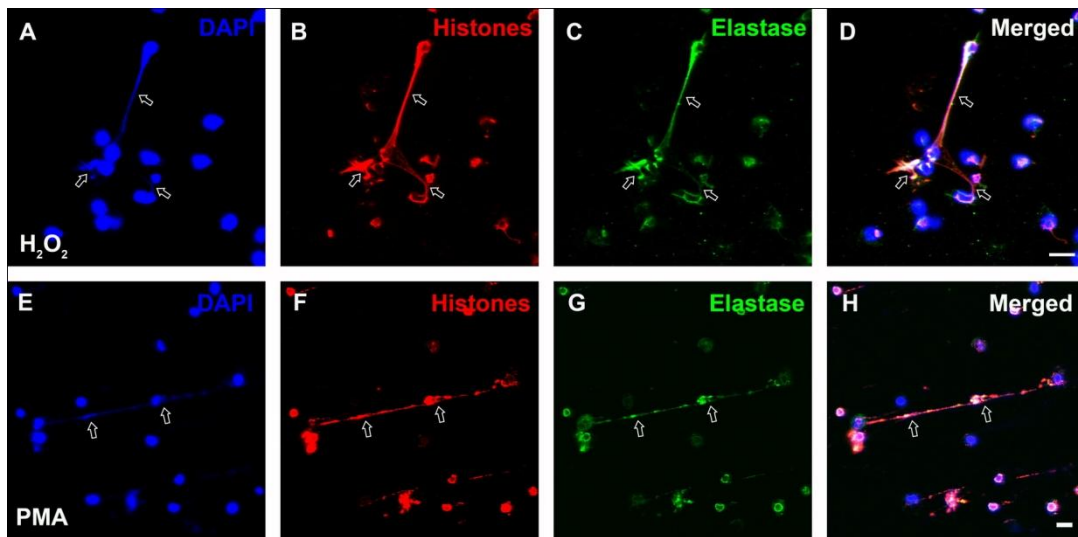**Fig.2.** DNA, histones and elastase co-localize in chicken HETs activated with PMA and $H_2O_2$. Isolated heterophils were activated with 0.15 mM/mL $H_2O_2$ (A–D) or 1 μg/mL PMA (E–H) for 180 min. Co-localization of DAPI-labeled DNA (A and E), DNA-histone H1 (B and F), and elastase (C and G) immunoreactivity was observed on HETs (arrows) using confocal microscopy and is shown in merged images (D and H). Scale bars: 10 μm.

### 3.3. Stimulation with PMA and hydrogen peroxide induced HETs release

Application of phorbol myristate acetate (PMA) or hydrogen peroxide ($H_2O_2$) *in vitro* induced release of HETs in heterophils (Figs. 1, 2). Both PMA and $H_2O_2$ significantly increased the HET-DNA release index *in vitro* compared to non-stimulated heterophils ($1.113 \pm 0.038$ and $1.552 \pm 0.101$, respectively), and $H_2O_2$ stimulation induced significantly higher HET-DNA release than PMA ($n = 19$, $P < 0.001$; Fig 1G).

Generation of oxygen radicals has been associated with NETs release in human neutrophils, and exogenous $H_2O_2$ generated from glucose oxidase can induce NETs release in mammals (Fuchs *et al*., 2007). Furthermore, inhibition of oxygen radical production with catalase and superoxide dismutase (SOD) interfered with release of NETs (Brinkmann and Zychlinsky, 2007). Chicken heterophils stimulated with PMA and exogenous $H_2O_2$ released significantly more HETs than non-stimulated controls, in accordance with reports suggesting that the oxidative burst pathway is related to NETs release in mammalian neutrophils (Brinkmann and Zychlinsky, 2007).

PMA acts on mammalian neutrophils and chicken heterophils as a potent inducer of oxidative burst compared to non-PMA-stimulated cells (Fuchs *et al*., 2007; He *et al*., 2003), which indicates that the mechanism involved in activation of NADPH oxidase and protein kinase C, and subsequent oxidative burst generation, is conserved between the two cell types (He *et al*., 2003). However, chicken heterophils have been shown to have limited capacity for PMA-induced oxidative burst compared to mammalian neutrophils (He *et al*., 2003), and the lack of myeloperoxidase in heterophils indicated the absence of the hydrogen peroxide-halide reduction pathway that exists in mammals (Giambelluca and Gende, 2008; Heiner *et al*., 2003).

Application of $H_2O_2$ enhanced the entry of calcium in neutrophils and changes in nuclear transcription factor activity (Heiner *et al*., 2003). The observed increase in intracellular calcium may be associated with the activation of neutrophil signaling pathways,  leading to an increase in oxidative radical production and potential stimulation of NET release (Heiner *et al*., 2003; Giambelluca and Gende, 2008). Stimulation of avian heterophils produced detectable quantities of $H_2O_2$ or superoxide anion, but failed to achieve the amount generated during oxygen-dependent mechanisms of oxidative burst in mammals (Harmon, 1998).

The HETs release observed in PMA-stimulated chicken heterophils indicates that the oxidative burst dependent mechanism of HETs release may be conserved in birds. Limited $H_2O_2$ production due to PMA stimulation, coupled with lack of MPO-facilitated halide reduction pathway could lead to accumulation of $H_2O_2$ and initiate HET-DNA release. The application of exogenous $H_2O_2$ induced a significant increase in HETs release compared to non-stimulated control and PMA stimulated heterophils, further suggesting a role for hydrogen peroxide in HETs release in chicken, in accordance with observed effects of $H_2O_2$ in mammals (Giambelluca and Gende, 2008; Heiner *et al*., 2003). It is suggested that $H_2O_2$ can be used as potent stimulant of chicken HETs production to facilitate *in vitro* studies of HETs.

Chicken Heterophil Extracellular Traps are described for the first time in the present study, using immunochemistry and confocal microscopy, indicating that HETs are made of DNA-Histone complexes, and are associated with granular enzymes. Quantification of HETs indicated that extracellular DNA was present in the supernatant of $H_2O_2$ and PMA stimulated cells, and that exogenous $H_2O_2$ was more effective in HETs-DNA production. Similarity of HETs to mammalian and fish NETs suggests that further studies of this phenomenon in

chicken heterophils from different genetic lines may increase our understanding of disease resistance or pathology of inflammatory processes observed in modern chicken production.

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# CHAPTER 4. HETEROPHIL FUNCTIONAL RESPONSES TO DIETARY IMMUNOMODULATORS VARY IN GENETICALLY DISTINCT CHICKEN LINES

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## Abstract

The effect of diet supplementation of immunomodulators on *in vitro* chicken heterophil function was investigated using three diverse genetic line of chickens (broiler, Fayoumi, and Leghorn). Dietary supplementation with β-glucan, ascorbic acid, and corticosterone was administered orally to chickens from 8 to 11 weeks of age. Heterophil function was evaluated weekly during supplementation using bacterial killing, phagocytosis, and heterophil extracellular traps (HETs-DNA release) assays. Fayoumis fed with basal diet had significantly higher HETs-DNA release ($P = 0.002$). A significant effect of genetic line (line effect) in chickens fed with immunomodulator supplemented diets was observed in bacterial

killing and HETs-DNA release ($P < 0.001$), and a significant diet effect was observed on bacterial killing ($P < 0.001$) and HETs-DNA release ($P = 0.043$). Dietary supplementation of immunomodulators has shown potential for manipulation of the heterophil function in chickens. These results suggested the important role of genetic background in innate immune responses, and the possibility of increasing disease resistance using selection for specific genetic markers.

## 1. Introduction

An effective immune response against pathogens requires a highly orchestrated combination of good host genetics, availability of essential nutrients, and fully functional cellular defenses. The host defenses have to compete for metabolic resources with other physiological processes such as growth and reproduction. It has been reported that host selection to increase specific immune response parameters caused a reduction of desirable production traits in chickens (Cheng and Lamont, 2008; Lamont, 2003; Lamont et al., 2003). More information about genetic regulation of immune function is necessary to achieve balanced selection outcomes. The Fayoumi line (indigenous type) used in this study has a set of unique alleles missing from the broiler or Leghorn lines (domestic type) (Liu et al., 2003; Zhou and Lamont, 1999), which could contribute to its relative resistance against selected pathogens (Pinard-van der Laan et al., 1998). Comparison of the genetic lines that have been selected for commercial meat or egg production (broiler, Leghorn) with a line that has not undergone commercial selection (Fayoumi) can provide better understanding of the genetic relationships between production traits and immune response.

In chickens, heterophils represent key components of the cellular innate immune defenses and their biology resembles that of mammalian neutrophils (Harmon, 1998). Heterophils are major cell type involved in initial response to various pathogens and irritants (Andreasen et al., 1991; Chansoriya et al., 1993; Fulton et al., 1993; Kogut et al., 1998; Latimer et al., 1990; Latimer et al., 1988; Swaggerty et al., 2005), and play important role in pathogen recognition followed by initiation of cytokine and chemokine production to relay information to other immune cell types (Latimer et al., 1990; Latimer et al., 1988; Swaggerty et al., 2006; Swaggerty et al., 2004). Pattern recognition receptors (PRRs) on the surface of heterophils recognize pathogen-associated molecular patterns (PAMPs) of foreign microorganisms, stimulating phagocytosis and destruction of bacteria (Stabler et al., 1994). Transcription inactivity was earlier considered a consequence of terminal differentiation of granulocytes, including heterophils, but recent studies of gene expression have demonstrated that heterophils actively produce cytokines in response to bacterial stimulation of PRRs (Kogut, 2003; Swaggerty et al., 2006; Swaggerty et al., 2004), and therefore actively participate in the regulation of innate immune responses. Cytokine and chemokine signaling is essential for proper control and initiation of the adaptive immune response and information obtained from dissection of effector cell responses and signaling may be applied in genetic selection for improved resistance to pathogens (Fearon and Locksley, 1996; Lamont, 1998).

Nutritional modulation of immune responses can act in concert with genetic background to further enhance immune function or improve disease resistance (Klasing, 1998). Use of immunomodulators in feed has been shown to improve vaccine efficacy, reduce antibiotic usage, and minimize drug residues in chicken products (Lamont, 1998). The $\beta$-1, 3-1, 6-glucans are immunomodulatory complex polysaccharide components of cell walls found in a

large variety of organisms. Their stimulatory effects on immune responses, including granulocytes and mononuclear phagocytes have been documented (Ainsworth, 1994; Brown and Gordon, 2005; Brown et al., 2003; Chen et al., 2008; Engstad et al., 2002; Lowry et al., 2005; Palić et al., 2007). Use of highly purified particulate β-1,3-1,6-glucans from baker's yeast in diets stimulated cellular and humoral immune responses, and increased disease resistance in a number of species (Ainsworth, 1994; Engstad et al., 2002; Lowry et al., 2005). Soluble β-glucans can up-regulate leukocyte activity, both alone and in concert with LPS (Engstad et al., 2002). Glucan-specific receptors (Dectin-1) are present on phagocytic cell membranes of several species. When β-glucans bind with Dectin-1, they induce potent functional activation, such as an increase in phagocytosis and killing, in mammals, fish, and chickens *in vitro* and *in vivo* (Ainsworth, 1994; Brown and Gordon, 2005; Brown et al., 2003).

It has been established that ascorbic acid (AA) offers the important advantages of being nontoxic, economical and not conferring antibiotic resistance (Andreasen and Frank, 1999). The use of ascorbic acid to improve immune responses and protect against disease has been examined in poultry (Andreasen and Frank, 1999; Gross, 1992; McKee et al., 1997). Heterophil-mediated bacterial killing was found to be increased upon treatment with ascorbic acid. Furthermore, it has been suggested that heterophil function responses are dependent on an adequate concentration of ascorbic acid, since its depletion resulted in reduced phagocytosis and bacterial killing (Andreasen and Frank, 1999). High concentration of ascorbic acid accumulation in the cytosol appears to facilitate reduction of the tissue damage and lesions associated with Newcastle disease virus, *Mycoplasma gallisepticum* and *Escherichia coli* infections (Gross, 1992; Klasing, 1998). A decrease in ascorbic acid has

been associated with stress conditions; furthermore, the administration of ascorbic acid has been associated with a protective effect against cortisol-mediated immunosuppression in chickens (Gross, 1992; Kutlu and Forbes, 1993).

It has been suggested that heterophil functional capability appears to be related to ascorbic acid concentration. Glucocorticoids (GCs) decrease ascorbic acid content of neutrophils and inhibit neutrophil function (Gross, 1992; Kutlu and Forbes, 1993). In poultry, decreased ascorbic acid concentrations have been associated with stress, and administration of ascorbic acid has been associated with a protective effect against stress that leads to corticosterone release (Gross, 1992; Kutlu and Forbes, 1993). The mimicking effects from stressor on heterophil functions from healthy chickens can easily be *in vitro* assayed to evaluate cell competency after dietary corticosterone supplementation. Glucocorticoids are potent immunomodulators and produce potent anti-inflammatory effects by acting on the regulatory regions of glucocorticoid target genes. Activity of the transcription factor NF-κB on respiratory burst activity, proinflammatory cytokines, and immunoregulatory cytokines is affected by the action of GCs (Leung and Bloom, 2003; Luengo-Blanco et al., 2008; Weber et al., 2006). The corticosterone administration in chickens increased heterophil to lymphocyte (H/L) ratios (Shini et al., 2008a), and corticosterone supplementation changed heterophil morphology including size, shape, and granulation (Shini et al., 2008b). In regards to granulopoiesis, release of immature heterophils from bone marrow into peripheral blood circulation was observed when birds were exposed to corticosterone treatments (Shini et al., 2008b). The limited information about interactions of ascorbic acid, β-glucan, or corticosterone with genetically determined innate immune responses prompted their use as dietary immunomodulators in this study.

Heterophil function appears to be sex associated and genetically controlled (Swaggerty et al., 2003), and therefore it may be possible to select for enhancement of heterophil function using a variety of heterophil function assays (Andreasen and Frank, 1999; Chuammitri et al., 2009; Farnell et al., 2003; Kogut, 2003; Stabler et al., 1994; Swaggerty et al., 2003). The primary objective of this study was to evaluate heterophil function in genetically distinct chicken lines with and without dietary immunomodulator supplementation. Observed differences in heterophil responses in Fayoumi, broiler and Leghorn lines indicate potential for identification of genetic markers that can be used in selection of chickens with increased disease resistance.

## 2. Materials and methods

### 2.1. Chickens and Housing

The chicken lines used in this study have been previously defined for specific characteristics of disease resistance (Zhou and Lamont, 1999; Zhou et al., 2001). The birds were from outbred broiler, inbred Leghorn (Ghs-6) (Deeb and Lamont, 2002) and inbred Fayoumi (M15.2) lines (Deeb and Lamont, 2002) maintained at the Iowa State University Poultry Farm. The broiler line is characteristic of outbred birds used for meat production (Kaiser et al., 1998). Leghorn chickens are commonly used in egg production, and are the primary breed in commercial white egg-laying lines. The Fayoumi chicken is native to Egypt, and has not undergone commercial selection for improved meat or egg production. The Leghorn and Fayoumi lines used in the current study are highly inbred (Zhou and Lamont, 1999). Chicks were randomly selected from two hatches, tagged at hatch to identify pedigree, and received no vaccinations. Birds were housed in a controlled environment room

in floor pens and had *ad libitum* access to commercial starter diet and water to 8 weeks of age.

## 2.2. Experimental Design

*Experiment 1* was designed to determine if three genetic lines of chickens differed significantly in selected parameters of heterophil function. Hatchlings from Leghorn, broiler and Fayoumi lines were acclimated to housing conditions and handling by human beings until 8 weeks of age, when the experiment was conducted. Chickens were housed at the Iowa State University Laboratory Animal Facility in floor pens with fresh litter and 16-hour light and 8-hour dark cycles and received a standard diet. Animal care and use requirements were met throughout the procedures. Heterophils were collected from 54 birds from each chicken line, samples from groups of three individuals were pooled within line, and functional assays performed as described below.

*Experiment 2* was designed to determine effects of immunomodulation (via addition of ascorbic acid and β-glucan in feed) and stress (via addition of exogenous corticosterone in feed) on heterophil function in distinct genetic lines of chickens: broiler, Leghorn, or Fayoumi. A total of 168 chicks per line were selected for this experiment, coming from six replicate hatches. Chicks were raised in flood pens, separated by hatch and line, and fed a standard commercial diet until 8 weeks of age. At 8 weeks, birds from each line were randomly assigned to diet treatment groups, receiving a basal diet, or a diet supplemented with 0.1% β-glucans (basal diet with 0.1% w/w of 1,3-1,6 β-glucan from *Saccharomyces cerrevisiae* as MacroGard Feed Ingredient, Biotec Pharmacon, Tromsø, Norway), 0.1% ascorbic acid (basal diet with 0.1% ascorbic acid as ROVIMIX Stay-C 35, DSM Nutritional

Products, Basel, Switzerland), or 0.01% corticosterone (basal diet with 0.01% anhydrous

corticosterone from Sigma-Aldrich, St. Louis, MO). The diets with immunomodulator were

fed for three weeks, whereas the corticosterone supplemented diet were fed for two weeks

followed by one week without supplementation. Diets were mixed by the Animal Science

Department at Iowa State University using a Hobart feed mixer. Body weight and feed

consumption were monitored for the duration of experiment. During the experimental period

(8-11 weeks of age), birds were housed in floor pens separately by line and diet treatment.

### 2.3. Blood Collection, Pooling, and Chicken Heterophil Isolation

Whole blood was collected from the jugular vein of each bird on days 1, 3, 7, 14, and 21

after the start of diet treatment. Samples were collected in syringes and mixed with EDTA

solution (1% final concentration; Sigma-Aldrich; Blood: EDTA v/v ratio was 10:1). Blood

samples were pooled within line and diet treatment group ($n = 4$ birds per pooling group;

experiment 2) to produce a total of 144 pooled blood samples for heterophil isolation. The

heterophil functional assays were done in triplicate.

Chicken heterophils were isolated from collected whole blood using a previously

described technique with modifications (Andreasen and Frank, 1999; Chuammitri et al.,

2009). Briefly, one volume of chicken blood was gently mixed with one volume of 1%

methylcellulose (Sigma-Aldrich), centrifuged (15 x $g$, 15 min), plasma and buffy coat layer

above the erythrocytes collected and transferred to a different tube. The remaining interface

above packed erythrocytes was gently rinsed to collect remaining heterophils using

disposable pipette with 3 mL of Hank's Balanced Salt Solution without calcium, magnesium

and phenol red (HBSS$^{CMF}$, 1X, Cellgro, Mediatech, Manassas, VA) supplemented with 0.1%

fetal bovine serum (FBS, Altanta Biologicals, Lawrenceville, GA). The heterophil collections was diluted in HBSS$^{CMF}$ w/FBS, centrifuged (370 x $g$, 10 min), supernatant discarded, and the cell pellets resuspended in HBSS$^{CMF}$ w/FBS. Cell suspension was placed on discontinuous density gradient (Histopaque, Sigma-Aldrich; specific gravity of 1.077 over 1.119 g/mL), centrifuged (500 x $g$, 30 min), the 1.077/1.119 g/mL interface and 1.119 g/mL bands were collected, resuspended using HBSS$^{CMF}$ w/FBS, and washed (370 x $g$, 10 min). The cell pellets were resuspended in red blood cell (RBC) lysis buffer (1 g/L KHCO$_3$; Fisher Scientific, Pittsburgh, PA; and 0.87 g/L NH$_4$Cl; Sigma-Aldrich; in deionized water) and RBCs lysed for 3 min. After lysing, cells were washed with HBSS$^{CMF}$ and centrifuged (370 x $g$, 5 min). The supernatant was discarded and the cell pellet resuspended with HBSS$^{CMF}$. Heterophil viability was determined to be >95% using hemocytometer slide (Neubauer) and 0.1% trypan blue solution. Cells were counted by particle counter (Model Z1, Beckman Coulter, Fullerton, CA) and cell suspension adjusted to 5 x 10$^6$ cells/mL. The ratio of heterophil to non-heterophil cells was determined to be >90% using Diff-Quik (Dade Behring, Deerfield, IL) stained cytospin preparations of cell suspensions.

## 2.4. Phagocytosis

Phagocytosis of *Salmonella* Enteritidis (SE) was used to determine phagocytic activity of heterophils via flow cytometry detection of fluorescent bacteria in heterophils by previously published methods with modification (Andreasen et al., 2001; He et al., 2007). In short, fluorescent SE was prepared by growing SE to log phase, heat killed at 60 °C for 90 min, centrifuged and bacterial pellet mixed with 5 mL of Sytox (Sytox Green, Molecular Probes) in HBSS, vortexed to mix, and incubated at room temperature for 10 min. Sytox Labeled SE

was adjusted to 5 x $10^7$ CFU/mL with HBSS, opsonized with 10% heat-inactivated chicken serum (Atlanta Biologicals) and stored until use. For the phagocytosis assay, heterophils and prepared labeled SE were added to duplicate wells for each sample at 1:10 ratio, respectively, and supplemented with HBSS w/ 1% heat-inactivated chicken serum (Atlanta Biologicals). Control wells were loaded with heterophils (2.5 x $10^5$ cells) and HBSS/w 1% heat-inactivated chicken serum, or with labeled SE without cells (2.5 x $10^6$ bacteria). Plates were centrifuged at 400 x $g$, 5 min, 4 $^o$C and incubated at 41 °C with 5% $CO_2$ for 2 h. After incubation, plates were washed at 400 x $g$, 5 min, 4 $^o$C, supernatant was discarded and cell pellets resuspended with 1% paraformaldehyde (Polysciences, Warrington, PA) in PBS. Aliquots of preserved cells were transferred to 5 mL polystyrene tubes and were analyzed by BD FACSCan flow cytometer (BD Biosciences, San Jose, CA). Flow cytometry data were analyzed with FlowJo version 6.0 software (TreeStar, Ashland, OR). The region of background fluorescence was set at 100 units for cut-off value. The phagocytic activity was reported as mean fluorescence intensity (MFI).

## 2.6. Bacterial Killing

Heterophil bacterial killing was evaluated using a poultry nalidixic acid–resistant strain of virulent *Salmonella* Enteritidis (SE) phage type 13a (kindly provided by Dr. H.M. Opitz, University of Maine, Orono, ME) in a formazan metabolic conversion in XTT colorimetric assay as previously described with modifications (Stevens and Olsen, 1993; Tunney et al., 2004). SE was freshly prepared for each assay by colony picking method and propagation in LB broth until optical density ($OD_{595}$) of 0.5 or greater was reached. Bacterial concentration (CFU/mL) was determined by the growth curve regression equation: *y = (OD $_{595}$)(5.09 X $10^9$)*

$+ (- 8.3 X 10^8)$. SE broth culture was diluted with HBSS, aliquoted into 1.5 mL eppendorf tube and pelleted (14,000 RPM, 2 min). The supernatant was discarded and bacterial pellets were opsonized with 10% heat-inactivated chicken serum (Atlanta Biologicals) for 20 min at $37^o$ C. Opsonized SE was diluted to final concentration (5 x $10^7$ CFU/mL to be used in standard curve determination; and 2 x $10^7$ CFU/mL to be used in assay wells). Standard for determination of CFU number was prepared by two-fold serial dilutions of 5 x $10^7$ CFU/mL opsonized bacteria. Actual viable bacterial concentrations were verified by seeding standard dilutions on media plates and plate colony counts.

The positive control wells were seeded with opsonized SE only ($10^6$ CFU per well) and test wells for each sample were seeded with heterophils and SE in 1:2 ratio, respectively (5 x $10^5$ heterophils to $10^6$ CFUs of opsonized SE). All control and test wells were done in duplicate. Plates were centrifuged (400 x $g$, 5 min, 4 $^o$C) and incubated at $41^o$C with 5% $CO_2$ for 45 min. After incubation, plates were centrifuged (1000 x $g$, 5 min, 4 $^o$C) supernatant removed from control and test wells, and 120 µL of deionized water was added to lyse heterophils for 3 min. After lysing, 150 µL of XTT working solution (1 mg/mL in LB broth, filter sterilized, 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt, Sigma-Aldrich; supplemented immediately prior loading with 1% Menadione sodium bisulfite; Sigma-Aldrich) solution was added to all wells, plates were rocked for 1 min, and then incubated at 41 $^o$C for 90 min for color change. Plates were centrifuged (1,000 x $g$, 5 min, 4$^o$C), 200 µL of supernatant from each well was transferred to a clear plate and optical density in each well was determined at $OD_{450-650}$ using a microtiter plate spectrophotometer (V-Max, Molecular Devices) with SOFTmax PRO 4.0 software, and the

XTT assay was validated by counting colony forming units from control and test wells. The percent killing was calculated using the following formula:

$$\% \text{ Killing} = 100 - \left\{ \frac{OD_{\text{test well}}}{OD_{\text{control Well}}} \times 100 \right\}$$

## 2.7. Heterophil extracellular traps-DNA release

Heterophil extracellular traps (HETs)-DNA release was quantified by measuring extracellular DNA with fluorometric assay as previously described (Chuammitri et al., 2009). In short, $2.5 \times 10^5$ heterophils were seeded into 96-well plates and stimulated with hydrogen peroxide in HBSS ($H_2O_2$; Fisher Scientific) at final concentration of 0.15 mM or HBSS (non-stimulated control). The cells were incubated at 41°C with 5% $CO_2$ for 2 h. HETs-DNA released from stimulated heterophils were digested with Micrococcal Nuclease (MNase; 500 mU/mL Worthington Biochemical, Lakewood, NJ) for 20 min at 37 °C. The MNase activity was inactivated with 5 mM EDTA, followed by centrifugation at 400 x $g$, 5 min, 4 °C, and 50 µL of supernatant from each well were collected for double-stranded DNA quantification using Quant-iT PicoGreen dsDNA Reagent and Kits (Molecular Probes) according to manufacturer's instructions. dsDNA quantification plates were read in fluorescence plate reader (SpectraMAX Gemini XS, Molecular Devices; excitation 492 nm, emission 520 nm; with SOFTMax PRO software 4.0). At any given assay dates, DNA Standard Curve (both high-range and low-range standard curve were prepared according to manufacturer's instructions, along with samples. The HETs-DNA release was calculated using the following formula:

HETs-DNA release (ng/mL) = DNA $_{\text{stimulated cells}}$ - DNA $_{\text{non-stimulated cells}}$

## 2.8. Statistical Analysis

In experiment 1, a total of 18 pooled samples (bacterial killing) or 9 pooled samples (the remaining assays) were used, with each pool containing the sample from 3 individual birds. The data analysis was performed using one-way ANOVA and $p < 0.05$ was considered statistically significant. Student's $t$ test was later used to examine the significant difference between pairs. The percent of bacterial killing, mean fluorescence intensity (MFI) of stimulated heterophils, and the difference in HETs-DNA release (ng/mL) were used in one-way ANOVA.

For experiment 2, the main effects used in statistical analysis were chicken lines (Line), dietary supplementations (Diet), and time effect (Week) of either β-glucan, ascorbic acid, or corticosterone. The time effect (Week) of diet supplementation was examined for weeks 0 (days 1 and 3), 1 (day 7), 2 (day 14), and 3 (day 21). For analysis, data from bacterial killing, phagocytosis, and HETs-DNA release were analyzed using three-way and two-way factorial ANOVA by JMP version 7.0 (SAS Institute, Cary, NC). The model included chicken lines, diets, and weeks with all interactions

The models for all effects tested were calculated with Least Square (LS) means and data presented as LS mean ± standard error (SE). The differences in activity were determined by ANOVA. $p < 0.05$ was considered significant. Student's $t$ test was used to determine individual rankings of classes within the significant main effects (Line, Diet) and between interactions (Line*Diet). Graphs were generated using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA).

### 3. Results

### 3.1. Response of heterophils from different chicken lines to *in vitro* stimulation

Heterophils from different chicken lines showed different responses to the stimulation *in vitro* (Table 1). The Fayoumi line demonstrated significantly higher HETs-DNA release ($P$ = 0.002) when compared to the broiler and Leghorn lines (Table 1). Mean fluorescence intensity (MFI) of phagocytosis assay for broiler, Fayoumi, and Leghorn heterophils were 175.69 ± 31.01, 163.08 ± 31.01, and 75.31 ± 31.01 respectively ($P$ = 0.063; Table 1). HETs-DNA release by hydrogen peroxide was significantly higher in Fayoumi heterophils (123.52 ± 20.16) when compared to Leghorn and broiler heterophils (8.53 ± 21.39 and 32.76 ± 21.39; Table 1). Leghorn chickens had the highest bacterial killing activity among the three chicken lines, although it was not statistically significant (66.52 ± 7.91% in Leghorn; Table 1).

### 3.2 Response of heterophils to *in vivo* immunomodulation

It was observed in this study that different immunomodulators caused variable changes in heterophil function. Comparison of the chicken lines fed the basal diet (no immunomodulators) and diet supplemented with immunomodulators showed significantly higher HETs-DNA release in the Fayoumi line ($P$ < 0.001; Table 2; Line; Fig. 1a). The significant difference in corticosterone induced immunomodulation was observed in HETs-DNA release activity (Fig. 1b). Phagocytic activities also differed among lines ($P$ < 0.069; Table 3; Line). The Fayoumi heterophils had lower phagocytic activity compared to broiler and Leghorn chickens (LS mean of 1,150.43 ± 54.30, 1,238.05 ± 54.30, and 1,336.25 ± 58.63, respectively; Table 3; Line, Fig. 1e). Leghorn heterophils also showed significantly higher percentage killing than broiler and Fayoumi heterophils (LS mean of 43.11 ± 2.82,

42.95 ± 2.54, and 22.23 ± 2.61, respectively; *P* < 0.001, Table 4; Fig. 1c). The significant effect of genetic line was observed in HETs-DNA release and bacterial killing.

Immunomodulatory diet treatments significantly enhanced the release of HETs-DNA in all genetic lines (161.39; 184.62; 265.86 ng/mL; β-glucan, ascorbic acid, and corticosterone respectively) when compared to basal diet (106.86 ng/mL, Table 2; Diet). The corticosterone treatment significantly increased HETs-DNA release compared to basal diet (*P* = 0.043; Table 2; Diet; Fig. 2a-b). The effect of immunomodulators (β-glucan and ascorbic acid) in different chicken lines was not observed in heterophil bacterial killing (Table 4; Diet). Chickens fed with corticosterone had lower bacterial killing activity compared to basal, β-glucan, and ascorbic acid diets within the same chicken lines (Table 4; Diet and Fig. 2c-d).The overall phagocytic activities of all chickens fed corticosterone diet were lower than chickens fed basal diet (Table 3; diet and Fig.1f). Even though Fayoumis had higher DNA release, they were the lowest in bacterial killing and phagocytosis (Table 3, 4; Line and Fig. 2c, e). The associations between genetic lines and diet effects were significant in bacterial killing (*P* = 0.012) and phagocytosis (*P* = 0.001).

The differences of heterophil responses among diets (basal, β-glucan, ascorbic acid and corticosterone) within the same line (Leghorn or broiler or Fayoumi) were also observed. The increasing trend in DNA release was observed in all chicken lines fed with corticosterone compared to basal diet. Bacterial killing ability in chickens fed dietary corticosterone was lower compared with the chickens fed basal diet (Fig. 2c-d). There were no differences in phagocytic activity among chicken lines fed immunomodulators. Broilers fed corticosterone diet had the lowest phagocytic activity. Leghorns fed with ascorbic acid had decreased phagocytosis compared to other two lines (Fig. 2f). Phagocytic activity in Fayoumis fed with

ascorbic acid was increased compared to the basal diet (Fig. 2f). Differences between diets within Leghorn chickens were observed in phagocytosis (Fig. 2f). Broilers (Fig. 2b, d, f) and Fayoumis (Fig. 2b, d, f) showed significant effect of diet within each line for all three functional assays.

## 4. Discussion

The immune gene expression profiles of different chicken genetic lines investigated in day-old chicks challenged with *Salmonella* indicated that observed differences in immune responses between breeds can be further explored using chickens of different ages (Cheeseman et al., 2007). Limited information about effects of genetic background on heterophil function in chickens beyond an early age prompted the present study, designed to investigate functional responses of heterophils in genetically distinct chicken lines fed diets with and without dietary immunomodulators. Heterophil function, measured as heterophil extracellular trap (HET) release, phagocytosis, and bacterial killing, was significantly different in 8 weeks old chickens with different genetic backgrounds (broiler, Leghorn and Fayoumi; Experiment 1), and significant differences in effects of dietary immunomodulators on heterophil function among the genetic lines were observed (Experiment 2).

The broiler, Leghorn, and Fayoumi lines included in this study are distinct at the DNA level, as genetically characterized by microsatellite markers (Kaiser and Lamont, 2002; Kaiser et al., 2000; Zhou and Lamont, 1999). In experiment 1, heterophil function was compared between the three distinct chicken lines (broiler, Leghorn, and Fayoumi), at similar ages (8 weeks), revealing that DNA release was significantly higher in Fayoumis than in broilers or Leghorns (Table 1). Differences in specific immune response, measured as

antibody production to TNP-KLH (trinitrophenyl-conjugated keyhole limpet hemocyanin) as antigen without adjuvant, between layer and broiler type chickens of similar age were observed by Koenen et al. (2002) indicating the existence of variation in acquired defense mechanisms between the two chicken types (Koenen et al., 2002).

Fayoumi chickens have not been subject to stringent selection for production traits and, therefore, likely retain pre-industrial characteristics of the species, including alleles for disease resistance (Redmond et al., 2009). The Fayoumis used in this study originated from Egyptian domestic fowl, then were highly inbred, and have been reported as less susceptible to Marek's disease, tumor development (general and Rous sarcoma virus), more resistant to avian leukosis, coccidiosis and colonization by *Salmonella* Enteritidis (Pinard-van der Laan et al., 1998; Zhou et al., 2002). Therefore, observed line differences in heterophil function from chickens fed basal diet (Experiment 1) may be related to the genetic background of the birds used in the experiment. The genetic distinction of Fayoumis from the production-type broiler and Leghorn lines can contrast the immune function between commercial type and indigenous-type lines and help reveal genes involved in disease resistance and dietary immunomodulation to be later used in enhancement of existing stocks (Deeb and Lamont, 2002).When compared immunomodulator diets to basal diet within the same chicken line, and between lines (Fig. 2), the immunomodulators affected on heterophil function in all lines to the different degree. Due to complex interactions between lines and diets, each dietary immunomodulator is discussed separately.

Application of β-1,3-1,6-glucan from *Saccharomyces cerevisiae* at 0.1% w/w in the diet enhanced HETs-DNA release, but not bacterial killing and phagocytosis compared to basal diet. Beta-glucans have been reported to have significant immunomodulatory effect on innate

and adaptive immunity (Engstad et al., 2002). Soluble β-glucan alone, or synergistically with bacterial lipopolysaccharide (LPS), can up-regulate leukocyte activity (Engstad et al., 2002). Enhanced neutrophil oxidative burst, increased bacterial killing, and direct activation of nuclear transcription factors (NF-κB) in the absence of inflammatory cytokines have also been reported for β-glucan activity (Lowry et al., 2005; Wakshull et al., 1999).

The mechanism of β-glucan action is linked to Dectin-1, Fc-γ receptor, complement receptor 3 (CR3), and toll-like receptors (TLRs), especially TLR-2 and MyD88 signaling in mammals (Ainsworth, 1994; Brown, 2006; Brown et al., 2003). Recently, Dectin-1-like β-glucan receptor on chicken heterophils has been reported (Nerren and Kogut, 2009). A function of Dectin-1-like β-glucan receptor on chicken heterophils may not be fully capable as Dectin receptor of mammalian neutrophils. Heterophil function in β-glucan treated chickens in the present study was similar to or slightly lower compared to the chickens fed with the basal diet in bacterial killing and phagocytosis. On the contrary, extracellular killing mechanism (HETs-DNA release), increased in all three chicken lines fed with β-glucans. This suggested that the decrease in intracellular killing might be compensated with a novel and unexplainable extracellular killing phenomenon. The β-glucan induced activation of nuclear transcription factors (NF-κB-like) could explain the effect on HETs-DNA release and bacterial killing but the exact mechanism remains unknown (Engstad et al., 2002). The concentration of β-glucan (0.1%) used in the current experiment was selected based on the report of similar experiments with different species, lines and age groups. Effects of glucans on bird heterophil function have not been extensively studied. The presence of Dectin-1- like β-glucan receptor on heterophils was demonstrated using functional analysis (Nerren and

Kogut, 2009). Our experiments used healthy chickens, thus it is possible that the effects of β-glucan dietary supplementation may differ during the disease condition.

As an immunomodulator, ascorbic acid has increased the protection against combination of Newcastle disease virus and *Mycoplasma gallisepticum* infection, and it was also protective against secondary and primary *Escherichia coli* infection and *Staphylococcus aureus* (Andreasen and Frank, 1999; Gross, 1992; Klasing, 1998). Ascorbate recycling is one of many antioxidant properties of ascorbic acid (Wang et al., 1997). Ascorbate recycling in neutrophils, which occurs when extracellular ascorbate is oxidized, may prevent oxidative damage caused by bacterial infection. The supplementation of the diet with 0.1% ascorbic acid induced changes in heterophil activity compared to chickens fed the basal diet. The Fayoumi heterophils increased HETs-DNA release, phagocytosis, and bacterial killing after introduction of ascorbic acid. Fayoumi heterophils were most responsive to ascorbic acid supplementation, showing significant increase in DNA release, phagocytosis and higher bacterial killing after starting supplementation. In Fayoumis, data of phagocytosis and bacterial killing, which were closely related functions and also well-regulated, showed that the activation of one effector function can initiate another down-stream function. Taken together, the results of the current study support earlier findings that ascorbic acid can be used as dietary immunomodulator in chickens. Fayoumis were more responsive to ascorbic acid immunomodulation effects than Leghorn and broilers. This specific chicken line has a potential to get advantage of this immunomodulator.

When 0.01% dietary corticosterone was fed to healthy Leghorn, Broiler and Fayoumi chickens, the effects on heterophil function were surprising. Both suppressive and stimulating effects of corticosterone were observed in heterophil functional assays. The

corticosterone treatment caused significant suppression of phagocytosis and bacterial killing whereas significant increase in HETs-DNA release in all three chicken lines. Fayoumi heterophils appeared most responsive to this corticosterone effect, exhibiting significantly higher HET-DNA release. On the contrary bacterial killing was suppressed in Fayoumis more than other lines. The mechanisms behind stimulation and suppression observed in the present study remain unknown. The down-regulation of bacterial killing and phagocytosis may be explained by inactivation of NF-κB and impaired NADPH oxidase pathway (Gauss et al., 2007; Luengo-Blanco et al., 2008) but further studies are required to fully explain the pathways. The observed suppression of heterophil function is in accordance with existing evidence of suppressive effects of glucocorticoids on mammalian neutrophils (Brown and Gordon, 2005; Burton et al., 1995; Weber et al., 2006; Weber et al., 2004). NF-κB is closely associated with impairment of NADPH oxidase activation and reduction in oxidative burst in neutrophils (Gauss et al., 2007; Luengo-Blanco et al., 2008). Therefore, observed reduction of phagocytosis in heterophils from corticosterone treated chickens may be associated with suppression of NF-κB activation pathways. Many pro-inflammatory genes, like interleukins, cytokines and adhesion molecules, have NF-κB binding sites in their promoters. These genes are induced upon activation of NF-κB (Schaaf and Cidlowski, 2002). In mammalian neutrophils, glucocorticoids inhibit the expression of pro-inflammatory and immunoregulatory cytokines (Almawi and Melemedjian, 2002). The observed increase in HETs-DNA release in heterophils from corticosteroid-treated chickens has not been previously described. The release of DNA found in HETs is likely an end result of granulocyte-specific process of cell death, which is different from apoptosis and necrosis, thus coined NETosis (Fuchs et al., 2007).

Glucocorticoids have been reported to delay the process of apoptosis of neutrophils through regulation of *Fas* gene expression (Burton et al., 2005).Our results of the present study suggested that the release of DNA from stimulated heterophils took longer time than the process of phagocytosis and bacterial killing. The extension of heterophil half-life in blood circulation under corticosterone supplement may have enhanced the extracellular killing than the intracellular ones because of more time availability. The action of corticosterone on circulating heterophils might have shifted the apoptosis process to NETosis-like mechanism. By unknown mechanism, corticosterone might have initiated transcriptions of the gene or group of genes that are necessary for altering DNA conformation, unwinding chromatin, dissolving nuclear membrane, and released nuclear materials to extracellular space in the form of HETs. In mammals corticosteroids lead to accumulation of aged neutrophils that cannot perform bactericidal function and are no longer useful to host immune defense (Burton et al., 2005). Thus the accumulation of aged heterophils may explain a reduction in phagocytosis and bacterial killing activities in our present study.

The rising public concerns about antibiotic residues in meat and poultry products, and increased occurrence of multiple drug resistant bacterial strains present the poultry industry with the incentive to search for the alternatives to conventional use of antibiotics in the intensive egg and meat production. Genetic differences between chicken lines and subsequent different responses to immunomodulation with β-glucan and ascorbic acid can act as the source of the tools to enhance disease resistance in production setting. To better understand the mechanisms involved in heterophil function responses, studies of gene expression and disease challenges in different chicken lines treated with dietary

immunomodulators need to be performed. The present study clearly indicated that genetic background has significant effect on heterophil function, and that use of immunomodulators can alter heterophil responses in healthy chickens.

## Acknowledgments

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**Table 1**

Heterophil function is different in three distinct genetic chicken lines (*P*-values, One-way ANOVA)**.**

| Functional Assay | Line | Mean ± S.E.M | | | *P*-value |
|---|---|---|---|---|---|
| HETs- DNA release [a] | Leghorn [B] | 8.53 | ± | 21.39 | 0.002 |
| | Broiler [B] | 32.76 | ± | 21.39 | |
| | Fayoumi [A] | 123.52 | ± | 20.16 | |
| | | | | | |
| Phagocytosis [b] | Leghorn | 75.31 | ± | 31.01 | 0.063 |
| | Broiler | 175.69 | ± | 31.01 | |
| | Fayoumi | 163.08 | ± | 31.01 | |
| | | | | | |
| Bacterial Killing [c] | Leghorn | 66.52 | ± | 7.91 | 0.910 |
| | Broiler | 63.61 | ± | 7.91 | |
| | Fayoumi | 61.67 | ± | 7.91 | |

[a] Values in HETs-DNA release = DNA (ng/mL), Mean ± Standard Error of Mean (Mean ± S.E.M)

[b] Values in Phagocytosis = Mean Fluorescence Intensity (MFI), Mean ± S.E.M

[c] Values in Bacterial Killing = % bacterial killing, Mean ± S.E.M

[A,B] Lines not labeled with the same letter are significantly different.

**Table 2**

Effect of genetic lines (line) and dietary supplementations (diet) on heterophil extracellular trap (HETs)-DNA release (*P*-values, ANOVA).

| Variables | LS Mean ± Std Error | | | Categorical Effects | | *P*-value |
|---|---|---|---|---|---|---|
| *Line* | | | | *Main Effect* | | |
| Leghorn [B] | 84.40 | ± | 36.77 | | Line | <0.001 |
| Broiler [B] | 124.07 | ± | 33.27 | | Diet | 0.043 |
| Fayoumi [A] | 330.58 | ± | 33.27 | | | |
| | | | | *Interaction* | | |
| *Diet* | | | | | Line*Diet | 0.962 |
| Basal [B] | 106.86 | ± | 39.68 | | | |
| β-glucan [AB] | 161.39 | ± | 40.20 | | | |
| Ascorbic acid [AB] | 184.62 | ± | 39.68 | | | |
| Corticosterone [A] | 265.86 | ± | 39.68 | | | |

Values = DNA release (ng/mL), Least Square Mean ± Standard Error (LS Mean ± SE), 95% Confident Interval and Mean.

[A,B] Lines or Diets not labeled with the same letter are significantly different.

**Table 3**

Effect of genetic lines (line) and dietary supplementations (diet) on heterophil phagocytosis (*P*-values, ANOVA).

| Variables | LS Mean ± Std Error | | | Categorical Effects | | *P*-value |
|---|---|---|---|---|---|---|
| *Line* | | | | *Main Effect* | | |
| Leghorn | 1336.25 | ± | 58.63 | | Line | 0.069 |
| Broiler | 1238.05 | ± | 54.30 | | Diet | 0.235 |
| Fayoumi | 1150.43 | ± | 54.30 | | | |
| | | | | *Interaction* | | |
| *Diet* | | | | | Line*Diet | 0.001 |
| Basal | 1342.48 | ± | 64.99 | | | |
| β-glucan | 1260.99 | ± | 64.99 | | | |
| Ascorbic acid | 1187.88 | ± | 63.83 | | | |
| Corticosterone | 1174.96 | ± | 63.83 | | | |

Values = Fluorescence (Mean Fluorescence Intensity; MFI), Least Square Mean ± Standard Error (LS Mean ± SE), 95% Confident Interval and Mean.

**Table 4**

Effect of genetic lines (line) and dietary supplementations (diet) on heterophil bacterial killing (*P*-values, ANOVA).

| Variables | LS Mean ± Std Error | | | Categorical Effects | | *P*-value |
|---|---|---|---|---|---|---|
| *Line* | | | | *Main Effect* | | |
| Leghorn [A] | 43.11 | ± | 2.82 | | Line | <0.001 |
| Broiler [A] | 42.95 | ± | 2.54 | | Diet | 0.105 |
| Fayoumi [B] | 22.23 | ± | 2.61 | | | |
| | | | | *Interaction* | | |
| *Diet* | | | | | Line*Diet | 0.012 |
| Basal [A] | 41.50 | ± | 3.05 | | | |
| β-glucan [AB] | 36.30 | ± | 3.08 | | | |
| Ascorbic acid [AB] | 35.97 | ± | 3.05 | | | |
| Corticosterone [B] | 30.62 | ± | 3.12 | | | |

Values = % bacterial killing, Least Square Mean ± Standard Error (LS Mean ± SE), 95% Confident Interval and Mean.

[A,B] Lines or Diets not labeled with the same letter are significantly different.
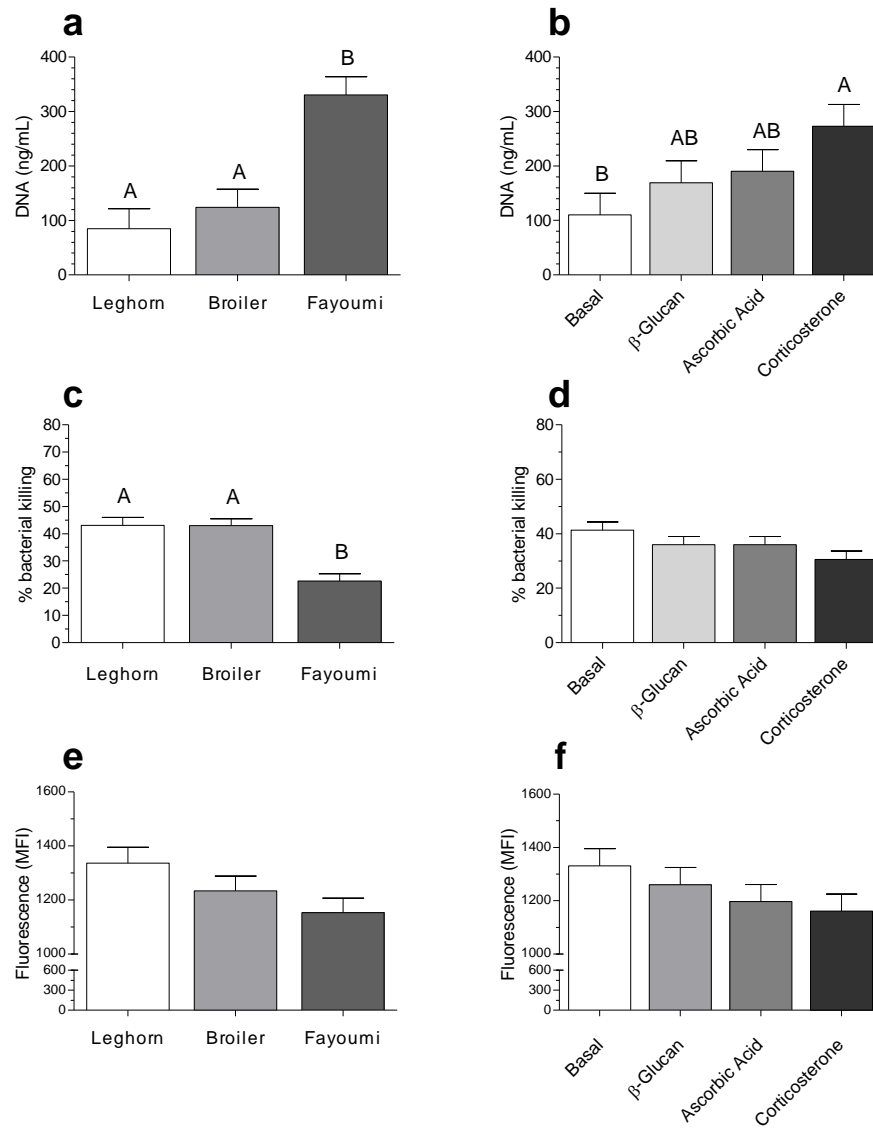
**Fig. 1.** Effects of genetic line and dietary immunomodulators on heterophil function. **a-b:** Heterophil extracellular trap (HETs)-DNA release. HETs-DNA release was significantly different between Fayoumis and other two chicken lines **(a)** and corticosterone diet had significantly higher DNA release compared to basal diet **(b)**. **c-d:** Bacterial Killing. Bacterial killing is significantly different between Leghorns, Broilers, and Fayoumis **(c). e-f**: Phagocytosis. Phagocytosis was not significantly different in both line and diet effects. Data are presented as ng/mL of released DNA (a-b), % bacterial killing (c-d), and mean fluorescence intensity (MFI; e-f). All data are mean ± S.E.M. from n ≥ 13. Different letters above bars (A, B) represent *P < 0.05*.
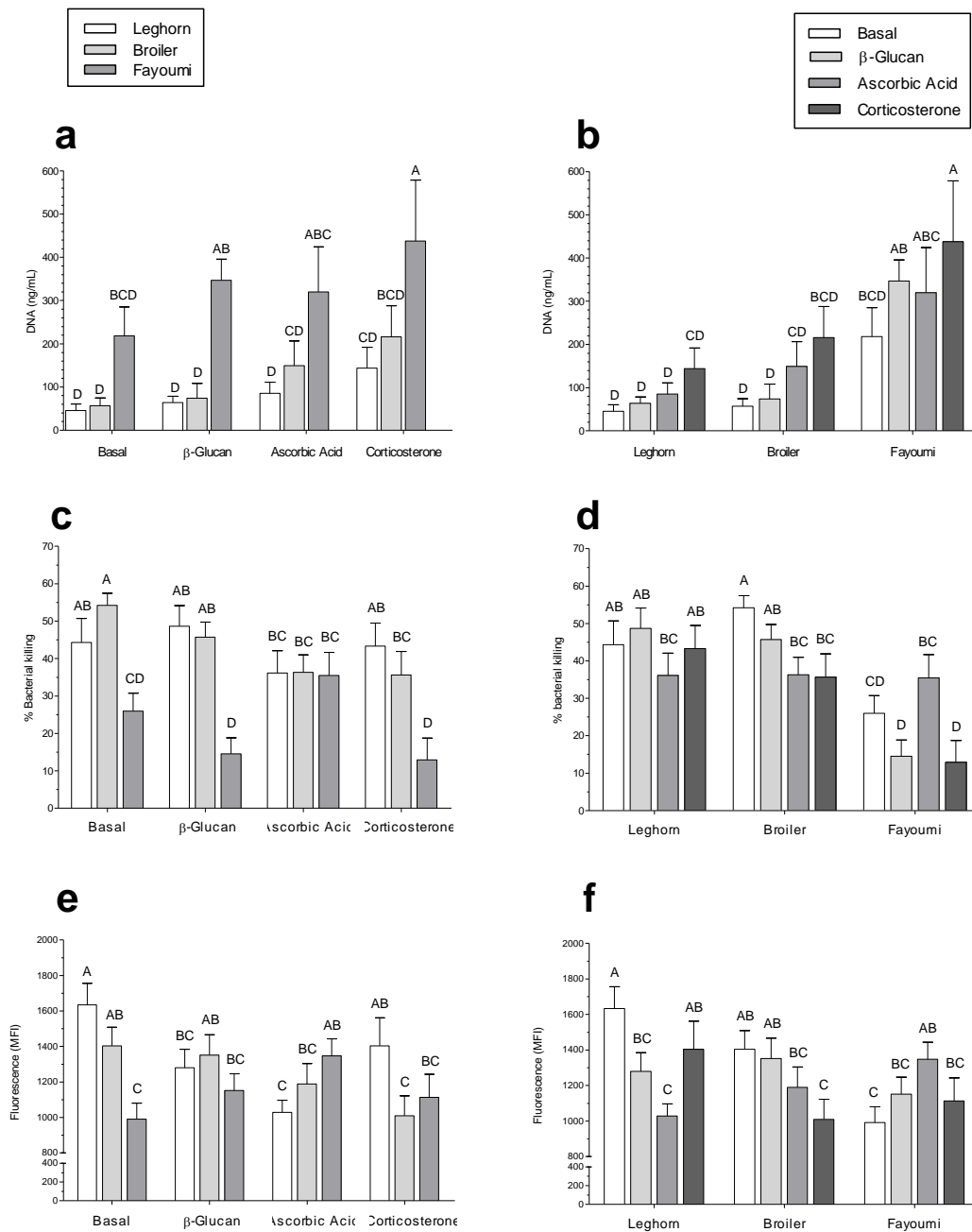
**Fig. 2.** Comparisons of genetic lines within the same dietary immunomodulators **(a, c, e)** and comparisons of dietary immunomodulators within lines on heterophil function **(b, d, f). a-b:** Heterophil extracellular trap (HETs)-DNA release. **c-d:** Bacterial Killing. **e-f**: Phagocytosis. Data are presented as ng/mL of released DNA (a-b), % bacterial killing (c-d), and mean fluorescence intensity (MFI; e-f). All data are mean ± S.E.M. from n ≥ 13. Different letters above bars (A, B, C, D) represent *P < 0.05*.

# CHAPTER 5. EFFECT OF GENETIC BACKGROUND AND DIETARY IMMUNOMODULATORS ON HETEROPHIL FUNCTION AND CLEARANCE OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS IN MATURE CHICKEN HENS

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## Abstract

Poultry salmonellosis is an important food safety concern and *Salmonella enterica* serovar Enteritidis (SE) is a major serotype that contaminates eggs and chicken meat products. Chicken heterophils are instrumental in controlling and preventing this foodborne pathogen from colonization of the gastrointestinal tract and viscera. A better understanding

of genetic background and dietary immunomodulation in enhancing chicken heterophil function would help control *Salmonella* colonization and fecal shedding, thus significantly reduce this microbial contamination problem. We examined the effects of genetic background and dietary immunomodulation on heterophil function and clearance of SE. Hens from three genetically distinct lines (Leghorns, Fayoumis and broilers) were randomly assigned to basal or enhanced diet (basal diet supplemented with dietary β-glucan and ascorbic acid). Within each diet group, hens were further divided into non-challenged or challenged with SE. Heterophil functions were evaluated using phagocytosis, bacterial killing, and heterophil extracellular traps (HETs)- DNA release assays. Bacteriological evaluation of SE clearance was conducted using fecal samples after challenge as well as visceral organs obtained at necropsy. Chicken genetic line and SE challenge status had significant effects on all parameters of heterophil function. The dietary immunomodulation influenced HETs-DNA release and phagocytic activities post SE challenge. Differences in genetic lines on heterophil function were also significant in most functional assays in both challenge groups. Enhanced diet with immunomodulators reduced overall SE fecal shedding and visceral organ colonization. An increase in SE clearances by enhanced diets was also detectable in broilers and Leghorns. The results suggest that resistance to *Salmonella* in poultry may be improved by both dietary immunomodulators and genetic selection.

## 1. Introduction

Salmonellosis is a common bacterial foodborne illness in the United States, leading to significant disease outbreaks (CDC, 2009). *Salmonella enterica* serovar Enteritidis (SE) is a chicken pathogen of considerable economic importance that causes human foodborne

illnesses and acute gastroenteritis (Thesmar, 2009). *Salmonella* Enteritidis was the most prevalent of all *Salmonella* serotypes causing disease among 6,750 *Salmonella* isolates in 2008, which affected 3 out of 100,000 U.S. inhabitants on average (CDC, 2009). In addition to the typical enteric locations, SE often colonizes the chicken ovary and oviduct, causing egg contamination and increased risk of human illness both by meat and eggs (Keller et al., 1995; Keller et al., 1997). Although safe handling and cooking of potentially contaminated poultry products can prevent most disease incidence, contamination with SE remains a problem in intensive food production, processing and packaging, and in commercial food service.

A genetic basis for resistance against *Salmonella* infection in poultry has been established (Bumstead and Barrow, 1993; Kaiser et al., 2005). Regarding pathogenic SE, colonization of the intestinal tract and visceral organs of chickens has been studied in outbred broilers (Kramer et al., 2003) and the first filial (F1) generation from crossing outbred broilers and highly inbred Leghorn and Fayoumi lines (Abasht et al., 2009; Kramer et al., 2003; Malek et al., 2004). The results from those studies demonstrated a significance of genetic line on the expression of immune response genes that associated with SE load and resistance. The broiler line is characteristic of outbred birds used for meat production. Leghorns are the major breed contributing to many commercial egg-laying lines. The Fayoumi chicken is native to Egypt, and has not undergone selection for improved meat or egg production. Chickens have been intensively selected to increase meat or egg production, and industry requirements often placed improvement in disease resistance second to production characteristics, leading to increased losses because of disease (Pinard et al., 1998; Zekarias et al., 2002).Due to increases in food borne illnesses originating from poultry products, and

associated costs of disease control, commercial lines genetically selected for a balance between production traits and disease resistance may be favored by both producers and consumers.

Chicken heterophils have an important role as the first line of defense against bacterial infections, including SE. In recent literature, information about the characterization of genetic mechanisms, gene expression pattern, and functional efficiency of chicken heterophils in response to SE stimulation has been documented (Chiang et al., 2008; Kogut et al., 2003; Redmond et al., 2009). Genetic selection with dietary immunomodulation of chicken heterophil responses can be a valuable component of industry programs to reduce and control SE contamination in poultry products. We have recently reported differences in heterophil function responses from genetically distinct chicken lines fed β-glucan and ascorbic acid (Dissertation chapter 4). Other studies have evaluated immunomodulator effects in chickens, for example, β-glucans (Chen et al., 2008; Lowry et al., 2005; Morales-Lopez et al., 2009), ascorbic acid (Lohakare et al., 2005; Pardue et al., 1985), and dietary folic acid (Hebert et al., 2005).

Supplementation of diets with ascorbic acid (AA) can increase neutrophil adherence, chemotaxis and phagocytic activity (Erickson et al., 2000), suppress the production of free oxygen radicals, and neutralize harmful oxidants and free radicals (Erickson et al., 2000; Padh, 1991; Wang et al., 1997). Furthermore, AA can inhibit NF-κB, a transcription factor for proinflammatory cytokines, keep metal ions ($Fe^{2+}$, $Cu^+$) in their reduced form, and stabilize procollagen mRNA (Erickson et al., 2000; Padh, 1991). It has been suggested that redox reactions of AA with oxygen radicals can act as an alternative source of $H_2O_2$ in neutrophils, and induce myeloperoxidase (MPO) independent bacterial killing mechanisms

(DeChatelet et al., 1972). Stress conditions, such as increased environmental temperature or bacterial infection, caused reduction or even depletion of internally produced AA in the chickens as reviewed Pardue and Thaxton, (1986). Dietary supplementation with AA increased chicken tolerance to heat stress and decreased heat associated mortality as well as improved growth rates and production parameters (Lohakare et al., 2005; Pardue et al., 1985). As reviewed by Pardue and Thaxton (1986), AA addition to the diet increased chicken resistance to coccidiosis, *Salmonella gallinarum* (fowl typhoid), Newcastle virus, and *Pasteurella multocida*.

Beta (β) 1,3-1,6-glucans from *Saccharomyces cerevisiae* can interact with a specific receptor, Dectin-1 Receptor, in mammals (Brown et al., 2003). Chickens possess a Dectin-1-like β-glucan receptor on immune cells which stimulate the immune system (Nerren and Kogut, 2009). Purified β-glucan feed additives significantly decreased the incidence of SE organ invasion in chickens and increased heterophil phagocytosis, bactericidal killing, and oxidative burst (Lowry et al., 2005). *In vitro* studies revealed that glucans can enhance cellular innate immune responses including phagocytosis, degranulation, oxidative burst, arachidonic acid metabolism, and cytokine production (Adams et al., 1997; Wakshull et al., 1999). Both soluble and particulate forms of β-glucan can activate NF-κB-like and NF-interleukin-6 (IL-6)-like transcription factors, and increase production of IL-8, effectively priming the immune system to respond to different stimuli (Adams et al., 1997; Engstad et al., 2002; Wakshull et al., 1999).

The differences in heterophil responses in juvenile Leghorn, broiler and Fayoumi chickens exposed to immunomodulators (Dissertation chapter 4), prompted the present study

of heterophil function and clearance of SE infection in mature hens using three genetically distinct chicken lines during dietary immunomodulator supplementation.

## 2. Materials and methods

### 2.1. Chickens and Housing

The chicken lines used in this study have been previously defined for specific characteristics of disease resistance (Zhou and Lamont, 1999). Females from broiler, Leghorn (Ghs-6) and Fayoumi (M15.2) lines maintained at the Iowa State University Poultry Farm were randomly selected from five hatches, tagged at hatch to identify pedigree, and received vaccinations for Marek's disease, chicken pox, Newcastle disease, and Infectious bronchitis disease. All chickens were acclimated to housing conditions and raised until 5-7 months of age at the Iowa State University Poultry Science Research Center. Birds were provided with fresh wood shavings bedding, standard light cycles, and *ad libitum* access to commercial diet and water before transfer to the Livestock Infectious Disease Isolation Facility (LIDIF), 4 days before starting each experiment trial.

### 2.2. Challenge with *Salmonella enterica* serovar Enteritidis

An isolate of *Salmonella enterica* serovar Enteritidis Phage Type 8 was obtained from USDA APHIS National Veterinary Services Laboratory (NVSL), Ames, IA (ID # SALM-08-2762). The SE phage Type 8 was used in the challenge as it is the most common SE phage type isolated from sporadic and outbreak cases of human and poultry salmonellosis (Lindell et al., 1994). The received isolate was plated on Luria-Bertani (LB) agar; colonies were picked and cultured in LB broth at 37°C with agitation until reaching an exponential growth phase. The concentration of bacteria in the stock solution was determined

spectrophotometrically using a standard curve at a reference wavelength of $\lambda = 595$ nm. SE inoculums at final concentration of $2 \times 10^8$ CFU/ mL were prepared and diluted in sterile HBSS prior to challenge. Each bird was inoculated orally with 1 mL of $2 \times 10^8$ CFU of bacterial suspension (or 1 mL of HBSS for non-inoculated control birds) using a syringe with an attached flexible inoculation teat.

### 2.3. Experimental design

Leghorn, Fayoumi and broiler hens, 5-7 months of age, from a breeder flock cultured negative for SE were housed individually in steel layer cages with manure collection trays in a Biosafety Level 2 (BL-2) facility at LIDIF; and acclimated four days before the start of experimental diets. The chickens were randomly assigned, within line, to treatment (diet with immunomodulators) or control (basal diet), as well as challenge or non-challenged groups (Table 1). The basal diet was a commercial corn-soy-based diet (Breeder #21-A HYI, Whiton Feeds,Perry, IA). The enhanced diet was the basal diet supplemented with 0.1% β-glucan (w/w; MacroGard Feed Ingredient, Biotec Pharmacon, Tromsø, Norway) and 0.1% ascorbic acid (w/w; ROVIMIX Stay-C 35, DSM Nutritional Products, Basel, Switzerland). This design was repeated three times in succession with a 1-week gap interval between each experimental replicate. The experimental schedule is summarized in Table 2. In brief, SE negative status was confirmed 3 days prior to the start of the experiment by negative culture of both feces and eggs. Hens were monitored daily, and egg production and clinical signs were recorded for the duration of the experiment. Dietary treatment was initiated on day 0 of the experiment. The SE challenge was performed on three consecutive days (days 5, 6, 7)

after start of dietary treatment. Blood was collected for heterophil function assays on days 0, 3, 6, 7, 10, 13, 17. Culture of SE from feces and eggs was performed 3 days before each experimental replicate was started on treatment diets and at day11 (feces only) of each experiment. The experiment was concluded on day 18 of the dietary treatment. All hens were euthanized, subjected to pathological examination, lesions from experiment chickens were scored, and samples for histopathology and bacteriology were collected.

## 2.4. Sampling for bacteriological analysis of *Salmonella enterica* serovar Enteritidis

Fecal samples (about 5 g/chicken) were collected from the manure trays below each individual cage within one hour after defecation, using two sterile cotton swabs, placed in tubes with tetrathionate broth, and stored in a 37°C incubator prior to analysis by the Veterinary Diagnostic Laboratory (VDL) at Iowa State University. Samples from non-challenged chickens from the same line and diet treatment were pooled together for assaying, and samples from challenged chickens were assayed individually.

Egg samples were collected three days prior to the start of the diet treatment (one day after transferring hens to LIDIF). Individual eggs were labeled, placed in egg cartons and transferred to the VDL for testing. Eggs from four chickens of the same diet/treatment/line were pooled for SE bacterial culture. Tissue samples from individual birds with or without lesions (cecum, ovary, oviduct, spleen and liver) were aseptically collected at necropsy and submitted for SE bacterial culture. SE isolation and detection was performed by VDL using their standard operating procedures (SOP). The specimen was inoculated into pre-enrichment media (tetrathionate broth). The media tubes were incubated at 42° C overnight (18-24 hours). The inoculum was streaked on selective agar plates (brilliant green (BGA) and

XLT4) and incubated at 42° C for 18-24 hours. The *Salmonella* suspect colonies on BGA and XLT4 were picked and lactose negative colonies transferred to Kligler's, SIM and Urea test, incubated at 42° C for 18-24 hours, for biochemical confirmation of *Salmonella* and serotyping.

## 2.5. Heterophil isolation and function assays

### 2.5.1. Cell isolation

Whole blood was collected from the jugular vein in syringes and mixed with EDTA solution (1% final concentration; Sigma-Aldrich; Blood: EDTA v/v ratio was 10:1). Chicken heterophils were isolated from collected whole blood as previously described (Chuammitri et al., 2009). Briefly, chicken blood was mixed with 1% methylcellulose, centrifuged (15 x *g*, 15 min) and buffy coat was collected, placed on discontinuous density gradient (Histopaque, Sigma-Aldrich; specific gravity of 1.077 over 1.119 g/mL) and then centrifuged (500 x *g*, 30 min). The 1.077/1.119 g/mL interface and 1.119 g/mL bands were collected, resuspended in HBSS$^{CMF}$ w/FBS, and washed (370 x *g*, 10 min). After lysing the red blood cells with RBC lysis buffer (KHCO$_3$ and NH$_4$Clin deionized water) for 3 min, isolated heterophils were washed, and finally adjusted to 5 x 10$^6$ cells/mL with HBSS (>95% heterophils, and viability).

### 2.5.2. Phagocytosis assay

Phagocytosis of SE was used to determine phagocytic activity of heterophils via flow cytometry using heat killed SE-FITC. Briefly, heat killed SE were labeled with FITC (Sigma-Aldrich), and opsonized with 10% chicken serum (Atlanta Biologicals, Lawrenceville, GA). Opsonized FITC-labeled SE (2.5 x 10$^6$ bacteria) were mixed with

heterophils ($2.5 \times 10^5$ cells) in U-bottom, 96-well plate, centrifuged ($400 \times g$, 5 min, 4°C), incubated (41°C with 5% $CO_2$ for 2 h), washed and fixed with 1 % paraformaldehyde (Polysciences, Warrington, PA), and analyzed by BD FACSCanto flow cytometer (BD Biosciences, San Jose, CA). Flow cytometry data were analyzed with FlowJo version 6.0 (TreeStar, Ashland, OR). The region of background fluorescence was set at 100 units for cut-off value. The phagocytic activity was reported as Mean Fluorescence Intensity (MFI).

### 2.5.3. Bacterial killing assay

Heterophil bacterial killing of SE was evaluated using a formazan metabolic conversion colorimetric assay as previously described with minor modifications. Freshly prepared SE bacterial pellets were opsonized with 10% chicken serum for 20 min at 37°C. Heterophils (5 $\times 10^5$ heterophils) and opsonized SE ($10^6$ CFUs) were seeded into microtiter plates, centrifuged ($400 \times g$, 5 min, 4°C) and incubated at 41°C with 5% $CO_2$ for 45 min. After incubation, supernatant was removed and 120 µL of deionized water was added to lyse heterophils for 3 min. After cell lysis, 150 µL of 2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) solution (Sigma-Aldrich) was added to all wells, plates were rocked for 1 min, and then incubated at 41°C for 90 min for color change. Optical density in each well was determined at $OD_{450-650}$ using a microtiter plate spectrophotometer (V-Max, Molecular Devices). The XTT assay was validated by counting colony forming units from control and test wells.

The percent killing was calculated with the following formula:

$$\% \text{ Killing} = 100 - \left\{ \frac{OD_{\text{test well}}}{OD_{\text{control well}}} \times 100 \right\}$$

**2.5.4. Heterophil extracellular traps (HETs) assay**

Release of heterophil extracellular traps (HETs) was quantified by measuring extracellular DNA with a fluorometric assay as previously described (Chuammitri et al., 2009). In brief, $2.5 \times 10^5$ heterophils were stimulated to release HETs with hydrogen peroxide (0.15 mM $H_2O_2$; Fisher Scientific, Waltham, MA) for 2 h at 41°C with 5% $CO_2$. HETs released from stimulated heterophils were digested with Micrococcal Nuclease (MNase; 500 mU/mL,Worthington Biochemical, Lakewood, NJ) for 20 min at 37°C. Double-stranded DNA was quantified using Quant-iT PicoGreen ds DNA Reagent and Kits (Molecular Probes) according to manufacturer's instructions. The relative fluorescent units (RFU) were measured (492/520 nm) using a fluorescence plate reader (SpectraMAX Gemini XS) and the HETs-DNA release was calculated using the following formula:

$$\text{HETs-DNA release (ng/mL)} = \text{DNA}_{\text{stimulated cells}} - \text{DNA}_{\text{non-stimulated cells}}$$

**2.6. Statistical Analysis**

Repeated measures analysis of variance (ANOVA) models were applied to analyze heterophil function using the MIXED procedure in SAS 9 (SAS Institute, Cary, NC). The repeated measures ANOVA models included chicken line, diet, time, and challenge status as fixed effects, and replicate as random effect. The individual hen was the subject of repeated measures. There were no significant differences between diet treatments of challenge and non-challenged groups of pre-challenge (days 0, 3) within the same chicken line.

If a fixed effect was found to be significant ($P < 0.05$), then multiple comparisons between group means with Tukey adjustment for *p*-values were performed. To determine the correlation of SE in feces or tissues, Fisher's exact test was used to test the dependency

between diets (basal or enhanced diet) after challenge with SE in each of the feces and tissues (cecum and oviduct), within genetic line. Any bird that was free-of-Salmonella in both feces and tissues was categorized as "negative cultures". Graphical presentations were generated using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA).

## 3. Results

### 3.1. Heterophil function differs among genetically diverse lines of hens exposed to dietary immunomodulation and SE challenge

The key findings of our study were: 1) Overall phagocytosis, bacterial killing, and HETs-DNA release by heterophils were significantly affected by genetic line and challenge status, but not diet (Table 3); 2) In challenged hens, HETs-DNA release and bacterial killing were significantly affected by genetic line (Table 4). During the pre-challenge period, no significant effect of diet on heterophil function was observed (Table 3); and 4) *Salmonella* colonization and fecal shedding decreased in chickens fed enhanced diet, and greater percentage of SE clearance was observed in immunomodulator supplemented diet than in the basal diet group ($P = 0.09$).

Heterophil functional responses in post-challenge chickens (Days 10-17) were significantly affected by genetic line (analysis with mixed model ANOVA; Table 4). HETs-DNA release and bacterial killing were affected significantly by the line. HETs-DNA release was significantly higher in Fayoumis than Leghorns ($P = 0.0001$; Table 4; Fig. 1a) using Tukey's adjustment for multiple comparisons. No differences in phagocytic activities were observed among three lines of hens. Bacterial killing was significantly affected by the

line. Fayoumis had significantly lower bacterial killing than broilers or Leghorns ($P$ <0.0001; Table 4; Fig. 1c) using Tukey's adjustment for multiple comparisons.

Among the non-challenged chickens fed with enhanced diet, line and diet effects were observed (Table 5; Fig. S1). Significant differences among lines were observed in heterophil bacterial killing and phagocytic response ($P$ < 0.0001 and $P$ = 0.014, respectively; Table 5; Line). Broilers had significantly higher bacterial killing than Leghorns and Fayoumis (Fig S1c). Broilers had also higher phagocytic activity than Fayoumis but not Leghorns (Fig S1e). All functional results were not affected by diet (Fig. S1b, d, f). Within enhanced diet group, bacterial killing of broilers had significantly higher activity than both Leghorns and Fayoumis (Fig. S2d). Broilers fed basal diet also had significantly higher bacterial killing than Fayoumis fed the same diet (Fig. S2d). No differences within or between lines of HETs-DNA release and phagocytosis were observed in non-challenged hens.

Heterophil function responses in challenged chickens varied by genetic lines and diets. Significant differences among the lines were observed post challenge in HETs-DNA release and bacterial killing, but not in phagocytosis (Fig. 1a, c, e). No significant increase in heterophil responses between diets were detected after challenge (Fig. 1 b, d, f). HETs-DNA release increased post-challenge within the Fayoumi line fed enhanced diet, and an increasing trend was also observed in broiler and Leghorn lines (Fig. 2a). Compared to Fayoumis fed either basal or enhanced diet, Leghorns with respective diet had significantly lower DNA release (Fig. 2a-b). Bacterial killing was significantly different between commercially selected lines (broilers and Leghorns) and pre-industrial line (Fayoumis; Fig. 1c). Although slight increase in bacterial killing in Fayoumis was observed by enhanced diet, overall this activity was significantly lower compared to Leghorns and broilers fed the same

diet (Fig. 2c-d). Phagocytic functions of hens fed either basal or enhanced diet had no significant changes across all chicken lines (Fig. 1e-f). Higher phagocytic activities were observed in Leghorns and Fayoumis fed with enhanced diet but it was not statistically significant (Fig. 2f).

## 3.2. Clearance of *Salmonella* Enteritidis infection is increased in genetically diverse hens with dietary immunomodulator supplementation

All challenged hens tested positive for SE in fecal samples at day 11 of the experiment (day 5 after first challenge), verifying the effectiveness of the challenge. All non-challenged hens tested SE negative. Bacterial cultures from viscera were collected at necropsy and SE status (positive/negative) was compared to fecal samples from the same bird. Culture negative in all necropsy samples (cecum, oviduct, liver, and other viscera if gross lesions were noted) in challenged hens were considered to have cleared the SE infection (Table 6). Overall, 21% of challenged hens fed diet enhanced with immunomodulators and 11% of hens fed the basal diet had cleared the SE infection by the necropsy day ($P = 0.091$, Fisher's exact test; Fig. 3). Challenged hens in each line demonstrated an increased percentage of SE clearance in enhanced versus basal diet groups (Leghorn 17% versus 8%; broiler 30% versus 11%; and Fayoumi 16% versus 13%). The SE clearance in Leghorn and broiler hens fed the enhanced diet was at least two-fold higher than in the basal diet group, but no significant differences between diets were observed using Fisher's exact test (Table5, Fig. 3).

## Discussion

We have found the strong evidence that genetics and diet have influence on heterophil function and clearance in SE.  Our results agree with the former research of SE organ

invasion and effects of immunomodulatory substances and diets, which suggested that immune function could be enhanced with breed selection and dietary supplementation (Bumstead and Barrow, 1993;Keller et al., 1995; Kogut, 2002; Lowry et al., 2005; MacKinnon et al., 2009; Malek et al., 2004; Redmond et al., 2009). Use of F1 population from crossing outbred broiler with inbred Leghorn or Fayoumi chickens as models in immunogenetic studies of SE resistance was recently described in conjunction with several gene loci that were linked to the SE resistance (Lamont et al., 2002; Malek et al., 2004; Malek and Lamont, 2003).The unique disease resistance phenotypes of these chicken lines were associated with effects of T-cell specific surface protein (CD28), TLR4/MD-2, caspase-1, inhibitor of apoptosis protein-1 (IAP-1), Prosaposin (PSAP), MHC class I, natural resistance-associated macrophage protein 1 (NRAMP1), tumor necrosis factor related apoptosis inducing ligand (TRAIL), transforming growth factor $\beta3$ (TGF-$\beta3$), and SAL1 on SE bacterial load and colonization (Lamont et al., 2002; Liu and Lamont, 2003; Malek et al., 2004; Malek and Lamont, 2003).

Diet treatment with $\beta$-glucans and AA significantly increased the amount of HETs-DNA release and phagocytic activity in the post-challenge period. This finding is in accordance with earlier observations of increased phagocytosis, bacterial killing, oxidative burst, proinflammatory mediators and cytokine production induced by application of $\beta$-glucans (Adams et al., 1997; Lowry et al., 2005). The presence of a specific $\beta$-glucan receptor (Dectin-1) for detection of fungal infections has been confirmed in macrophages, neutrophils (Brown et al., 2003) and chicken heterophils as Dectin-1-like $\beta$-glucan receptor (Nerren and Kogut, 2009). Several signaling pathways could be connected to $\beta$-glucan activity, including pathways involving protein kinase C (PKC), phospholipase A2 (PLA2), production of pro-

inflammatory mediators, protein tyrosine kinase (PTK) and mitogen activated protein kinase (MAPK) (Adams et al., 1997). As immunostimulators, β-glucans are capable of cross-linking of CD11b/CD18 (CR3) for increasing the secretion of IL-8, also known as neutrophil chemotactic factor (Engstad et al., 2002; Xia et al., 1999). β-glucan enhanced bactericidal activity and myeloid and megakaryocyte progenitor proliferation, which in turn accelerated the release of mature neutrophils from the bone marrow to blood circulation and finally to the site of infection, improving bacterial clearance at the site of infection (Wakshull et al., 1999).

The chickens fed the immunomodulator enhanced diet demonstrated the significant increase in HETs-DNA release and other heterophil functions, which might be attributed to the addition of ascorbic acid, or synergistic action of ascorbic acid and β-glucan, rather than the action of β-glucans alone. Multiple studies have indicated the potential for ascorbic acid (AA) to be used as modulator of heterophil function in chickens, including increase in bactericidal activity (Andreasen and Frank, 1999). While acting as a scavenger of free oxygen radicals, AA was reported to provide an alternate method of creating hydrogen peroxide ($H_2O_2$) which is an important substrate for MPO in ROS reactions (DeChatelet et al., 1972). The potential for HETs-DNA release to be manipulated by the action of $H_2O_2$ was demonstrated recently indicating a potential pathway for induction of HETs via ascorbic acid related intracellular changes (unpublished data). Because avian heterophils lack myeloperoxidase (Penniall and Spitznagel, 1975), which is important for generation of ROS of downstream pathways in oxidative burst and phagocytosis, HETs-DNA release may be used by heterophils as a substitute for the oxygen-dependent killing mechanisms.

HETs-DNA release was significantly higher in the chicken heterophils from birds fed the enhanced diet. The release of extracellular nuclear DNA associated with histones and

proteolytic granules can partially limit the spreading and colonization of *Salmonella*

organisms in and around the areas of heterophil recruitment in tissues or the capillary

network (Clark et al., 2007). The observed reduction in SE shedding via fecal content and SE

colonization of digestive and reproductive tract in chickens fed the immune modulator

enhanced diet coincided with the increase in HETs-DNA release, indicating potential for

increased ability of hens fed the immunomodulators to cope with the presence of live

bacteria, both in circulation and localized invasion of tissues. Extracellular antimicrobial

killing ability alone may not be sufficient to combat with live SE after challenge. With the

help of intracellular antimicrobial properties such as phagocytosis and oxidative burst,

bacterial killing of invading SE may have increased in heterophils from hens fed with

immunomodulators. SE spreading could have been restricted at some parts of intestine

because of the enhanced heterophil activities and newly released heterophils from the bone-

marrow could have eliminated localized bacteria completely.

   Heterophil phagocytic activity did not significantly change in our present study.

Phagocytosis is the first mechanism of intracellular bacterial killing that is activated by the

presence of live SE. Phagocytosis may have already reached to the point where further

increase was difficult to achieve even with the presence of live SE. Heterophils in our studies

have been exposed *in vivo* to priming inflammatory mediators in bloods or tissues, for

example, LPS, IL-6, IL-8, fMLP or C5a, for a certain period that could have an attenuated

antimicrobial function after isolation and when activated *in vitro* (van Eeden et al., 1999).

Data from Lowry et al.(2005) indicated that β-glucan is a potential priming agent for chicken

heterophils and a significant decrease in SE organ invasion was achieved by the action of a

purified β-glucan in immature chickens. In our current study, chicken heterophils could have

been primed by the effects of β-glucan before the presence of live SE from the challenge, resulting in significantly increased function after the SE challenge. The indirect evidence supporting the above scenario is observed in as increase in HETs-DNA release in all lines, and increased bacterial killing and phagocytic activity of Leghorn and Fayoumi chickens. These increased heterophil activities may have facilitated the overall SE clearance. As the number of SE negative hens increased at necropsy, it is possible that there was an increase in the release of heterophils from the bone marrow or increased migration of heterophils from adjacent tissues/areas to the site of infection by β-glucan as previously mentioned.

Enhanced diet could increase the overall SE clearance but some chickens could not completely clear the SE infection at a time of necropsy. As suggested by Lindell et al. (1994) reinfection by horizontal transmission or superinfection during a diminishing innate immune response may account for positive cultures at necropsy regardless of the beneficial outcomes from diets (Lindell et al., 1994). Therefore, the short term dietary supplementation with immunomodulators used in this study might have not been of sufficient duration to eliminate or minimize organ invasion and fecal shedding. The focus in the present study for *in vitro* cellular analysis was on heterophil function; however, the SE clearance data likely reflect the complex interplay of all cell types in the chicken immune system, allowing for macrophages, dendritic cells, and lymphocytes interaction with heterophils. The initial innate immune response by heterophils was indeed at some instances tapering off to a level that may have allowed reinfection and recolonization of remaining SE (Lindell et al., 1994).

The effect of dietary immunomodulators on SE clearance was significantly affected by the genetic background of the chickens used in this study, suggesting that genetics and dietary immunomodulators acted in conjunction to provide birds with increased bacterial

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resistance. Fayoumi lines, genetically distant from broiler and Leghorn lines have no history for production selection and have higher natural diversity in their genetic background that may interact differently with the presence of pathogens than commercially selected chicken lines. Our observations in Fayoumis also reaffirmed the influences of genetics on cellular function. In our findings, Fayoumi chickens fed with enhanced diet showed increase in both phagocytosis and bacterial killing. Fayoumis may have granted the advantages of either action of β-glucans or ascorbic acid or both for driving the downstream functions after heterophil phagocytosis (e.g. oxidative burst, degranulation) over two commercial chicken lines. Fayoumis had a narrower range of bacterial clearance compared to other two lines but it also need to be noted that the numbers of animals used for the present experiment were considerably smaller compared to an industrial level. The observed effect of enhanced diet on SE clearance indicates potential for improvement, and an industrial scale study of immunomodulators may be required prior to definitive conclusion about increased SE resistance in hens.

The current study suggests that the genetic background underlying the innate cellular immunological functions of heterophils could be enhanced by feeding dietary immunomodulators, leading to partial reduction in SE shedding and colonization in chickens. The increased level of functional activity is a good indicator of chicken heterophil competency in innate immune response. The combined approach of genetic selection and dietary immunomodulation could be used to assist the poultry industry in controlling and reducing risk of public health risk associated with SE contamination of chicken products.

## Acknowledgments

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**Table 1**

Study design to evaluate effect of genetic line (Leghorn, broiler and Fayoumi) and dietary immunomodulator supplementation (β-glucan and ascorbic acid) on chicken heterophil function during challenge with *Salmonella enterica* serovar Enteritidis (SE). Non-challenge: chickens orally administered sterile phosphate buffered saline (PBS) solution; Challenge: chickens orally administered SE in HBSS; Basal diet: a commercial corn-soy based diet (Breeder #21-A HYI, Whiton Feeds, Perry, IA); Enhanced diet: basal diet with addition of 0.1% β-glucan and 0.1% ascorbic acid; $n$: number of individual hens assigned to each diet and challenge group in each replicate trial. There were three trial replicates, for a total of 72 non-challenged and 144 challenged hens.

| | Non-challenge ($n= 24$ per replicate) | | Challenge ($n= 48$ per replicate) | |
|---|---|---|---|---|
| | Basal Diet | Enhanced Diet | Basal Diet | Enhanced Diet |
| Leghorn | $n = 4$ | $n = 4$ | $n= 8$ | $n= 8$ |
| Broiler | $n = 4$ | $n = 4$ | $n= 8$ | $n= 8$ |
| Fayoumi | $n = 4$ | $n = 4$ | $n= 8$ | $n= 8$ |

**Table 2**

Schedule of activities and sample collection during challenge experiment.

Hens were kept at Biosafety Level 2 (BL-2) of experimental animal housing facility beginning four days prior to dietary treatment to acclimate. **T**: Diet treatment (basal, or β-glucan and ascorbic acid) was initiated on Day 0 and stopped at Day 17 (feed was withdrawn the night before necropsy) of the experiment; **C**: Challenge with *Salmonella enterica* serovar Enteritidis (SE) phage type 8 by oral administration of 1 mL of innoculum containing $2 \times 10^8$ colony forming units (CFU) on each of three consecutive days (Days 5, 6 and 7, after initiation of the diet treatment). Hens from non-challenged groups received PBS; **HF**: Whole blood samples were collected for heterophil cellular function; **DxSE**: Diagnostic testing for presence/shedding of SE in feces; **Egg:** Diagnostic testing for presence/shedding of SE inside laid eggs.

| Day | -3 | 0 | 3 | 5 | 6 | 7 | 10 | 11 | 13 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Activity | | T (start) | | C | C | C | | | | T (end) | Necropsy |
| Sampling | DxSE, Egg | HF | HF | | HF | HF | HF | DxSE | HF | HF | Tissues |

**Table 3**

The main effects and interactions of *Salmonella enterica* serovar Enteritidis (SE) challenge, genetic line and diet post infection on heterophil function. Heterophil function varied significantly after challenge depending on chicken genetic line (line) and dietary treatment (diet) in the mixed model ANOVA (*P*-values).

|  | HETs-DNA Release | Bacterial Killing | Phagocytosis |
|---|---|---|---|
| **Line** | 0.0586 | <.0001 | 0.0391 |
| **Diet** | 0.1465 | 0.8045 | 0.6804 |
| **Challenge** | 0.0036 | <.0001 | 0.0005 |
| **Line*Diet** | 0.7581 | 0.8067 | 0.6351 |
| **Line*Challenge** | 0.0328 | 0.0069 | 0.6182 |
| **Diet*Challenge** | 0.0830 | 0.3124 | 0.8991 |
| **Line*Diet*Challenge** | 0.6408 | 0.0749 | 0.4019 |

**Table 4**

The main effects of *Salmonella enterica* serovar Enteritidis (SE) challenge, genetic line and diet post infection on heterophil function. Heterophil function varied significantly after challenge depending on chicken genetic line (line), and dietary treatment (diet) in the mixed model ANOVA (*P*-values).

|  | **HETs-DNA Release** | **Bacterial Killing** | **Phagocytosis** |
|---|---|---|---|
| **Line** | 0.0001 | <.0001 | 0.574 |
| **Diet** | 0.360 | 0.919 | 0.527 |
| **Line*Diet** | 0.914 | 0.250 | 0.444 |

**Table 5**

The main effects of genetic line and diet on heterophil function of non- *Salmonella enterica* serovar Enteritidis (SE) challenged hens. Heterophil function varied significantly depending on chicken genetic line (line) and dietary treatment (diet) in the mixed model ANOVA (*P*-values).

|  | HETs-DNA Release | Bacterial Killing | Phagocytosis |
|---|---|---|---|
| **Line** | 0.889 | <.0001 | 0.014 |
| **Diet** | 0.068 | 0.987 | 0.756 |
| **Line*Diet** | 0.659 | 0.339 | 0.603 |

**Table 6**

Clearance of *Salmonella enterica* serovar Enteritidis (SE) in hens from genetically different lines subjected to different dietary treatments. [*] SE clearance: ceca, cecal contents, and oviducts were negative for bacterial culture. [†]Fisher's exact test was used to test the dependency between supplemented diets (basal or enhanced diet) after challenge and bacteria in each of the feces and tissues (cecum and oviduct), with chicken lines controlled.

| | SE Clearance [*] | | |
|---|---|---|---|
| | Individuals with negative cultures / total individuals (Percentage of clearance) | | |
| | Basal Diet | Enhanced Diet | *P*-value[†] |
| Diet (total) | 7/63 (11.11%) | 15/71 (21.13%) | 0.091 |
| Line | | | |
| Leghorn | 2/23 (8.7%) | 4/24 (16.67%) | 0.354 |
| Broiler | 2/18 (11.11%) | 7/23 (30.43%) | 0.134 |
| Fayoumi | 3/22 (13.64%) | 4/24 (16.67%) | 0.551 |
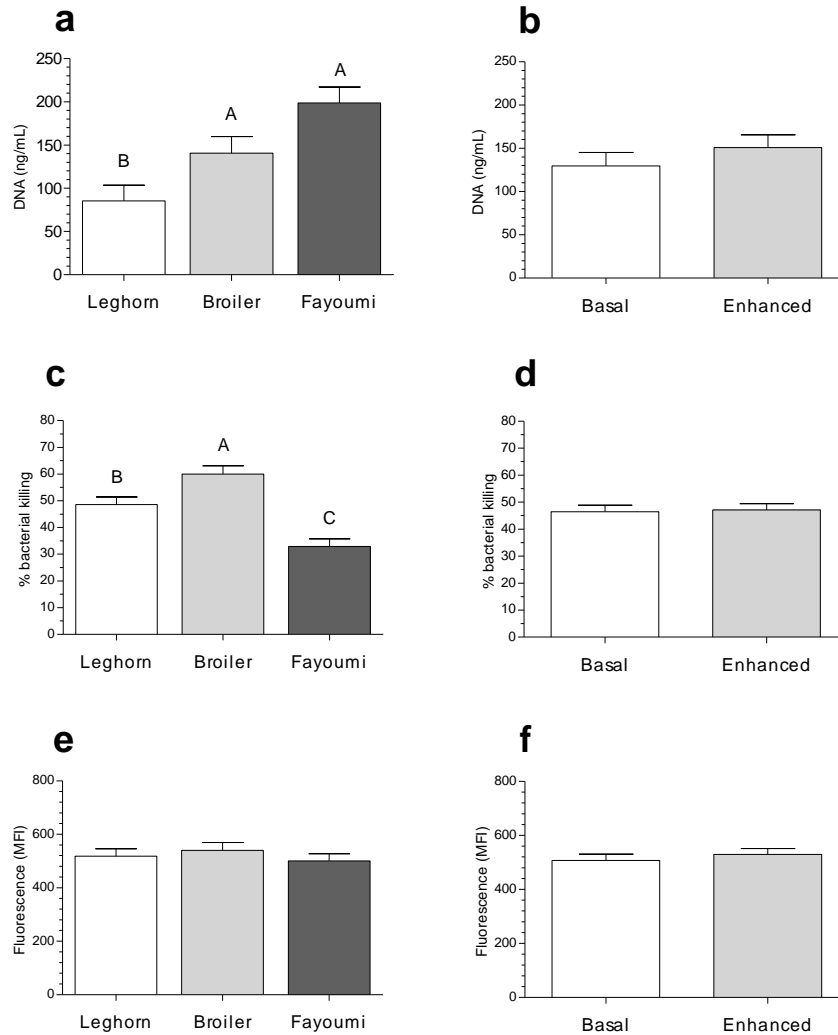
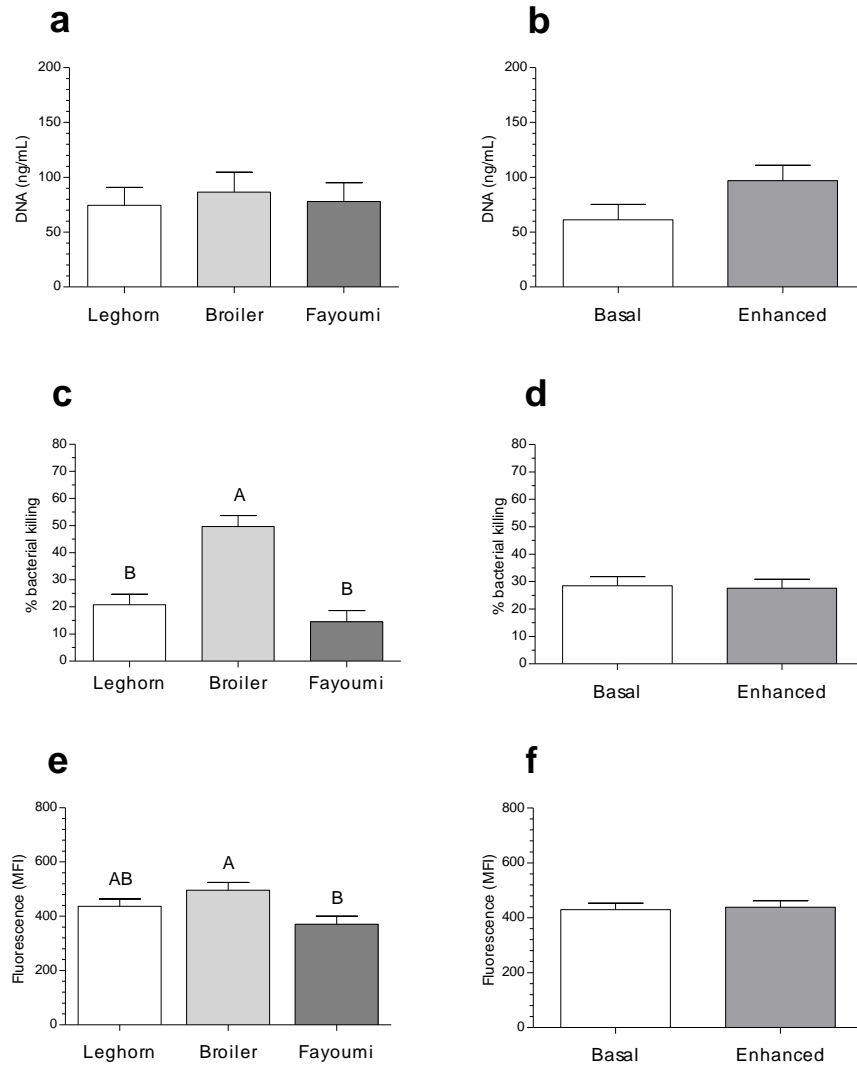**Fig.1**.Effects of genetic line and dietary immunomodulators on heterophil function in challenged chickens. **a-b**: HETs-DNA release; **c-d**: bacterial killing; **e-f**: phagocytosis. Data are presented as ng/mL of released DNA (a-b), % bacterial killing (c-d), and mean fluorescence intensity (MFI; e-f). All data are expressed as mean ± S.E.M. from n ≥ 30. Different letters above bars (A, B) indicate significant difference (*P< 0.05).*
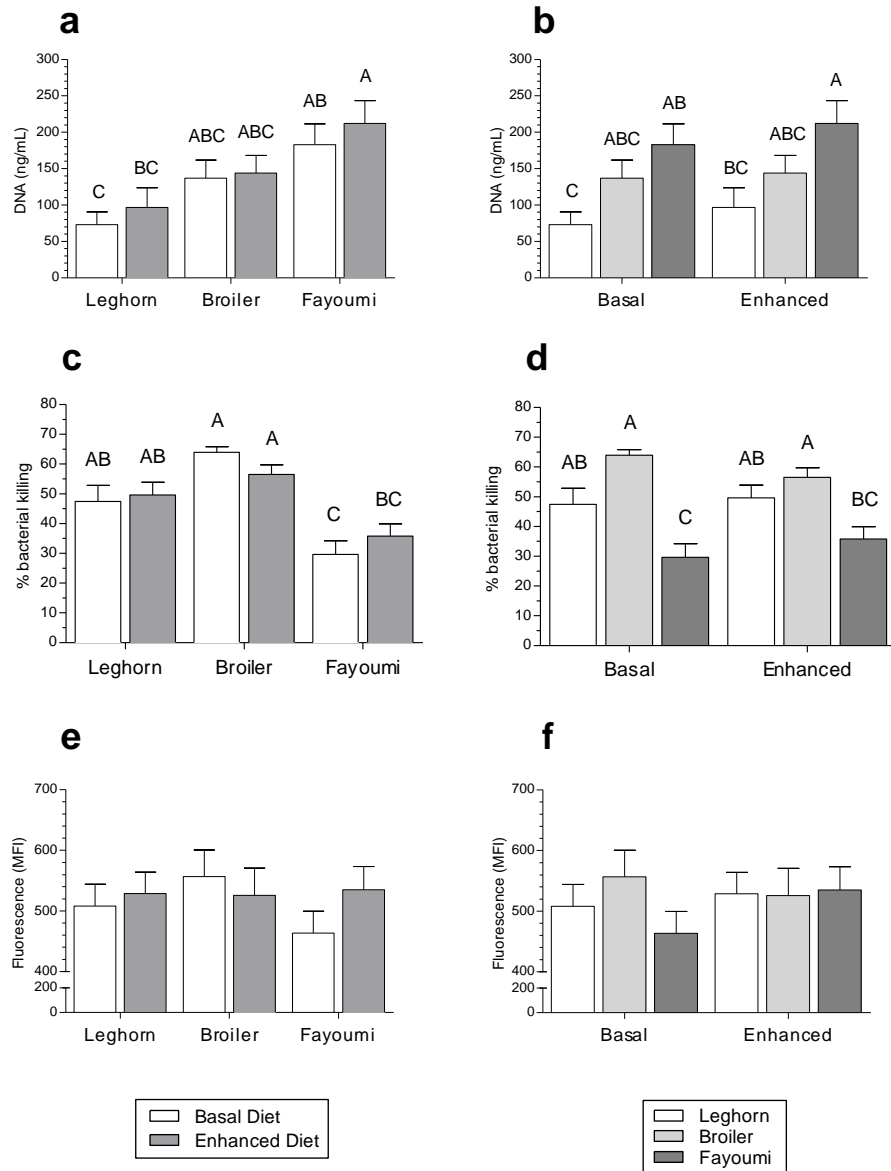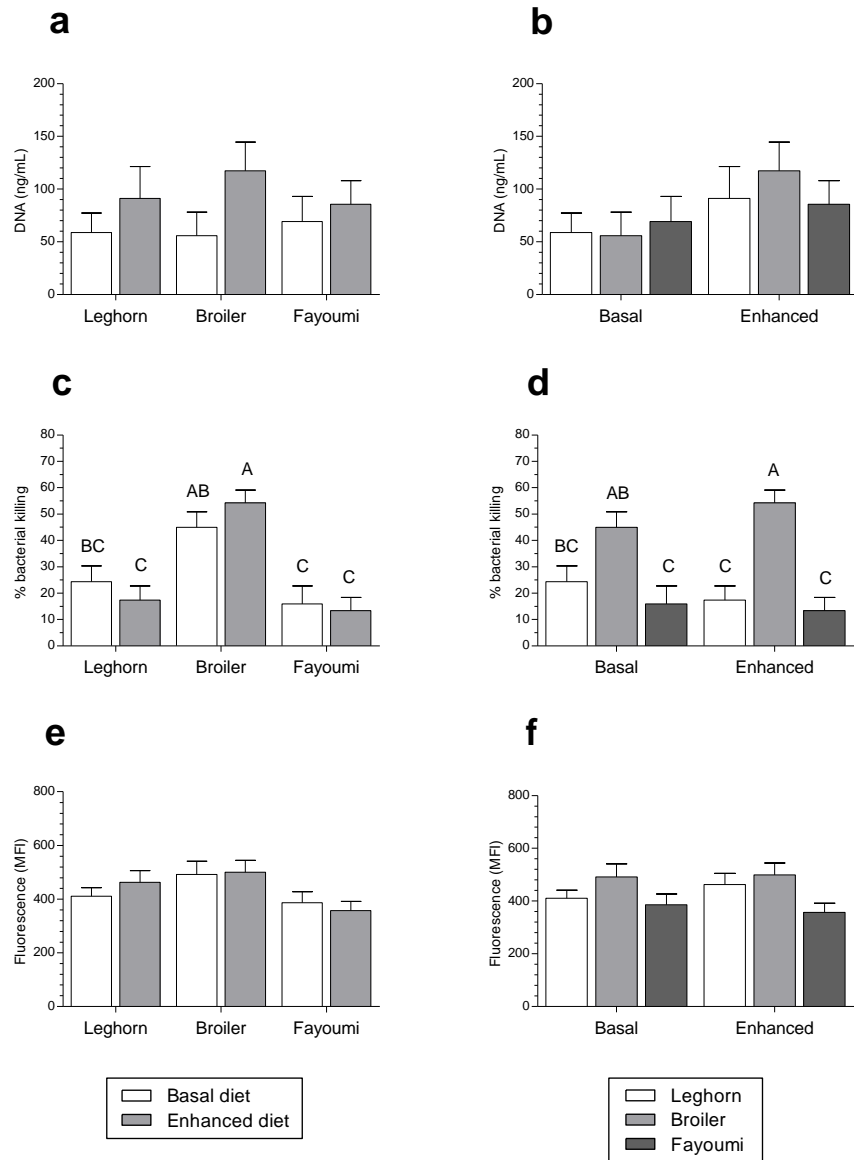
**Fig.2.**Comparisons of genetic lines within the same diet **(a, c, e)** and comparisons of dietary immunomodulators within the same line **(b, d, f)** on heterophil function in *Salmonella*-challenged chickens**. a-b:**HETs-DNA release. **c-d:**Bacterial Killing. **e-f**: Phagocytosis. Data are presented as ng/mL of released DNA (a-b), % bacterial killing (c-d), and mean fluorescence intensity (MFI; e-f). All data are expressed as mean ± S.E.M. from n ≥ 30. Different letters above bars (A, B, C) indicate significant difference (*P< 0.05)*.
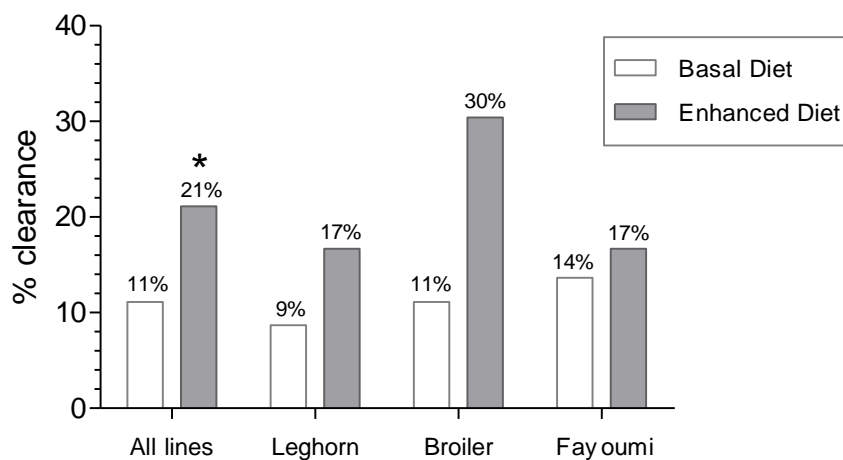
**Fig.3.** The clearance of *Salmonella enterica* serovar Enteritidis (SE) in hens from genetically distinct chicken lines fed basal or immunomodulator enhanced diets. The percentage of SE clearance for basal and enhanced diets was calculated with Fisher's exact test. Overall effect of diet on SE clearance was observed at statistical significance level of 0.09 (*).

Supplemental data:



**Fig.S1**.Effects of genetic line and dietary immunomodulators on heterophil function in non-challenged chickens. **a-b**: HETs-DNA release; **c-d**: bacterial killing; **e-f**: phagocytosis. Data are presented as ng/mL of released DNA (a-b), % bacterial killing (c-d), and mean fluorescence intensity (MFI; e-f). All data are expressed as mean ± S.E.M. from n ≥15. Different letters above bars (A, B) indicate significant difference (*P< 0.05)*.

**Fig. S2.**Comparisons of genetic lines within the same diet **(a, c, e)** and comparisons of dietary immunomodulators within lines **(b, d, f)** on heterophil function in non-challenged chickens**. a-b:** HETs-DNA release. **c-d:** Bacterial Killing. **e-f**: Phagocytosis. Data are presented as ng/mL of released DNA (a-b), % bacterial killing (c-d), and mean fluorescence intensity (MFI; e-f). All data are expressed as mean ± S.E.M. from n ≥15. Different letters above bars (A, B, C) indicate significant difference (*P< 0.05)*.

Supplemental Data S3

## Composition of basal diet

| Ingredient | Percent as fed |
| --- | --- |
| Corn, ground | 59.252 |
| Soybean meal 47% | 26.184 |
| Limestone | 4.751 |
| Limestone/#12 | 4.041 |
| Fat AVI-VEG | 2.819 |
| Dicalphos 22C18.58 | 2.186 |
| Salt | 0.392 |
| Breeder | 0.250 |
| Methionine DL 99 | 0.125 |

The basal diet was a commercially produced corn-soy mixture (Breeder #21-A HYI, Whiton

Feeds, Perry, IA). The enhanced diet added 0.1% β-glucan (w/w; MacroGard Feed

Ingredient, Biotec Pharmacon, Norway) and 0.1% ascorbic acid (w/w; ROVIMIX Stay-C 35,

DSM Nutritional Products, Switzerland).

# CHAPTER 6. GENERAL CONCLUSIONS

## CONCLUSIONS

In the present studies, we determined that chicken heterophils use phagocytosis, bacterial killing, or heterophil extracellular traps (HETs) to respond to the presence of *Salmonella enterica* serovar Enteritidis. Heterophil function was modulated by the application of immunomodulators, β-glucan, ascorbic acid or corticosterone. The enhancement of heterophil functions occurred with or without *Salmonella* challenge. Genetic background of the chicken breed or line was demonstrated to play a vital role in heterophil function responses in birds fed with immunomodulators.

An important step before investigating the effects of dietary supplementation of immunomodulators was to define the presence of well-established functional capabilities of heterophils in each chicken line. It was therefore necessary to determine the base line response.

1) We successfully characterized the presence of heterophil extracellular traps in avian heterophils (chapter 3) that have potential role in extracellular trapping and killing of pathogens. We have also established the technique to quantitate the amount of DNA released during HETs formation.

2) We utilized newly defined HETs-DNA release along with assay of other intracellular killing mechanisms to evaluate a base-line response from three diverse chicken lines (broiler, Leghorn and Fayoumi). This evaluation revealed that genetic background of chickens has significant influence on heterophil function responses. The responses of

Fayoumis significantly differed from broilers and Leghorns in HETs-DNA release ($p =$ 0.002; Chapter 4).

3) The significant difference in heterophil responses from different chicken lines exposed to immunomodulators (β-glucan, ascorbic acid and corticosteroids) *in vivo* was observed (Chapter 4). The genetic line significantly affected bacterial killing ($p < 0.001$) and HETs-DNA release ($p = < 0.001$). Moreover, diet effect played a major role in HETs-DNA release ($p = 0.043$).

4) The increase in *Salmonella* Enteritidis infection clearance in hens fed diet supplemented with immunomodulators was observed (Chapter 5). The heterophil function under supplemented diet conditions is highly affected by genetic background of chicken line (chapter 5) and the effect of dietary immunomodulators was observed in HETs-DNA release assay of challenged chickens.

Collectively, we have presented evidence herein that the genetic background and dietary immunomodulation have affected the overall chicken heterophil function in response to challenge with pathogenic *Salmonella enterica* serovar Enteritidis. We conclude that heterophil function in chickens, which is required for disease resistance, is not only critically dependent on the diet but also strongly influenced by genetic components of each line.

## RECOMMENDATIONS FOR FUTURE RESEARCH

The findings summarized in this dissertation constitute advance understanding the roles of heterophils in chicken research; however, extensive additional studies are needed to determine the beneficial roles of this granulocyte in more detail. Using gained knowledge from current studies could potentially expand the depth and scope of the forthcoming

research. As noted by other involved research results generated by our group, some immune response genes are very likely to involved in SE colonization and infection of particular chicken genetic lines (for instance; IL-6, GM-CSF, TGF-β4, and IL-10). The global pattern of cytokine and/or chemokine gene expressions of activated heterophils under both diet supplementation and SE challenge is one of the remaining questions. Thanks to the emergence of DNA microarray technology, a large number of expressed genes under *Salmonella* exposure and immunomodulatory effects can be surveyed quicker than ever before. As more information from this technology accumulates, certain pathways involved in heterophil functional activity, for instance HETs, would be another potential feature to study. To determine a big picture of complex immunity, how heterophils interact with other cells in immunity especially macrophages and lymphocytes would be a point to be investigated. Research from this immunological standpoint could be beneficial in fulfilling the missing piece of information.

As recognized in this and other studies that β-glucan and ascorbic acid are two of many immunostimulants, it is advised to focus on another potential immunostimulants that could improve efficiency of innate cellular immunity. Other promising immunomodulators (e.g. green tea and other herbal extracts) that provide potent increase in heterophil functions could be addressed and tested in the future research.

# ACKNOWLEDGEMENTS