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Effects of Dietary Copper, Zinc and Manganese Source and Level on the Acute Inflammatory Response of Broilers

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Effects of Dietary Copper, Zinc and Manganese Source and Level on the Acute Inflammatory Response of Broilers

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Abstract

Trace minerals, particularly copper, zinc and manganese, play a role in ensuring optimal immune function. To examine the effects of diets containing different levels and sources of trace minerals on the inflammatory response, Cobb 500 broilers were fed four experimental diets containing 5 mg/kg Cu and 45 mg/kg of Zn and Mn or 10 mg/kg Cu and 90 mg/kg Zn and Mn in either the sulfate or hydroxychloride form. When the broilers were 5 weeks of age, 16 μg of lipopolysaccharide (LPS) was injected into the pulp of growing feathers (GF; 16 GF per chicken; 1 μg LPS per GF). Injected GF and blood were sampled before (0 hour) and at 6- and 24-hours post-GF-injection of LPS. Samples were used to determine leukocyte infiltration profiles in LPS-injected pulps, alterations in concentrations and proportions of blood leukocytes (WBC), concentrations of thrombocytes, red blood cells (RBC) and other RBC-related measurements, reactive oxygen species (ROS) generation, and superoxide dismutase (SOD) activity. A time effect ($P \leq 0.05$) was observed for most of the aspects examined. In GF, LPS-injection into the pulp resulted in heterophil and monocyte/macrophage infiltration that reached maximal levels at 6- and 24-hours, respectively. Additionally, levels of ROS generation were greatly increased at 6 hours post-pulp injection of LPS. In the blood, GF injection of LPS increased heterophil and monocyte concentrations at 6 hours, thrombocyte concentration and plasma SOD activity at 24 hours, and decreased lymphocyte and RBC concentrations at 6 hours. While few dietary effects were observed, birds fed lower trace mineral levels had lower SOD activity in the GF pulp (but not in the plasma) than the chickens fed higher levels of trace minerals. Using the GF as a minimally invasive, cutaneous test-site, together with periodic sampling of injected GF and blood for laboratory analyses, the early phase of LPS-induced inflammatory response could be observed both at the local (GF-pulp) and systemic (peripheral blood) level in the same broiler

chickens. Furthermore, inclusion of the GF-injection approach revealed effects of dietary supplementation on the inflammatory activities in the inflamed tissue that were not detected in the blood.

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Table of Contents

List of Tables and Figures

Literature Review

Introduction

The current commercial poultry industry is constantly researching and developing new methods to rear chickens in the most efficient and safest ways possible. With the constant increase in human population numbers, food consumption is bound to also increase, which means that agricultural producers must strive to meet demands. The modern day broiler chicken is one of the most efficient sources of protein in terms of production, and the broiler plays a major part in human food security (Mottet and Tempio, 2017).

When considering the impact of the broiler chicken as a protein source, it is of utmost importance to keep these birds healthy and productive. Any efforts to better understand the chicken's immune system and improve their immune function should be prioritized. A major input in commercial poultry production is the feed provided to the birds. Optimization of the broiler's nutrition offers a major window of opportunity that will help the chicken grow and produce in the most efficient and healthiest way.

Trace minerals and the form they are fed in can play a role in altering immune system functions. Copper, zinc and manganese in particular play a role in multiple biological systems in the body including immune function, and also serve as cofactors for important enzymes such as superoxide dismutase, an important antioxidant. Different forms of copper, zinc and manganese have been fed to chickens over the years and traditionally these micronutrients have been fed in the form of inorganic salts. In an attempt to increase stability and bioavailability of these trace minerals, the industry is now looking at feeding them in the form of hydroxychlorides (Miao and St. Clair, 2009; Naz et al., 2016; Perez et al., 2017).

The acute inflammatory response is a common area of study in research examining nutrient requirements (e.g., trace minerals) for optimal immune system development and function. Lipopolysaccharide (LPS) of Gram-negative bacteria is a well-known stimulant of innate immune system activities, including induction of the inflammatory process. Inflammation involves the recruitment of leukocytes from the blood to the site of infection, activation of leukocytes to eliminate or contain the infection, as well as tissue healing and reestablishing homeostasis (Abbas et al., 2014). This is achieved by complex signaling between the affected tissue and the periphery. Therefore, in order to fully assess the acute inflammatory response to LPS in an individual, examination of activities at both the local LPS injection site and the peripheral blood circulation is needed. For chickens there is a unique opportunity to accomplish this dual approach using minimally invasive procedures. Specifically, the pulp of growing feathers (a skin derivative) has been developed as a tissue testing site to monitor the local inflammatory activities initiated by pulp injection of LPS (Erf and Ramachandran, 2016). For this, LPS can be injected directly into the pulp of multiple growing feathers of a chicken to initiate inflammation, and later the growing feathers can be collected at separate time points to examine inflammatory activities (e.g. leukocyte infiltration) taking place in situ over time in the same animal (Byrne, 2016; Erf and Ramachandran, 2016). When combined with concurrent sampling of the blood, inflammatory activities can be assessed at both the local tissue site (pulp) and in the periphery. For this experiment, we'll use this approach for the first time to study the effects of feeding experimental diets containing different levels and sources of trace minerals on the quality of the LPS-induced acute inflammatory responses in broiler chickens.

Broilers

The broiler is a chicken raised for meat production. Today's broiler has been genetically selected to produce a high quality carcass in the most efficient way possible. Poultry plays a major role in food security and nutrition, and poultry meat offers a quality source of protein, energy and many other nutrients for humans. Globally, poultry meat is also one of the most common animal source foods consumed (Mottet and Tempio, 2017). As the world's population continues to grow, the demand for poultry meat will also continue to increase, and today's broiler chicken is an invaluable resource. In order to meet consumer demands and customer requests, we must continue to develop new ways to raise our birds in the safest, healthiest and most efficient way.

The poultry industry is constantly exploring innovative alternatives for improving flock health and efficiency. Looking into what the chickens eat, and how this plays a role in their immune system response is just one way to combat health issues in broiler flocks.

Following current industry trends, the term antibiotic-free poultry is a very popular and demanded product label. In recent years there has been a public push to raise commercial chickens without the use of any antibiotics, which has posed threats to both bird health and production efficiency. Today's consumer is concerned about overuse of drugs in food animals and the role it plays in developing antibiotic-resistant bacteria (Centner, 2016). Antibiotic resistance is one of the most serious health threats in the United States according to the US Centers for Disease Control and Prevention (CDC) (Centers of Disease Control and Prevention, 2013). If we can better understand the avian immune system and which feed additives optimize its function, we can eventually produce poultry meat raised without any antibiotics more easily.

Researchers are constantly trying to find new ways to improve flock health and performance through means other than antibiotics or drugs. A huge window of opportunity to better bird health and performance lies in their diet. "Almost all nutrients in the diet play a fundamental role in sustaining an optimal immune response, such that deficient and excessive intakes can have negative consequences on immune status and susceptibility to a variety of pathogens" (Kogut and Klasing, 2009).

One of the most important parts of the broiler industry in particular, is the bird's nutrition. Feed costs represent the greatest input cost in poultry production, and their nutrition directly affects the bird's performance and more importantly, the meat that it is capable of producing (Fanatico, 2003). Feeding high quality ingredients are a must, and the industry is always trying to find new forms or types of ingredients to make production more efficient overall.

Trace Minerals

Copper, zinc and manganese are essential trace minerals that play a role in various biological activities in the body including bone and joint health, growth performance, feather development, feed efficiency, metabolic functions, immune functions and more (Miao and St. Clair, 2009; Naz et al., 2016; Perez et al., 2017). These trace minerals also are co-factors for many important enzymes such as the antioxidant enzyme superoxide dismutase (SOD).

Over time, new technology has been introduced and has changed the way that these trace minerals can be produced as commercial feed additives. Significant advances have been made to establish more bioavailable forms of copper, zinc and manganese.

Copper, zinc and manganese have traditionally been fed to poultry in the form of inorganic salts, commonly known as sulfates, oxides and carbonates (Singh et al., 2015).

However, in more recent years, the industry has started to feed these minerals in the form of hydroxychlorides due to advances made in their in bioavailability, absorption and stability (Naz et al., 2016). More specifically, the Intellibond® sources of copper, zinc and manganese are hydroxychloride trace minerals that have three-dimensional crystalline structures. This crystalline structure provides optimal stability and helps to prohibit interactions with unwanted antagonists (Cohen and Steward, 2012). It also allows for more availability and protection of the minerals, and prolongs the release of these minerals so that they have a longer amount of time to be absorbed in the gut (Olukosi et al., 2019). When compared to sulfates, the hydroxychloride trace minerals have covalent chemical bonds, which also aid in efficiently transferring metals to the appropriate receptors for absorption (Cohen and Steward, 2012).

When considering different aspects of copper, zinc and manganese, it is also important to consider potential environmental impact that these minerals could have. The NRC set requirements for copper, zinc and manganese in 1994, however with advances in poultry genetics, technology, environmental elements, and other factors, many wonder whether these original requirements are still accurate for today's broiler (NRC, 1994). Current industry trends lean towards providing a large safety margin for trace minerals by formulating higher amounts than what is necessarily needed. This results in significant mineral excretion, which can then take a toll on the environment when the poultry litter is spread as fertilizer (Bao et al., 2007; Singh et al., 2015). By determining the amount of minerals required by broilers and using the most bioavailable option possible, we can lower the amount of minerals that are fed to the birds and in turn, lower the amount excreted.

Immune System

The avian immune system is comprised of two major parts, innate and adaptive immunity. Innate immunity is the first to respond and defend against unwanted microbes or antigens. The innate immune system is stimulated by pathogen-associated molecular patterns (PAMPs) and different types of microbes have different PAMPs that are recognized by soluble factors and cells of innate immunity. The innate system is comprised of three main defense mechanisms that help protect against microbes. These include the protection of an organism by its epithelial surfaces to prevent entry of microbes, and phagocytic cells and various blood proteins that work within the organism. If a microbe comes into contact with an organism's epithelial surface, both physical and chemical defense mechanisms will begin. If a microbe makes it past the epithelial barriers and makes it into the body, it will then encounter the cells of the innate immune system. In chickens, the cells of the innate immune system include granulocytes, such as heterophils, eosinophils, basophils and mast cells, as well as monocytes/macrophages and innate lymphoid cells like natural killer cells, all of which are leukocytes. These cells may produce inflammatory cytokines and antiviral proteins in addition to killing microbes. In contrast to adaptive immunity, innate immune cells begin working within minutes to hours after initial contact with a pathogen (Kumar et al., 2011; Abbas et al., 2014).

The inflammation reaction is initiated by the entry of a microbe into the tissue. Local tissue macrophages sense the infection and recruit the appropriate leukocytes from the blood stream to the site of the infection and tissue injury. The primary leukocytes that accumulate, activate and eventually destroy microbes are phagocytes, specifically heterophils, a granulocyte, and monocytes/macrophages (Abbas et al., 2014).

As in mammals, heterophils, the avian counterpart of the mammalian neutrophils, are the first line of defense in infections by extracellular microbes, especially bacteria, arriving within hours in large numbers in infected tissues. The heterophils are very active phagocytes, but are relatively short-lived and die within 24-48 hours of arrival. The rapid infiltration of heterophils is however accompanied by a slower infiltration of monocytes. Once in the infected tissue, monocytes differentiate further into macrophages and become activated phagocytes. Unlike heterophils, which reach peak infiltration levels within hours of initiation of the acute inflammatory response, recruitment, activation and function of macrophages are prolonged, continuing through elimination/containment of the infection. Moreover, while both heterophils and monocytes are phagocytes, meaning that they can be characterized as cells that ingest microbes to kill them in a process called phagocytosis, macrophages are the more effective phagocytes, capable of activating more sophisticated killing mechanisms than heterophils. They also help in cleaning up the damaged tissue as they ingest dead cells (including heterophils), and initiate tissue repair (Harmon, 1998; Abbas et al., 2014; Byrne, 2016).

Depending on the type of infection, other granulocytes such as basophils and eosinophils may also participate in acute inflammation, although they specialize in killing larger invaders (helminths) by releasing the toxic contents of their granules rather than removal of the microbe by phagocytosis. Basophils and eosinophils circulate in the blood and can be recruited to sites of infection in tissue by similar mechanisms as those for other leukocytes.

In addition to cellular components, acute inflammation also involves soluble components of innate immunity such as proteins of the complement system that are present in the plasma and tissue fluids and are activated by the presence of microbes. Once activated these soluble components can help in the recruitment of more leukocytes to the site of infection, facilitate

leukocytes binding to microbes for more effective phagocytosis and directly lyse microbes without cell walls by disrupting membrane integrity (Abbas et al., 2014).

Acute inflammation can develop rapidly in just minutes and can continue for hours and even days in some cases. Acute inflammation is initiated in tissues by cytokines and chemokines produced by local cells of innate immunity. The most important and abundant proinflammatory cytokines are tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-6 and the chemokine IL-8. These soluble signaling molecules aid in inducing inflammation and mainly stem from activated macrophages. T cells, heterophils, epithelial cells, and endothelial cells also serve as a source of some proinflammatory cytokines (Abbas et al., 2014).

Leukocytes such as macrophages recognize PAMPs of microbes with pattern recognition receptors (PRR). Binding of PAMPs to PRRs then activates the macrophages to initiate inflammation and fight the microbial infection. Toll-like receptors (TLRs) are an example of PRRs that are associated with cell membranes (i.e. plasma membrane and the membranes of endosomes and phagosomes). These TLRs can recognize different microbial ligands (i.e. PAMPs) that are shared by a large group of microbes and needed for the survival of the microbes. For example, TLR4 is expressed on the plasma membrane of macrophages and is activated by lipopolysaccharide (LPS), and important cell wall component common of Gram-negative bacteria. Activation of macrophages via TLR4 results in an intracellular signaling cascade that initiates phagocytic processes, gene expression, production and release of cytokines and chemokines, activation of enzymes and generation of reactive oxygen species. Lipopolysaccharide, also known as endotoxin, is a potent stimulator of inflammation, and, therefore, it is often used in research to examine the inflammatory response. In chickens, previous studies have demonstrated that injection of LPS results in heterophil and macrophage

infiltration to the site of injection typical of the classic acute inflammatory response profiles described for mammals (Smith et al., 1975; Yasui et al., 1994; Farnell et al., 2003; Kogut et al., 2003; Qureshi, 2003; Parham, 2005; Abbas et al., 2014; Byrne, 2016; Perez et al., 2017).

Ultimately, LPS stimulates neutrophils/heterophils and macrophages, and upon activation, these cells convert molecular oxygen into highly reactive oxygen species (ROS) in a cascade of elevated oxygen consumption known as the respiratory burst. These ROS can destroy both microbes and other cells that they come into contact with (Yasui et al., 1994; Abbas et al., 2014; Perez et al., 2017). High levels of ROS can induce oxidative damage to the cells and lead to oxidative stress in the affected tissue. Antioxidant systems, such as the enzyme superoxide dismutase are crucial in minimizing oxidative stress and damage. Superoxide is a common reactive oxygen species, and superoxide dismutase (SOD) protects against the toxicity of oxygen. "SOD catalyzes the conversion of two superoxide anions into a molecule of hydrogen peroxide (H_2O_2) and oxygen (O_2)" (Held and Instruments, 2015) and from here, peroxide can be broken down further into oxygen and water and is no longer harmful (Marklund et al., 1982). In eukaryotic cells, there are two main types of SODs found, i.e., SOD 1 (Cu/Zn-SOD) and SOD 2 (Mn-SOD). Copper, zinc and manganese are cofactors for SOD, which is also known as a metalloenzyme. Cu/Zn-SOD is primarily found in the cytosol, and Mn-SOD is primarily found in mitochondria. Many different cell types produce superoxide radicals. However, the oxidative burst by activated phagocytes, which is designed to kill the phagocytosed microbe, causes excessive amounts of ROS to be generated which requires SOD activity to avoid damage to the phagocyte (Marklund et al., 1982; Karlsson and Marklund, 1987; Griess et al., 2017; Perez et al., 2017). Additionally, previous research has shown that SOD activity levels increase after LPS injection.

Scientific information on the effects of trace minerals on the inflammatory response in chickens is limited. Perez et al. (2017) recently conducted a study examining the effects of low- and high- levels of Mn and Zn supplemented in either a hydroxychloride or sulfate form on the SOD activities in heterophils and monocytes isolated from blood of both layer and broiler chickens 24 h after they were injected with LPS. According to their findings, SOD activity levels in heterophils and monocytes from LPS stimulated broiler chickens were lower when chickens were fed marginally lower levels of trace minerals than conventional supplementation levels, independent of the form of the trace minerals. Additionally, there were no differences in SOD activity of heterophils and monocytes from LPS injected broilers when trace minerals were provided in the sulfate or hydroxychloride form, independent of the supplementation level (Perez et al., 2017).

Feather Injection

During inflammation, leukocytes are recruited from the blood to the site of infection in the tissue, and upon activation, begin processes to eliminate the microbes (Abbas et al., 2014). To fully examine the acute inflammatory response in an individual, it is important to consider both the area of infection in the tissue as well as the systemic responses in the peripheral blood. When studying the avian immune responses to PAMPs in vivo, common practices usually involve injecting PAMPs into skin derivatives such as the chicken wattle or wing web (Awadhiya et al., 1981; Cotter et al., 1987). When using these skin derivatives to study inflammation, measurements are usually limited to measuring the time-course and extent of the swelling response, because sample collection for ex vivo examination involves very invasive procedures or euthanasia of the experimental animal, thus prohibiting evaluation of local activities over time in the same individual. A novel method developed by Erf (US Patent

8,216,551; Licensed by Nanomatronix, LLC) utilizes the growing feather (GF) as a far less invasive testing site for in vivo analysis of both tissue and cellular activities initiated by intradermal injection of test material into the GF pulp (Byrne, 2016; Erf and Ramachandran, 2016; Erf et al., 2017; Sullivan and Erf, 2017). The GF of a bird has a portion of living integument in the form of the feather pulp. The pulp consists of a column of vascularized dermis (8-10 mm in height x 2-3 mm diameter) that is enveloped by a layer of epidermis, and resides inside a hard, keratinized sheath. The pulp can be injected with a test material (e.g. LPS) to stimulate an acute inflammatory response. Multiple GFs may be injected at the same time but collected at different time points afterwards and can be accompanied by concurrent blood sample collection. By doing this, both the local and systemic inflammatory responses can then be examined via ex vivo analyses such as preparing single cell suspensions from the GF pulps for immunofluorescent staining and determination of leukocyte infiltration profiles by flow cytometry and by analysis of blood cell profiles using automated hematology (Erf and Ramachandran, 2016).

Hypothesis

Based on previous research studies, we hypothesize that when experimental diets containing different levels and sources of copper, zinc and manganese are fed to broiler chickens, the local and systemic inflammatory responses following an intradermal LPS injection into the pulps of growing feathers will differ depending on the source and level of the trace mineral supplementation.

Objectives

The purpose of this study is to examine the effects of feeding broiler chickens diets containing conventional and marginally lower levels of hydroxychloride and sulfate forms of copper, zinc and manganese on the in vivo inflammatory response in pulp and blood initiated by intradermal injection of LPS into the pulps of growing feathers. A 24 h time-course study will be conducted to determine local (GF dermal tissue) and systemic (blood) inflammatory activities such as pulp infiltration profiles of leukocytes, alterations in the concentrations and proportions of blood leukocytes, concentrations of thrombocytes and red blood cells (RBC), as well as various other RBC-related measurements. The generation of reactive oxygen species by pulp cell suspensions as well as SOD activities in the pulp homogenates and plasma from pulp and blood samples will also be examined. For all analyses, pulp and blood samples collected before and 6 h and 24 h after pulp injection of LPS will be used. To examine leukocyte infiltration in GF pulps, pulp cell suspensions will be prepared, immunofluorescently stained using chicken leukocyte-specific antibodies and subjected to leukocyte population analysis by flow cytometry. Automated hematology will be used for determination of different cell populations in the blood following LPS pulp-injection. To determine generation of ROS by pulp cell suspensions, samples will be used in a kinetic fluorescence assay and SOD activity levels in pulp homogenates and plasma will be determined using a superoxide dismutase assay.

MATERIALS AND METHODS

Experimental Animals and Diets

The University of Arkansas IACUC committee approved all protocols and procedures involving animals used in this trial (IACUC approval number 16054). Fifty-five Cobb MVx500 straight run broiler chicks from a commercial hatchery were obtained on day of hatch. All chicks were tagged for identification before leaving the hatchery facility. Chicks were placed in a temperature-controlled, biosecure room at the Arkansas Experiment Station's Poultry Health Laboratory, Division of Agriculture, University of Arkansas, Fayetteville. All birds were provided ad libitum access to both feed and water. For the first week of life, all chicks were penned together in a 3 ft. x 6 ft. floor pen bedded with wood shavings. The birds were checked and facility cleaned daily. A common starter diet meeting the primary breeder nutrient recommendations was fed to chicks until 7 d post-hatch. A lighting schedule and specific temperature targets were implemented and adjusted according to management guidelines set forth by the primary breeder. At 2 d post-hatch, a small sample of blood (2-3 μL) was collected from the wing vein and used for PCR testing to determine gender by molecular methods in Dr. D. Rhoads Laboratory in the Department of Biological Sciences, University of Arkansas, Fayetteville (Mozdziak et al., 2005).

A basal corn-soybean meal-based grower diet was formulated to meet primary breeder nutrient recommendations except for Cu, Zn and Mn concentrations. Copper, zinc and manganese were fed as either inorganic sulfate or hydroxychloride (Intellibond, Micronutrients, LLC) forms and at two different concentrations. Four experimental diets were formulated by mixing a portion of this initial basal diet with either 5 mg/kg Cu and 45 mg/kg of Zn and Mn from either sulfate or hydroxychloride sources, or 10 mg/kg Cu and 90 mg/kg of Zn and Mn

from either sulfate or hydroxychloride sources. At 7 d post-hatch, birds were randomly separated into 12 pens (3 ft. x 3 ft.) with 4 birds per pen, while making sure that there was at least one male and one female per pen. Each pen of birds was then randomly assigned one of the four experimental diets with varying concentrations and sources of Cu, Zn and Mn. Experimental diets were provided ad libitum in a mash form from d 7 to d 42. Samples of experimental diets were obtained for proximate composition and mineral concentration analyses by the Division of Agriculture's Analytical Laboratory, University of Arkansas, Fayetteville (Table 1). On d 42, all birds were euthanized by intravenous injection of sodium pentobarbital (Somnasol, Henry Schein, OH).

Initiation of a Local Inflammatory Response in the Dermis of Growing Feathers by Pulp-injection of Lipopolysaccharide

To ensure availability of uniform growing feathers for lipopolysaccharide (LPS) injection, growing feathers were plucked from the breast tracts of each bird when the birds were 16 days of age and allowed to regenerate for 18 days. For this, each bird was restrained by Velcro straps around their legs and laid on their side with one wing underneath their body to prevent movement. A solution of 70% ethanol was sprayed on the breast of the bird to wet down the feathers and sanitize the breast tract area. Once the breast tract feathers were located, a row of 10 to 12 growing feathers running parallel to the keel bone of the bird were plucked by applying gentle pressure on the skin with one hand and pulling the growing feather with the other hand. This method was applied to both the right and left breast feather tracts of each bird.

When the chicks were 34 days of age (18 days post plucking), a row of growing feathers (**GFs**), all at the same stage of development, was present on each breast feather tract of the birds.

For LPS injection, a LPS solution was prepared using LPS from *Salmonella typhimurium* (Sigma, Chemical Company, Saint Louis, MO) diluted in endotoxin-free Dulbecco's Phosphate-buffered Saline (PBS; Sigma, Chemical Company, Saint Louis, MO) to a final concentration of 100 μg LPS/mL. Disposable 0.3 mL syringes with a 0.01 mL graduation and 8 mm length by 31 gauge needles (BD; Becton, Dickinson, and Company, Franklin Lakes, NJ) were aseptically filled (Erf et al., 2017-JAT graphene paper) in a NuAir Biological Safety Cabinet (NuAir, Plymouth, MN) with 200 μL of LPS solution (100 μg LPS/mL), one syringe per chicken, and transported to the Poultry Health Lab animal room for injections. One male and one female from each pen were selected at random to be used for GF injection with LPS. For GF injection, each chicken was restrained as previously described, and their breast feathers were sprayed down with 70% ethanol solution. Once the row of 18-day-old GFs was found, 8 GFs on each breast tract were prepared for injection (16 total per bird). For this, the barbs emerging from the sheath were cut off with scissors leaving about 3 mm of sheath above the pulp epidermis. Once each GF was cut, the injection process began. Using the pre-filled 0.3 mL syringes, each GF's pulp was carefully injected with 10 μL of LPS solution by inserting the needle through the top of the GF and into the center of the column. To ensure proper delivery of LPS in the middle of the pulp within the column, the needle was fully inserted into the center of the pulp until the syringe head was resting on the sheath, hence stabilizing the injection and avoiding unnecessary damage to the pulp.

Sample Collection

Three time points were assessed in this study. Samples were collected prior to LPS injection (0 h sample), six hours post LPS injection (6 h sample) and twenty-four hours post LPS

injection (24 h sample). For each time point, the samples were collected and analyzed in the same way.

At each time point, a total of 6 growing feathers and one blood sample were collected from the male and female broiler from each of the twelve pens. Two growing feathers were plucked (un-injected for 0 h time point, injected for 6 and 24 h time points) and placed into a tube of ice cold endotoxin-free Dulbecco's Phosphate-buffered Saline (PBS; Sigma, Chemical Company, Saint Louis, MO) and kept on ice until use in the laboratory. The other 4 growing feathers were plucked and placed inside an aluminum foil, labeled pouch that was immediately placed in liquid nitrogen to snap freeze these samples for later analyses. Approximately 2 mL of blood was collected from the brachial (wing) vein via 1 inch x 25 gauge needle into a heparinized 3 mL syringe to prevent coagulation. Once all blood and GF samples were collected at a time point, samples were taken immediately back to the laboratory. The 2 GFs in PBS were used for same day preparation of pulp cell suspensions for immunofluorescent staining and leukocyte population analysis by flow cytometry as well as analysis of reactive oxygen species generation (ROS) by kinetic fluorescence assay. Blood was used for isolation of plasma and whole blood cell population analyses by automated hematology. The 4 GFs that were snap frozen in liquid nitrogen were placed in a box inside a freezer at -80°C for later preparation of pulp homogenates.

Preparation of Pulp Cell Suspensions

Single-cell suspensions from the pulps of the sampled GFs were made for both cell population analysis and determination of ROS activity on the day of collection. To isolate the pulp from GFs, the sheath was cut longitudinally, and the entire pulp was pulled out of the sheath

with a pair of forceps (Erf and Ramachandran, 2016). The pulp was then placed in 1 mL D-PBS containing 0.1% collagenase (type IV, Life Technologies, Carlsbad, CA) and 0.1% dispase II (Boehringer Mannheim, Mannheim, Germany) and incubated in a 40°C water bath for 10 minutes. After incubation, the 2 pulps from each bird were gently pushed through 60 μm nylon mesh along with ice-cold D-PBS to make a single-cell suspension. The pulp cells were then washed by centrifugation at 250 x *g* for 8 minutes at 4°C two times. The final pellet of cells was re-suspended to a final volume of 0.5 mL with D-PBS and kept on ice until use for immunofluorescent staining or ROS kinetic fluorescence assay.

Immunofluorescent Staining of Pulp Cell Suspensions and Cell Population Analysis by Flow Cytometry

Pulp cell suspensions were immunofluorescently stained (Erf and Ramachandran, 2016) using multiple fluorescently labeled mouse monoclonal antibodies (mAb) specific for chicken leukocyte markers (Southern Biotechnology Associates, Inc. Birmingham, AL). All cell suspensions were dual labeled with either 1) chicken pan leukocyte marker CD45-specific mAb conjugated to spectral red fluorochrome (SPRD) together with phycoerythrin (PE)-conjugated mAb specific for the chicken monocyte/macrophage marker KUL01 (KUL01-PE), or 2) with mAb specific for the chicken B cell marker Bu-1 conjugated to PE (Bu-1-PE) together with the mAb specific to the chicken T cell marker CD3 (CD3-SPRD). The isotype of all mouse monoclonal antibodies used was IgG1. The leukocyte-specific antibodies, along with PE- and SPRD-conjugated isotype controls (mouse IgG1 with irrelevant specificity; Southern Biotech) to identify non-specific binding of monoclonal antibodies, were diluted 1:100 in PBS+ (DPBS, 1% bovine serum and 0.1% sodium azide). For immunofluorescent staining, 50 μL of cell suspension

and 50 μL of antibodies were added to wells of a 96-well round-bottom plate. A pool of cell suspensions was also added in separate wells and combined with 50 μL of isotype control antibodies. Two-color cell population analysis was conducted as described in Erf and Ramachandran (2016) using a BD C6 Accuri flow cytometer (BD; Becton Dickinson Immunocytometry Systems, San Jose, CA) and FlowJo software (Flow Jo, LLC, Ashland, OR). The heterophil (avian neutrophil) population was identified based on size (FSC) and granularity (SSC) characteristics of CD45+ leukocytes (Seliger et al., 2012). All data were expressed as the percentage of stained cells in the total pulp cell suspension (% pulp cells).

Reactive Oxygen Species Activity

Reactive oxygen species (ROS) generation was determined for each pulp cell suspension in three wells of a 96-well flat-bottom plate. For this, 100 μL of cell suspension was added to 100 μL of room temperature D-PBS to each well to make a 1:1 solution used for the 0 h (before LPS injection) samples and then incubated for one hour at 37°C. Due to increased ROS generation levels following LPS injection, 6 h pulp cell suspension samples were diluted 1:4 with D-PBS, and 24 h pulp cell suspensions samples were diluted 1:2 with D-PBS. Meanwhile, 1 mg of 2', 7'-dichlorofluorescin-diacetate (DCF-DA, Sigma, St. Louis, MO) was dissolved in 100 μL of 100% ethanol to make a stock solution. This DCF-DA stock solution was immediately covered and protected from light while it was thoroughly mixed for 20-30 minutes. After the mixing process of DCF-DA and ethanol was complete, 2.30 μL of this DCF-DA stock solution was added to 1 mL of PBS. After the 1 h incubation of the pulp cell suspensions, 20 μL of DCF-DA solution was pipetted into each well containing sample. Controls were added to each plate to rule out any background fluorescence activity, and then the plate was immediately taken to a

plate reader. The plate reader was set at 37°C with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Generation of fluorescence from the samples was analyzed via kinetic read for 1.5 h at 10-minute intervals. Data were transformed by making graphs of each sample's kinetic readings, and from there a line of best fit was calculated. The slope of each line of best fit's equation was used to report the extent of ROS generation (Rath et al., 1998).

Blood Profile Analysis via Automated Hematology (CellDyn)

Half of the blood sample (1 mL) collected from all 24 birds at each time point was taken to the automated hematology analyzer (Cell-Dyn; Abbott Diagnostics, Abbott Park, IL) kindly provided by the USDA-ARS Poultry Production and Product Safety Research Unit housed in the Center of Excellence for Poultry Science, Fayetteville, AR. Data acquired included blood leukocyte (heterophils, monocytes, lymphocytes, basophils, and eosinophils) concentrations (K/μL) and percentages, concentrations of thrombocytes (K/μL), concentration of red blood cells (Millions/μL), as well MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), and RDW (red cell distribution width).

Superoxide Dismutase Activity

A Superoxide Dismutase (SOD) assay kit was obtained from Cayman Chemical (Item No. 706002, Ann Arbor, Michigan) to determine activity in both GF pulps and plasma samples. Multiple assays were performed to determine the optimal procedures and concentrations for both pulp homogenates and plasma samples.

The optimized procedure was as follows: Feather pulp SOD activity was determined using the previously frozen samples collected from all 24 birds at each time point. Each pulp was

removed from the feather sheath as previously described in (Erf and Ramachandran, 2016) and weighed. The pulp was then placed in a 1.5 mL microcentrifuge tube and cold 20 mM HEPES buffer (pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) was added at 1 part pulp weight to 10 parts of buffer volume (e.g. 30 mg plus 300 μL HEPES buffer). A volume of zirconium oxide beads (0.5 mm) equivalent to the pulp weight (e.g. 30 mg pulp plus 30 mg of beads) were then added to the pulp tissue-buffer mixture. Tubes containing feather pulps, HEPES and beads were then added to a Bullet Blender® STORM (Next Advance, Troy, NY) and blended for a total of 10 minutes at speed 12 to make pulp homogenates at 5°C. Upon completion of blending, homogenates were centrifuged at 10,000 x *g* for 15 minutes. Supernatant was collected and diluted 1:8 with HEPES buffer. Seven superoxide dismutase standards (ranging from final SOD activity levels of 0 to 0.05 U/mL) were made fresh (no more than 2 hours before samples were added to the assay plate) according to Cayman Chemical's protocol and kept on ice until the SOD assay was performed. All reagents provided in the kit (assay buffer, sample buffer, radical detector, SOD standard) were added to the assay plate according to the kit protocol along with 10 μL of diluted pulp homogenates. Finally, 20 μL of xanthine oxidase was added to each well to generate reactive oxygen species. The assay plate was then incubated on a plate shaker for 30 minutes at room temperature. During this time, xanthine oxidase reacts with superoxide anions and tetrazolium salt (radical detector) to produce Formazan dye (yellow in color). Following the 30-minute incubation, the assay plate was spectrophotometrically evaluated at an absorbance of 450 nm using the Gen5 plate reader.

SOD activity was also measured on plasma samples from each bird at each time point. The previously collected plasma was removed from -80°C to room temperature for a thawing process of approximately 30 minutes. During this time SOD standards were prepared as directed

by the Cayman Chemical protocol and described above. Labeled tubes previously prepared with 192 μL of sample buffer in each were used to make plasma dilutions. Based on preliminary optimization studies, 8 μL of each sample was added to 192 μL sample buffer to make a 1:25 dilution. Diluted plasma samples were then added to a 96-well flat bottom plate provided with the kit. All reagents were added as described above following the Cayman Chemicals assay protocol. To determine SOD activity, color development was determined using a Gen5 plate reader at an absorbance of 450 nm.

Statistical Analyses

The experimental unit was the individual chicken with 6 chickens per dietary treatment (5 mg/kg Cu and 45 mg/kg of Zn and Mn in the sulfate form, 5 mg/kg Cu and 45 mg/kg of Zn and Mn in the hydroxychloride form, 10 mg/kg Cu and 90 mg/kg of Zn and Mn in the sulfate form or 10 mg/kg Cu and 90 mg/kg of Zn and Mn in the hydroxychloride form). Data were analyzed by a three-way Repeated Measures Analysis of Variance (RM ANOVA) using JMP 12 software (SAS Institute Inc., Cary, NC) to determine significant effects of time, source, and level, and source by level, source by time, level by time, and source by level by time interactions, for all measurements. In all cases, statistical significance was considered at $P \leq 0.05$. In the absence of interactions, main effect means are shown.

RESULTS

Feed Analysis

Feed analyses were completed for each experimental diet by the Central Analytical Laboratory, Poultry Science Center, University of Arkansas, Fayetteville (Table 1b). Copper content was analyzed at 12.2 mg/kg for the low-level sulfate diet and 17.0 mg/kg for the high-level sulfate diet with a 4.8 mg/kg difference between the two. Copper content was analyzed at 17.1 mg/kg for the low-level Intellibond diet and 16.5 mg/kg for the high-level Intellibond diet with a 0.6 mg/kg difference between the two diets. Manganese content was analyzed at 63.9 mg/kg in the low-level sulfate diet and 91.7 mg/kg in the high-level sulfate diet, with a difference of 27.9 mg/kg between the two diets. Manganese content was analyzed at 68.0 mg/kg in the low-level Intellibond diet and 104.0 mg/kg in the high-level Intellibond diet with a 36 mg/kg difference. Finally, Zinc content was analyzed at 86.6 mg/kg in the low-level sulfate diet and 112.0 mg/kg in the high-level sulfate diet, with a difference of 25.4 mg/kg between the two diets. Zinc content was analyzed at 84.2 mg/kg for the low-level Intellibond diet and 121.0 mg/kg in the high-level Intellibond diet with a 36.8 mg/kg difference.

Effects of Intradermal Pulp Injection of Lipopolysaccharide on Inflammatory Activities at the Site of Injection and in the Peripheral Blood Circulation

For all aspects examined, three-way repeated measures analysis of variance (ANOVA) was conducted to determine the effects of source, level and time, as well as their 2 and 3-way interactions ($P \leq 0.05$) (Table 2-7). In the absence of interactions, main effect differences were determined using Tukey's multiple means comparison tests ($P \leq 0.05$). In the case of significant 2-way interactions, differences between interacting variables were determined by Tukey's

multiple mean comparisons using main effect means across the non-interacting variable ($P \leq 0.05$). In the case of a 3-way interaction, differences between individual source, level and time means were determined by Tukey's multiple mean comparisons ($P \leq 0.05$).

Leukocyte population profiles in the pulp of growing feathers following pulp injection with LPS

Intradermal injection of LPS into the pulp of GFs resulted in infiltration of heterophils and monocytes/macrophages from the blood into the pulp. Independent of dietary treatment, heterophil levels (% pulp cells) were higher ($P \leq 0.05$) in GFs collected at 6 h and 24 h after LPS injection compared to levels in GFs collected prior to injection (1.21 ± 1.07 %) (Mean ± SEM) (Table 2, Figure 1). At 6 h post-injection, heterophil levels had increased to 25.03 ± 1.07 % and then decreased ($P \leq 0.05$) to 17.83 ± 1.07 % by 24 h. There was a source by level interaction ($P = 0.0353$) for heterophils, however multiple means comparisons of individual dietary treatments averaged across time did not reveal differences between dietary treatments ($P > 0.05$). Analysis of macrophage infiltration only revealed a time effect (Table 2), with macrophage main effect levels increasing ($P < 0.05$) from 1.98 ± 0.25 % at 0 h, to 3.12 ± 0.25% at 6 h, and again to 8.43 ± 0.25% at 24 h (Figure 1). There was no infiltration of lymphocytes into the pulp of GFs in response to LPS injection (Figures 1 and 2). Although, there was a level by time interaction ($P = 0.0353$; Table 2), multiple means comparison between high and low levels of trace mineral supplementation within a time point revealed only a difference at 0 h, before LPS injection independent of source of the trace mineral supplement ($P = 0.0492$; 1.83 ± 0.18% vs. 1.05 ± 0.18% lymphocytes for high versus low supplementation level, respectively). Overall, there was a slight drop in lymphocyte levels (% pulp cells) following LPS injection. Analysis of B and T

lymphocyte populations (Figure 2), revealed a similar drop ($P \leq 0.05$) in their levels following LPS injection, whereby T cell levels also exhibited a level by time interaction ($P = 0.028$), with marginally higher ($P = 0.059$) T cell levels before LPS injection (0 h) for birds fed the high (1.55 ± 0.15% T cells) vs. the low (0.92 ± 0.15% T cells) levels of trace minerals, independent of source.

Concentrations of leukocyte populations in the peripheral blood following intradermal LPS injection into the pulp of growing feathers

Intradermal injection of LPS into the pulp of GFs affected the concentration (K/μL) of various blood leukocytes (Table 3, Figure 3). Statistical analysis revealed no interactions and a significant time effect for the concentrations of heterophils, monocytes and lymphocytes. Hence only the main effect means are shown in Figure 3. Similar to observations in the pulp, heterophil concentrations increased ($P \leq 0.05$) from 8.36 ± 1.34 K/μL at 0 h to highest levels (29.01 ± 1.34 K/μL) at 6 h post LPS injection, but then returned to pre-injection levels by 24 h (9.34 ± 1.34 K/μL). Monocyte concentration levels increased ($P \leq 0.05$) from 0.89 ± 0.17 K/μL at 0 h to highest levels (1.85 ± 0.17 K/μL) at 6 h post LPS injection, and decreased to pre-injection levels by 24 h (0.68 ± 0.17 K/μL). Additionally, monocyte concentrations were affected by the level of dietary trace mineral supplementation ($P = 0.0507$), whereby higher monocyte concentrations were observed with the low (1.34 ± 0.14 K/μL) compared to the high (0.94 ± 0.14 K/μL) supplementation levels, independent of source and time point. While a time effect was observed, lymphocyte concentrations decreased ($P \leq 0.05$) from 8.58 ± 0.72 K/μL at 0 h to 5.35 ± 0.72 K/μL 6 h post LPS injection, but then increased ($P \leq 0.05$) again (11.44 ± 0.72 K/μL) at 24 h to levels higher ($P \leq 0.05$) than before LPS injection. Concentrations (K/μL) of eosinophils were

not affected by dietary treatment or LPS injection (Table 3). There was a 3-way interaction (P = 0.0356) in the concentrations of basophils. Multiple means comparisons did not reveal any difference among basophil concentrations, independent of diet or LPS treatments (P > 0.05).

Proportions (%) among leukocyte populations in the peripheral blood following LPS injection into the pulp of growing feathers

Intradermal injection of LPS into the GF pulp affected the proportions (%) among leukocyte populations in the peripheral blood (Table 4, Figure 4). Statistical analysis revealed no interactions for the concentrations of heterophils, monocytes and lymphocytes, therefore only main effect means are shown in Figure 4. Additionally, a significant time effect was found for both heterophil and lymphocyte proportions (%). Heterophil proportions (%) produced the same trends observed for heterophil levels in the pulp and blood (Table 4), increasing (P ≤ 0.05) from 45.68 ± 1.85 % at 0 h to higher levels (78.06 ± 1.85 %) at 6 h post LPS injection and returning to pre-injection levels (41.49 ± 1.85 %) 24 h post LPS injection. Lymphocyte proportions decreased (P ≤ 0.05) from 44.55 ± 1.60 % at 0 h to 15.50 ± 1.60 % at 6 h post LPS injection, but then returned to pre-injection levels (50.13 ± 1.60 %) 24 h post LPS injection. A marginal level effect (P = 0.0700) was observed in lymphocyte proportions (%) between chickens fed low (34.97 ± 1.30 %) vs. high (38.47 ± 1.30 %) levels of trace minerals. While a time effect was not observed for the proportions of monocytes (%), there was a level effect (P = 0.0316) with the lower level resulting in higher proportions of monocytes than the high level of trace mineral supplementation (4.98 ± 0.48 % vs. 3.42 ± 0.48 %). Proportions (%) of eosinophils were not affected by dietary treatment and LPS injection (Table 4). For basophils, statistical analysis revealed 3-way

interaction ($P \leq 0.05$) and no differences between the percentage of basophils independent of source and level of dietary supplementation and time-post LPS injection (Table 4).

Total white blood cell, thrombocyte, and red blood cell concentrations in the peripheral blood following LPS injection into the pulp of growing feathers

Intradermal injection of LPS into the pulp of GFs affected the blood concentrations of total leukocytes (white blood cells), thrombocytes, and red blood cells (Table 5, Figure 5). Statistical analysis revealed no interactions and a significant time effect for the concentrations of white blood cells (K/μL), thrombocytes (K/μL) and red blood cells (Mill/μL), therefore only the main effect means are shown in Figure 5. Total white blood cell concentrations followed the same overall trends observed for the heterophil population with concentrations increasing ($P \leq 0.05$) from 18.56 ± 1.68 K/μL at 0 h to 36.50 ± 1.68 K/μL at 6 h post LPS injection, and returning to pre-injection levels (22.30 ± 1.68 K/μL) by 24 h. Thrombocyte concentrations were similar at 0 h (22.94 ± 1.49 K/μL) and 6 h (24.83 ± 1.49 K/μL) post LPS injection but increased ($P \leq 0.05$) to 31.03 ± 1.49 K/μL by 24 h. Red blood cell concentrations decreased ($P \leq 0.05$) from 2.44 ± 0.05 Mill/μL at 0 h to 2.19 ± 0.05 Mill/μL at 6 h post LPS injection, and returned to pre-injection levels (2.42 ± 0.05 Mill/μL) by 24 h. While statistical analysis also found a source by level interaction within red blood cell concentrations Mill/μL ($P = 0.0198$), multiple means comparisons of individual dietary treatments averaged across time did not reveal differences between dietary treatments ($P > 0.05$).

Hemoglobin and hematocrit (g/dL) concentrations in the peripheral blood following LPS injection into the pulp of growing feathers

Intradermal injection of LPS into the pulp of GFs affected the blood concentrations (g/dL) of hemoglobin and hematocrit (Table 5, Figure 6). Statistical analysis revealed no interactions and a significant time effect for the concentrations of both hemoglobin and hematocrit; hence only the main effect means are shown in Figure 8. Hemoglobin concentrations decreased ($P \leq 0.05$) from 7.77 ± 0.11 g/dL at 0 h to 7.13 ± 0.11 g/dL at 6 h post LPS injection, and returned to pre-injection levels (7.90 ± 0.11 g/dL) by 24 h. Hematocrit concentrations followed the same trend as hemoglobin concentrations, decreasing ($P \leq 0.05$) from 58.12 ± 0.93 g/dL at 0 h to 52.86 ± 0.93 g/dL at 6 h post LPS injection, and returning to pre-injection levels (57.82 ± 0.93 g/dL) by 24 h.

Additional red blood cell measurements in the peripheral blood following LPS injection into the pulp of growing feathers

Intradermal injection of LPS into the pulp of GFs affected red blood cell measurements in the peripheral blood (Table 6). A source by level effect was observed for both mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). MCV concentrations were higher ($P \leq 0.05$) when chickens were fed the low-level sulfate diet vs. the high-level sulfate diet (245.28 ± 1.34 vs. 237.50 ± 1.34) whereas MCV levels were not different ($P > 0.05$) between the high- and low-level Intellibond diets. Additionally, there was a marginal ($P = 0.055$) main effect of time, indicating a drop ($P \leq 0.05$) in MCV by 24 h post LPS-injection (242.42 ± 1.33, 241.21 ± 1.33, and 238.33 ± 1.33 at 0, 6 and 24 h). Analysis of the MCH source by level interaction using multiple mean comparisons did not reveal individual differences due to level or source of trace

mineral supplementation. There were no main effects or interactions for the mean corpuscular hemoglobin concentrations (MCHC) or for the red cell distribution widths (RDW) (Table 6).

Reactive oxygen species generation in pulp cell suspensions following LPS injection

Intradermal injection of LPS into the pulp of GFs affected the reactive oxygen species (ROS) generation in pulp cell suspensions (Table 7, Figure 7). Statistical analysis revealed no interactions and a significant time effect for ROS generation, therefore only main effect means are shown in Figure 7. ROS generation increased ($P \leq 0.05$) from 0 h (254.43 ± 336.24) to higher levels (3129.15 ± 336.24) at 6 h post LPS injection, and returned to pre-injection levels (446.62 ± 336.24) by 24 h.

Superoxide dismutase activity in pulp homogenates and plasma following LPS injection into the pulp of growing feathers

Intradermal injection of LPS into the pulp of GFs affected the superoxide dismutase (SOD) activity levels in both the pulp homogenates and the plasma samples (Table 7, Figure 8). Time main effect means are represented in Figure 8. Pulp homogenate SOD activity levels (U/mL) showed no time effect ($P \leq 0.05$). However, there was a source by level interaction (Table 7). Multiple means comparisons of individual dietary treatments averaged across time reveal no difference in pulp SOD activity in chickens fed the low supplementation level of trace minerals, independent of the source (5.17 ± 0.43 U/mL for the low-level sulfate diet and 5.52 ± 0.43 U/mL for low-level Intellibond diet). Supplementation of the high-level sulfate source resulted in higher pulp SOD activity levels (7.42 ± 0.43 U/mL) than either of the low-level supplementations, whereas pulp SOD activity with the high-level Intellibond supplementation

was intermediate (5.89 ± 0.43 U/mL) to levels observed with the high-level sulfate diet and the low-level supplementations. Plasma SOD activity levels changed with time post LPS administration, with similar activity levels observed at 0 h (9.08 ± 0.64 U/mL) and 6 h post LPS injection (10.04 ± 0.64 U/mL), and elevated ($P \leq 0.05$) plasma SOD activity levels (13.74 ± 0.64 U/mL) at 24 h.

Table 1a. Ingredient and calculated nutrient composition of basal diet fed to Cobb 500 broilers from 7 to 35 d post-hatch.

Ingredient %	
Corn	60.40
Soybean meal	32.51
Poultry oil	3.41
Sodium chloride	0.36
Limestone	0.91
Dicalcium phosphate	1.51
Vitamin premix	0.10
Choline chloride	0.08
L-lysine HCl	0.21
DL-methionine	0.31
L-threonine	0.10
Experimental mineral premix[1]	0.10
Calculated in premix	
CP, %	22.3
AME, kcal/kg	3,015
Ca, %	0.90
nPP%	0.45

[1]Mineral premixes were formulated and mixed to provide the appropriate source and level of Cu, Mn, and Zn when included in the diet at 0.01%.

Table 1b. Analyzed nutritional values of experimental diets fed to Cobb 500 broilers from 7 to 35 d post-hatch.

	50% (low concentration)		100% (high concentration)	
Analyzed[1]	Sulfate	Intellibond	Sulfate	Intellibond
DM%	93.4	92.4	92.7	93.1
CP, %	23.2	23.4	23.0	22.9
Ash, %	5.23	4.93	4.96	5.18
Cu, mg/kg	12.2	17.1	17.0 (4.8)	16.5 (-0.6)
Mn, mg/kg	63.9	68.0	91.7 (27.8)	104.0 (36)
Zn, mg/kg	86.6	84.2	112.0 (25.4)	121.0 (36.8)

[1]Values in parentheses represent increase (mg/kg) relative to respective 50% diet.

Table 2. Leukocyte profiles (% pulp cells) in the pulp of growing feathers (GF) after pulp injection of lipopolysaccharide (LPS) in 5-wk-old Cobb 500 broiler chickens.

Diet	Time (h)	Heterophils	Macrophages	Lymphocytes	B Cells	T Cells
Sulfate, low conc.[1]	0	0.97 ± 0.09	1.99 ± 0.32	1.04 ± 0.39	0.17 ± 0.08	0.88 ± 0.31
Intellibond, low conc.[2]	0	1.43 ± 0.16	2.07 ± 0.33	1.05 ± 0.29	0.11 ± 0.03	0.94 ± 0.26
Sulfate, high conc.[3]	0	1.14 ± 0.27	1.95 ± 0.10	1.94 ± 0.38	0.24 ± 0.05	1.70 ± 0.34
Intellibond, high conc.[4]	0	1.32 ± 0.21	1.90 ± 0.17	1.70 ± 0.46	0.24 ± 0.13	1.46 ± 0.33
Sulfate, low conc.[1]	6	26.73 ± 2.77	3.61 ± 0.78	0.94 ± 0.36	0.07 ± 0.05	0.87 ± 0.31
Intellibond, low conc.[2]	6	24.03 ± 2.72	3.28 ± 0.22	1.06 ± 0.09	0.14 ± 0.05	0.92 ± 0.04
Sulfate, high conc.[3]	6	21.67 ± 2.35	2.89 ± 0.27	1.08 ± 0.15	0.10 ± 0.05	0.98 ± 0.13
Intellibond, high conc.[4]	6	27.65 ± 4.66	2.66 ± 0.62	0.70 ± 0.07	0.08 ± 0.02	0.62 ± 0.06
Sulfate, low conc.[1]	24	20.88 ± 1.56	9.10 ± 0.52	0.85 ± 0.08	0.04 ± 0.01	0.81 ± 0.07
Intellibond, low conc.[2]	24	16.97 ± 2.57	7.30 ± 0.38	0.81 ± 0.11	0.09 ± 0.03	0.72 ± 0.11
Sulfate, high conc.[3]	24	14.56 ± 1.63	8.44 ± 0.97	0.90 ± 0.06	0.11 ± 0.00	0.79 ± 0.06
Intellibond, high conc.[4]	24	18.80 ± 0.65	8.91 ± 0.61	0.57 ± 0.06	0.06 ± 0.01	0.50 ± 0.06
Statistical Analysis[5] Effects (P-value)						
Source		0.5723	0.2736	0.3143	0.6216	0.2283
Level		0.4379	0.7214	0.1718	0.1467	0.2636
Source*Level		0.0353	0.2154	0.2211	0.8690	0.1967
Hour		<0.0001	<0.0001	0.0030	0.0179	0.0049
Source*Hour		0.8676	0.6659	0.9701	1.0000	0.9570
Level*Hour		0.7469	0.3042	0.0336	0.7198	0.0283
Source*Level*Hour		0.2610	0.2015	0.9210	0.6464	0.9141

The pulp of 16 growing feathers (GF) were injected (i.d.) with 10 µL of LPS (100 µg/mL); 1 µg/GF.
Time: Before (0 h) and 6 and 24 h post-pulp injection of LPS
[1] 5 Cu, 45 Mn and Zn (mg/kg), Source: Sulfate
[2] 5 Cu, 45 Mn and Zn (mg/kg), Source: Intellibond
[3] 10 Cu, 90 Mn and Zn (mg/kg), Source: Sulfate

[4] 10 Cu, 90 Mn and Zn (mg/kg), Source: Intellibond

[5]3-way repeated measures ANOVA with a compound symmetry covariance structure; significance considered at $P \leq 0.05$.

Pulp cell suspensions from two GF were prepared and immunofluorescently stained with a panel of fluorescence-conjugated mouse monoclonal antibodies to identify chicken leukocytes (CD45), macrophages (KUL-01) and lymphocytes (Bu-1 and CD3). Analysis of cell populations was carried out by flow cytometry. Percentages of heterophils were based on size (FSC) and granularity (SSC) characteristics of leukocytes (CD45+ cells).

Data are mean ± SEM; n = 6 birds per diet (one male and one female from each of 3 pens)

Table 3. Concentrations (K/μL of blood) of individual white blood cells in blood collected from 5-wk-old Cobb 500 broiler chickens after injection of lipopolysaccharide (LPS) into the pulp of growing feathers.

Diet	Time (h)	Heterophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Sulfate, low conc.[1]	0	7.89 ± 0.16	7.08 ± 0.57	0.59 ± 0.22	0.009 ± 0.00	0.70 ± 0.15
Intellibond, low conc.[2]	0	7.86 ± 0.23	7.30 ± 1.69	0.99 ± 0.16	0.013 ± 0.00	0.88 ± 0.39
Sulfate, high conc.[3]	0	8.99 ± 0.55	9.49 ± 0.35	1.09 ± 0.36	0.019 ± 0.01	1.04 ± 0.22
Intellibond, high conc.[4]	0	8.71 ± 0.95	10.45 ± 1.15	0.90 ± 0.25	0.014 ± 0.01	1.34 ± 0.07
Sulfate, low conc.[1]	6	31.22 ± 2.03	5.91 ± 0.25	2.47 ± 0.18	0.010 ± 0.01	1.00 ± 0.16
Intellibond, low conc.[2]	6	24.83 ± 2.95	4.66 ± 0.45	2.14 ± 0.86	0.027 ± 0.03	0.41 ± 0.09
Sulfate, high conc.[3]	6	26.30 ± 3.96	6.22 ± 1.96	1.17 ± 0.29	0.011 ± 0.00	0.32 ± 0.12
Intellibond, high conc.[4]	6	33.70 ± 6.98	4.60 ± 0.94	1.63 ± 0.12	0.014 ± 0.01	0.51 ± 0.12
Sulfate, low conc.[1]	24	9.41 ± 1.01	12.84 ± 2.08	0.65 ± 0.32	0.020 ± 0.01	0.92 ± 0.31
Intellibond, low conc.[2]	24	9.58 ± 1.75	9.28 ± 2.34	1.23 ± 0.38	0.018 ± 0.01	1.78 ± 0.71
Sulfate, high conc.[3]	24	9.63 ± 1.75	11.71 ± 1.64	0.52 ± 0.19	0.004 ± 0.00	1.22 ± 0.04
Intellibond, high conc.[4]	24	8.73 ± 0.77	11.92 ± 1.75	0.32 ± 0.07	0.029 ± 0.02	0.76 ± 0.11
Statistical Analysis[5]						
Effects (P-value)						
Source		0.9972	0.3232	0.5524	0.3071	0.6134
Level		0.5767	0.1571	0.0507	0.8837	0.5960
Source*Level		0.1922	0.4192	0.6356	0.9367	0.6665
Hour		<0.0001	<0.0001	0.0001	0.8654	0.0125
Source*Hour		0.9714	0.4877	0.9701	0.7234	0.4520
Level*Hour		0.8336	0.4126	0.0884	0.7473	0.1169
Source*Level*Hour		0.1101	0.5861	0.2298	0.3988	0.0356

The pulp of 16 growing feathers (GF) were injected (i.d.) with 10 μL of LPS (100 μg/mL); 1 μg/GF.
Time: Before (0 h) and 6 and 24 h post-pulp injection of LPS

[1] 5 Cu, 45 Mn and Zn (mg/kg), Source: Sulfate
[2] 5 Cu, 45 Mn and Zn (mg/kg), Source: Intellibond
[3] 10 Cu, 90 Mn and Zn (mg/kg), Source: Sulfate
[4] 10 Cu, 90 Mn and Zn (mg/kg), Source: Intellibond
[5]3-way repeated measures ANOVA with a compound symmetry covariance structure; significance considered at $P \leq 0.05$.
Blood leukocyte concentration determined by CellDyn automated hematology analyzer.
Data are mean ± SEM; n = 6 birds per diet (one male and one female from each of 3 pens)

Table 4. Proportions (%) among peripheral white blood cell populations in blood collected from 5-wk-old Cobb 500 broiler chickens after injection of lipopolysaccharide (LPS) into the pulp of growing feathers.

Diet	Time (h)	Heterophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Sulfate, low conc.[1]	0	49.97 ± 3.36	42.22 ± 2.24	3.70 ± 1.09	0.057 ± 0.01	4.09 ± 0.67
Intellibond, low conc.[2]	0	47.92 ± 5.02	41.57 ± 4.05	5.75 ± 0.48	0.083 ± 0.04	4.69 ± 1.56
Sulfate, high conc.[3]	0	44.20 ± 1.69	45.60 ± 2.25	5.10 ± 1.62	0.091 ± 0.06	5.02 ± 1.01
Intellibond, high conc.[4]	0	40.62 ± 0.34	48.80 ± 0.30	4.06 ± 0.86	0.061 ± 0.02	6.44 ± 0.87
Sulfate, low conc.[1]	6	76.28 ± 0.29	15.62 ± 0.91	5.62 ± 0.64	0.024 ± 0.01	2.45 ± 0.56
Intellibond, low conc.[2]	6	76.90 ± 2.35	15.43 ± 2.56	6.29 ± 2.03	0.108 ± 0.10	1.27 ± 0.22
Sulfate, high conc.[3]	6	77.27 ± 4.06	18.35 ± 4.49	3.38 ± 0.49	0.029 ± 0.01	0.98 ± 0.32
Intellibond, high conc.[4]	6	81.80 ± 4.10	12.61 ± 3.43	4.24 ± 0.73	0.026 ± 0.03	1.34 ± 0.44
Sulfate, low conc.[1]	24	40.47 ± 6.99	53.10 ± 4.95	2.64 ± 1.21	0.100 ± 0.05	3.71 ± 1.07
Intellibond, low conc.[2]	24	43.70 ± 5.08	41.92 ± 4.82	5.92 ± 2.23	0.084 ± 0.04	8.35 ± 3.85
Sulfate, high conc.[3]	24	42.30 ± 3.15	50.03 ± 2.40	2.12 ± 0.50	0.018 ± 0.01	5.52 ± 0.98
Intellibond, high conc.[4]	24	39.50 ± 1.08	55.45 ± 1.78	1.66 ± 0.34	0.159 ± 0.08	3.24 ± 0.49
Statistical Analysis[5]						
Effects (P-value)						
Source		0.9969	0.4172	0.2039	0.2407	0.4568
Level		0.4633	0.0700	0.0316	0.7019	0.6708
Source*Level		0.7782	0.1911	0.1190	0.9537	0.3451
Hour		<0.0001	<0.0001	0.0862	0.4786	0.0010
Source*Hour		0.5931	0.5701	0.8592	0.6319	0.6701
Level*Hour		0.2132	0.4112	0.3551	0.7980	0.3050
Source*Level*Hour		0.6406	0.0672	0.4653	0.1927	0.0722

The pulp of 16 growing feathers (GF) were injected (i.d.) with 10 µL of LPS (100 µg/mL); 1 µg/GF.
Time: Before (0 h) and 6 and 24 h post-pulp injection of LPS
[1] 5 Cu, 45 Mn and Zn (mg/kg), Source: Sulfate
[2] 5 Cu, 45 Mn and Zn (mg/kg), Source: Intellibond

[3] 10 Cu, 90 Mn and Zn (mg/kg), Source: Sulfate
[4] 10 Cu, 90 Mn and Zn (mg/kg), Source: Intellibond
[5]3-way repeated measures ANOVA with a compound symmetry covariance structure; significance considered at $P \leq 0.05$.
Blood leukocyte proportions determined by CellDyn automated hematology analyzer.
Data are mean ± SEM; n = 6 birds per diet (one male and one female from each of 3 pens)

Table 5. Concentrations of total white blood cells, thrombocytes and red blood cells, hemoglobin and hematocrit in blood collected from 5-wk-old broiler chickens after injection of lipopolysaccharide (LPS) into the pulp of growing feathers.

Diet	Time (h)	White Blood Cells (K/µL)	Thrombocytes (K/µL)	Red Blood Cells (Mill/µL)	Hemoglobin (g/dL)	Hematocrit (g/dL)
Sulfate, low conc.[1]	0	16.12 ± 1.14	20.60 ± 1.07	2.32 ± 0.06	7.81 ± 0.26	57.38 ± 2.04
Intellibond, low conc.[2]	0	16.85 ± 2.43	24.75 ± 2.00	2.65 ± 0.24	7.80 ± 0.22	59.63 ± 1.82
Sulfate, high conc.[3]	0	20.42 ± 0.82	23.68 ± 0.17	2.45 ± 0.11	7.84 ± 0.18	58.52 ± 2.45
Intellibond, high conc.[4]	0	20.85 ± 2.53	22.73 ± 2.17	2.34 ± 0.02	7.64 ± 0.04	56.93 ± 1.17
Sulfate, low conc.[1]	6	40.37 ± 1.66	24.80 ± 3.72	2.16 ± 0.03	7.10 ± 0.21	52.77 ± 1.36
Intellibond, low conc.[2]	6	31.18 ± 4.00	23.27 ± 2.55	2.26 ± 0.10	7.34 ± 0.30	54.33 ± 1.91
Sulfate, high conc.[3]	6	34.02 ± 4.69	24.75 ± 4.18	2.23 ± 0.10	7.22 ± 0.16	52.85 ± 1.96
Intellibond, high conc.[4]	6	40.43 ± 6.73	26.50 ± 4.02	2.12 ± 0.06	6.88 ± 0.16	51.48 ± 1.27
Sulfate, low conc.[1]	24	23.62 ± 1.89	31.53 ± 4.31	2.38 ± 0.07	7.95 ± 0.29	58.38 ± 2.41
Intellibond, low conc.[2]	24	20.72 ± 3.72	28.72 ± 3.27	2.48 ± 0.07	8.02 ± 0.13	57.95 ± 1.12
Sulfate, high conc.[3]	24	23.08 ± 3.36	30.67 ± 3.01	2.49 ± 0.12	7.92 ± 0.28	58.67 ± 2.76
Intellibond, high conc.[4]	24	21.76 ± 2.52	33.20 ± 2.21	2.36 ± 0.04	7.71 ± 0.17	56.28 ± 1.09
Statistical Analysis[5] Effect (P-value)						
Source		0.6214	0.7646	0.6037	0.5414	0.7653
Level		0.3242	0.4545	0.4296	0.2812	0.3848
Source*Level		0.1598	0.7357	0.0198	0.1591	0.1897
Hour		<0.0001	0.0021	0.0024	<0.0001	0.0007
Source*Hour		0.8429	0.9057	0.6367	0.9827	0.7754
Level*Hour		0.7063	0.9493	0.8371	0.9133	0.9603
Source*Level*Hour		0.2089	0.4365	0.6451	0.7919	0.9383

The pulp of 16 growing feathers (GF) were injected (i.d.) with 10 µL of LPS (100 µg/mL); 1 µg/GF.
Time: Before (0 h) and 6 and 24 h post-pulp injection of LPS
[1] 5 Cu, 45 Mn and Zn (mg/kg), Source: Sulfate
[2] 5 Cu, 45 Mn and Zn (mg/kg), Source: Intellibond

[3] 10 Cu, 90 Mn and Zn (mg/kg), Source: Sulfate
[4] 10 Cu, 90 Mn and Zn (mg/kg), Source: Intellibond
[5]3-way repeated measures ANOVA with a compound symmetry covariance structure; significance considered at $P \leq 0.05$.
The concentrations of blood cell populations, hemoglobin and hematocrit were determined by CellDyn automated hematology analyzer.
Data are mean ± SEM; n = 6 birds per diet (one male and one female from each of 3 pens)

Table 6. Red blood cell measurements in blood collected from 5-wk-old broiler chickens after injection of lipopolysaccharide (LPS) into the pulp of growing feathers.

Diet	Time (h)	MCV	MCH	MCHC	RDW
Sulfate, low conc.[1]	0	246.8 ± 2.9	33.60 ± 0.35	13.62 ± 0.08	11.42 ± 0.16
Intellibond, low conc.[2]	0	240.0 ± 2.2	31.40 ± 0.58	13.08 ± 0.14	11.62 ± 0.23
Sulfate, high conc.[3]	0	239.0 ± 1.5	32.05 ± 0.71	13.43 ± 0.26	9.92 ± 1.73
Intellibond, high conc.[4]	0	243.8 ± 2.6	32.75 ± 0.18	13.43 ± 0.21	11.48 ± 0.27
Sulfate, low conc.[1]	6	244.0 ± 4.0	32.93 ± 0.75	13.48 ± 0.12	11.15 ± 0.08
Intellibond, low conc.[2]	6	240.2 ± 1.7	32.42 ± 0.09	13.50 ± 0.10	11.38 ± 0.22
Sulfate, high conc.[3]	6	237.5 ± 2.5	32.48 ± 0.81	13.70 ± 0.22	11.25 ± 0.16
Intellibond, high conc.[4]	6	243.2 ± 0.7	32.48 ± 0.69	13.40 ± 0.26	11.27 ± 0.14
Sulfate, low conc.[1]	24	245.0 ± 3.2	33.40 ± 0.27	13.62 ± 0.07	11.48 ± 0.30
Intellibond, low conc.[2]	24	233.7 ± 2.0	32.37 ± 0.52	13.83 ± 0.15	11.72 ± 0.44
Sulfate, high conc.[3]	24	236.0 ± 1.0	31.90 ± 0.74	13.52 ± 0.25	11.70 ± 0.18
Intellibond, high conc.[4]	24	238.7 ± 0.4	32.75 ± 0.93	13.78 ± 0.30	11.13 ± 0.04
Statistical Analysis[5]					
Effect (P-value)					
Source		0.2811	0.3065	0.6244	0.3832
Level		0.1640	0.4274	0.8444	0.2977
Source*Level		0.0002	0.0189	0.6950	0.8561
Hour		0.0546	0.9288	0.1182	0.5874
Source*Hour		0.2859	0.7313	0.1768	0.3891
Level*Hour		0.9961	0.8537	0.8262	0.5548
Source*Level*Hour		0.7910	0.3943	0.3165	0.3653

The pulp of 16 growing feathers (GF) were injected (i.d.) with 10 µL of LPS (100 µg/mL); 1 µg/GF.
Time: Before (0 h) and 6 and 24 h post-pulp injection of LPS

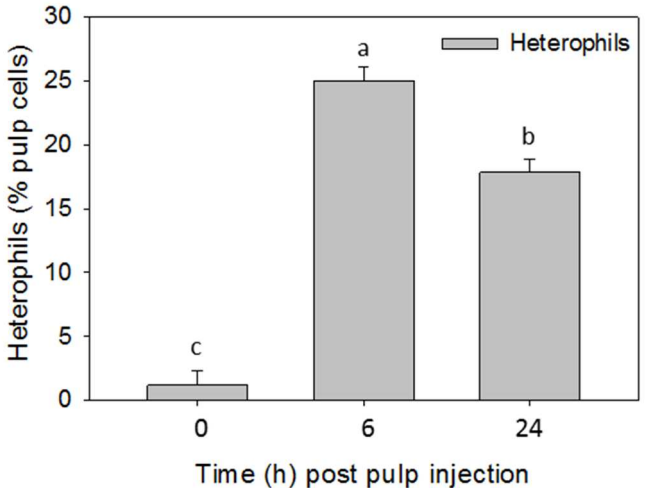
MCV=Mean Corpuscular Volume
MCH=Mean Corpuscular Hemoglobin
MCHC=Mean Corpuscular Hemoglobin Concentration
RDW=Red Cell Distribution Width
[1] 5 Cu, 45 Mn and Zn (mg/kg), Source: Sulfate
[2] 5 Cu, 45 Mn and Zn (mg/kg), Source: Intellibond
[3] 10 Cu, 90 Mn and Zn (mg/kg), Source: Sulfate
[4] 10 Cu, 90 Mn and Zn (mg/kg), Source: Intellibond
[5]3-way repeated measures ANOVA with a compound symmetry covariance structure; significance considered at $P \leq 0.05$.
Red blood cell measurements were determined by CellDyn automated hematology analyzer.
Data are mean ± SEM; n = 6 birds per diet (one male and one female from each of 3 pens)

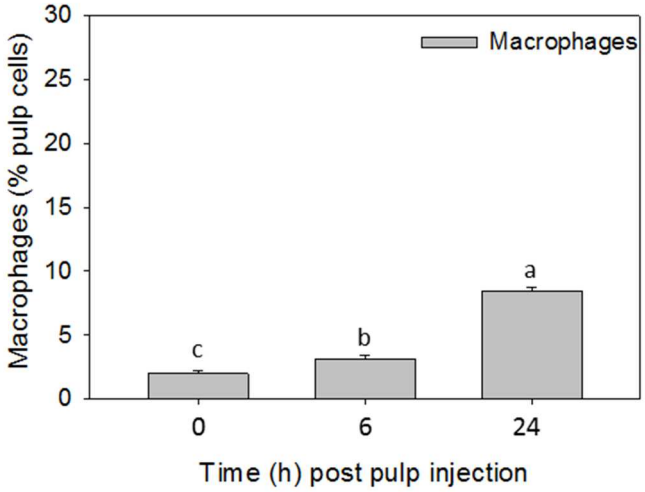
Table 7. Reactive oxygen species (ROS) generation in pulp cell suspensions and superoxide dismutase (SOD) activity in pulp homogenates and plasma after lipopolysaccharide (LPS) injection in the pulp of growing feathers of 5-wk-old Cobb 500 broiler chickens.

Diet	Time (h)	ROS Pulp	SOD Pulp	SOD Plasma
Sulfate, low conc.[1]	0	262.46 ± 24.92	4.44 ± 0.39	7.39 ± 1.51
Intellibond, low conc.[2]	0	329.68 ± 63.55	4.79 ± 0.31	10.00 ± 1.68
Sulfate, high conc.[3]	0	204.02 ± 33.71	7.34 ± 0.49	10.02 ± 1.50
Intellibond, high conc.[4]	0	221.51 ± 19.27	5.13 ± 0.38	8.92 ± 0.90
Sulfate, low conc.[1]	6	3,057.57 ± 1,154.44	5.70 ± 0.25	9.53 ± 1.32
Intellibond, low conc.[2]	6	2,959.69 ± 1,024.51	5.99 ± 0.51	11.37 ± 1.97
Sulfate, high conc.[3]	6	2,821.38 ± 605.10	7.18 ± 0.55	10.55 ± 1.13
Intellibond, high conc.[4]	6	3,677.93 ± 1,628.36	6.63 ± 1.18	8.74 ± 1.18
Sulfate, low conc.[1]	24	529.94 ± 126.77	5.35 ± 0.80	13.64 ± 0.25
Intellibond, low conc.[2]	24	436.81 ± 18.42	5.77 ± 0.39	13.71 ± 1.07
Sulfate, high conc.[3]	24	358.53 ± 62.27	7.78 ± 1.70	14.01 ± 0.74
Intellibond, high conc.[4]	24	461.16 ± 18.47	5.89 ± 0.65	13.64 ± 1.17
Statistical Analysis[5]				
Effects (P-value)				
Source		0.7175	0.1831	0.7765
Level		0.9430	0.0055	0.9642
Source*Level		0.6409	0.0378	0.0924
Hour		<0.0001	0.1867	<0.0001
Source*Hour		0.9105	0.7268	0.8641
Level*Hour		0.9277	0.8721	0.6795
Source*Level*Hour		0.8603	0.6855	0.5944

The pulp of 16 growing feathers (GF) were injected (i.d.) with 10 µL of LPS (100 µg/mL); 1 µg/GF.
Time: Before (0 h) and 6 and 24 h post-pulp injection of LPS
[1] 5 Cu, 45 Mn and Zn (mg/kg), Source: Sulfate
[2] 5 Cu, 45 Mn and Zn (mg/kg), Source: Intellibond

[3] 10 Cu, 90 Mn and Zn (mg/kg), Source: Sulfate
[4] 10 Cu, 90 Mn and Zn (mg/kg), Source: Intellibond
[5]3-way repeated measures ANOVA with a compound symmetry covariance structure; significance considered at $P \leq 0.05$.
Relative ROS generation in pulp cell suspensions was determined by kinetic fluorescence assay. SOD activity levels in plasma and pulp homogenates were measured by an assay kit purchased from Cayman Chemicals.
Data are mean ± SEM; n = 6 birds per diet (one male and one female from each of 3 pens)


Heterophils
Heterophils (% pulp cells)
30
25
20
15
10
5
0
a
b
c
0
6
24
Time (h) post pulp injection


Macrophages
Macrophages (% pulp cells)
30
25
20
15
10
5
0
a
b
c
0
6
24
Time (h) post pulp injection

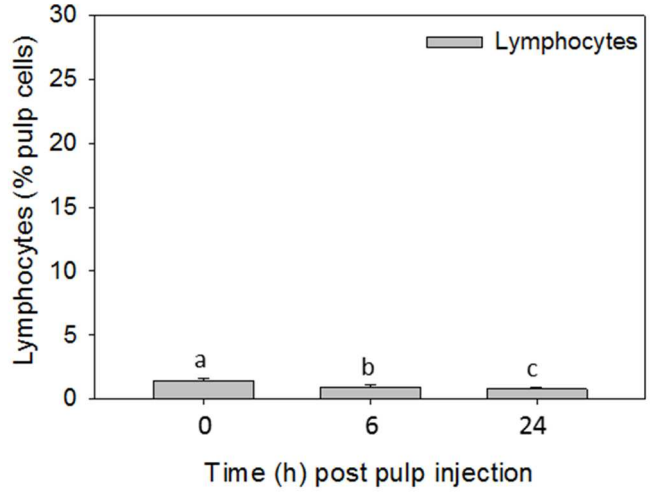

Lymphocytes
Lymphocytes (% pulp cells)
30
25
20
15
10
5
0
a
b
c
0
6
24
Time (h) post pulp injection

Figure 1. Infiltration of white blood cell populations into growing feathers after injection of lipopolysaccharide (LPS) into the pulp of growing feathers.
Sixteen growing feathers (GF) of 24, 5-wk-old Cobb 500 broilers were injected with 10 μL of LPS (100 μg/mL; 1 μg/GF). Two injected growing feathers from each chicken were collected at 0 (before injection), 6 and 24 h post-LPS injection for leukocyte population analysis. Pulp cell suspensions from GF were prepared and immunofluorescently stained with a panel of fluorescence-conjugated mouse monoclonal antibodies (Southern Biotech) to identify chicken macrophages (KUL-01) and lymphocytes (CD3 and Bu-1). Analysis of cell populations was carried out by fluorescence-based flow cytometry. Percentages of heterophils were based on size (FSC) and granularity (SSC) characteristics of leukocytes (CD45+). Data shown are time main-effect means ± SEM across dietary treatment; n = 24 birds (6 birds per diet). a-c For each cell type, means without a common letter are different ($P \leq 0.05$)

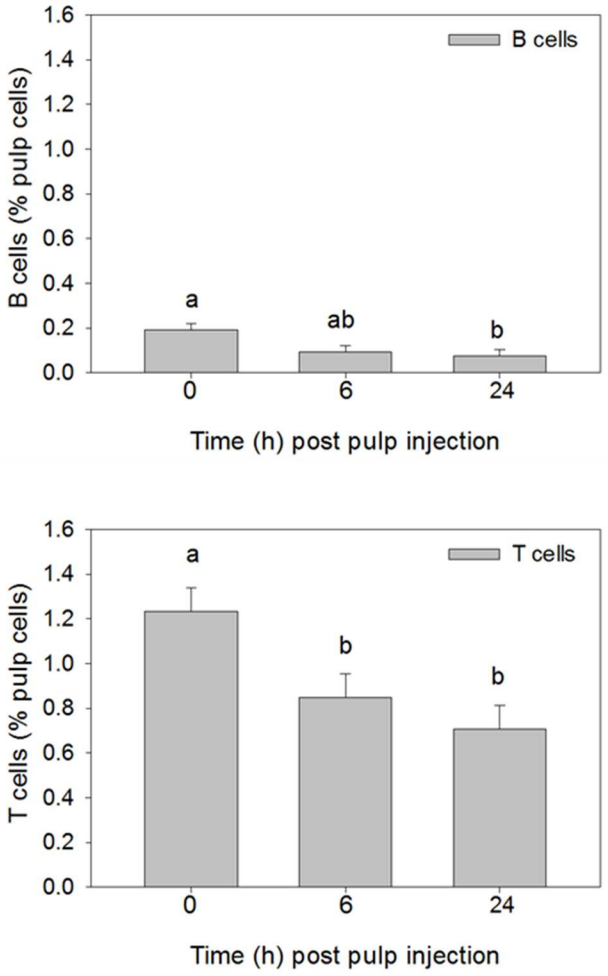
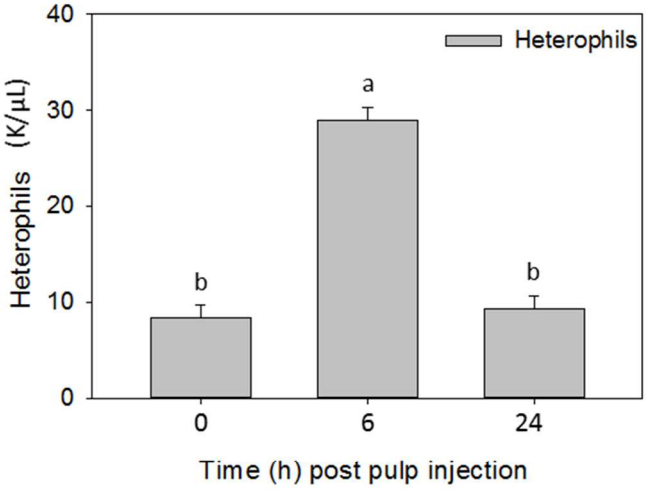
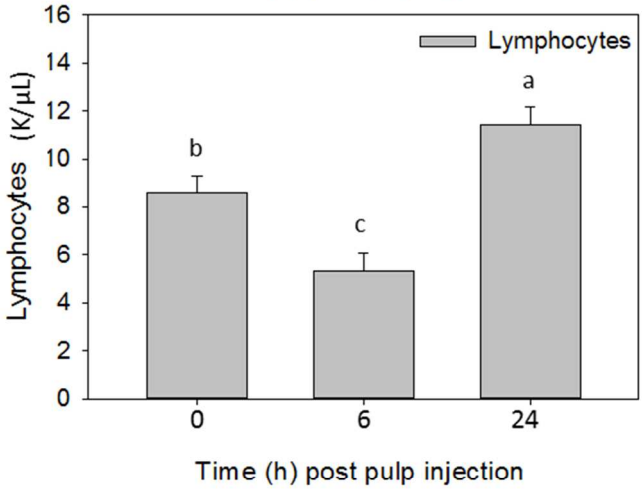


Figure 2. Infiltration of T and B-lymphocytes into growing feathers after injection of lipopolysaccharide (LPS) into the pulp of growing feathers.
Sixteen growing feathers (GF) of 24, 5-wk-old Cobb 500 broilers were injected with 10 µL of LPS (100 µg/mL; 1 µg/GF). Two injected growing feathers from each chicken were collected before- (0 h) and at 6 and 24 h post-LPS injection for leukocyte population analysis. Pulp cell suspensions from GF were prepared and immunofluorescently stained with chicken-specific B cell (Bu-1) and T cell (CD3) fluorescence-conjugated mouse monoclonal antibodies. Analysis of cell populations was carried out by fluorescence-based flow cytometry. Data shown are time main-effect means ± SEM across dietary treatment; n = 24 birds (6 birds per diet). a, b For each cell-type, means without a common letter are different ($P \leq 0.05$)


Heterophils
Heterophils (K/µL)
40
30
20
10
0
a
b
b
0
6
24
Time (h) post pulp injection


Lymphocytes
Lymphocytes (K/µL)
16
14
12
10
8
6
4
2
0
b
c
a
0
6
24
Time (h) post pulp injection

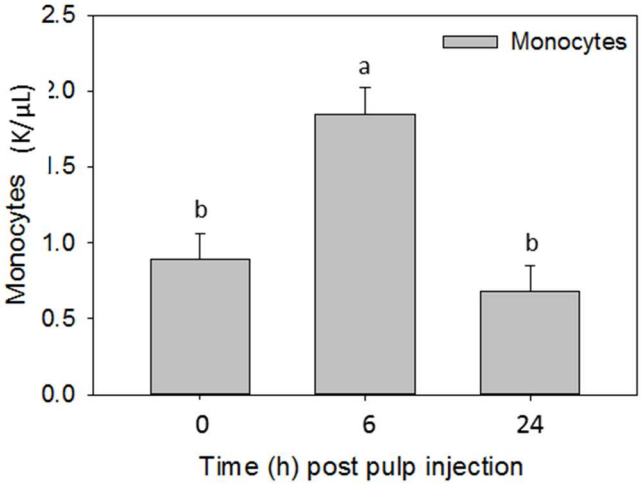
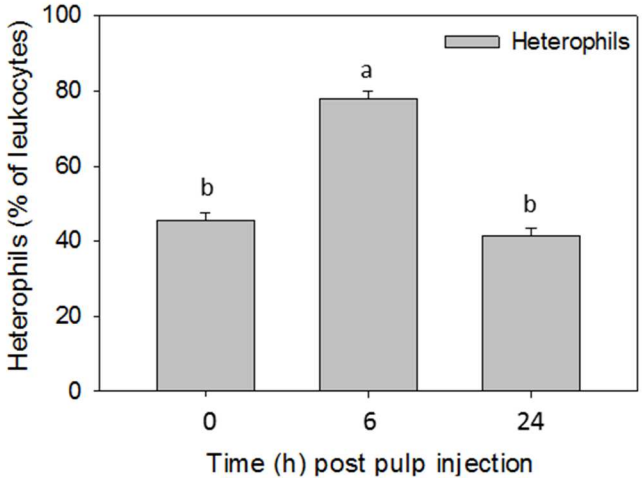
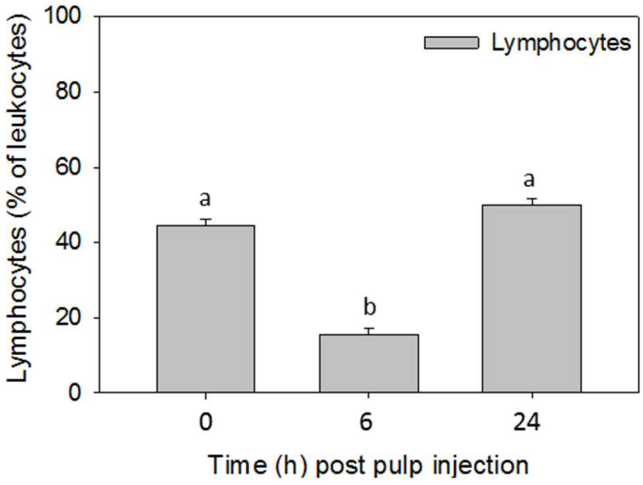

Monocytes
Monocytes (K/µL)
2.5
2.0
1.5
1.0
0.5
0.0
b
a
b
0
6
24
Time (h) post pulp injection

Figure 3. Concentration (K/µL) of heterophils, monocytes and lymphocytes in the peripheral blood after injection of lipopolysaccharide (LPS) into the pulp of growing feathers.

Sixteen growing feathers (GF) of 24, 5-wk-old Cobb 500 broilers were injected with 10 µL of LPS (100 µg/mL; 1 µg/GF). Heparinized blood (1 mL) was collected from each chicken before- (0 h) and at 6 and 24 h post-LPS injection for leukocyte population analysis. An automated hematology analyzer (Cell-Dyn) was used to determine cell concentrations (10^3 cells/µL). Data shown are time main-effect means ± SEM across dietary treatment; n = 24 birds (6 birds per diet). a-c For each cell-type, means without a common letter are different ($P \leq 0.05$)


Heterophils
Heterophils (% of leukocytes)
100
80
60
40
20
0
a
b
b
0
6
24
Time (h) post pulp injection


Lymphocytes
Lymphocytes (% of leukocytes)
100
80
60
40
20
0
a
b
a
0
6
24
Time (h) post pulp injection

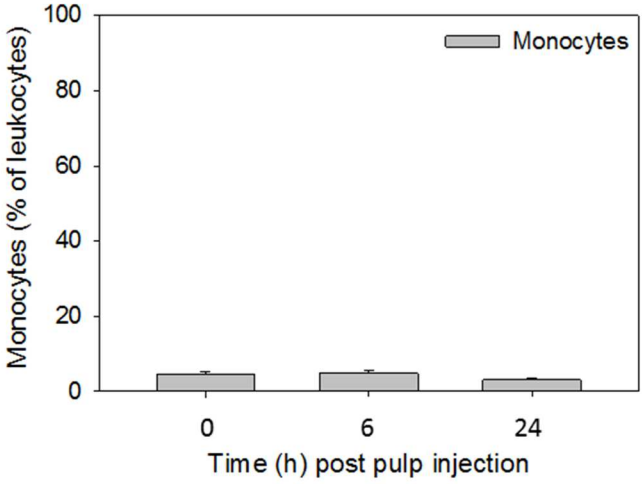
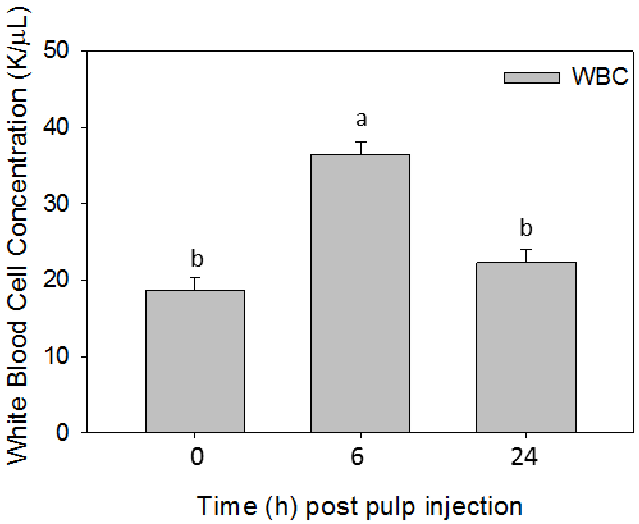
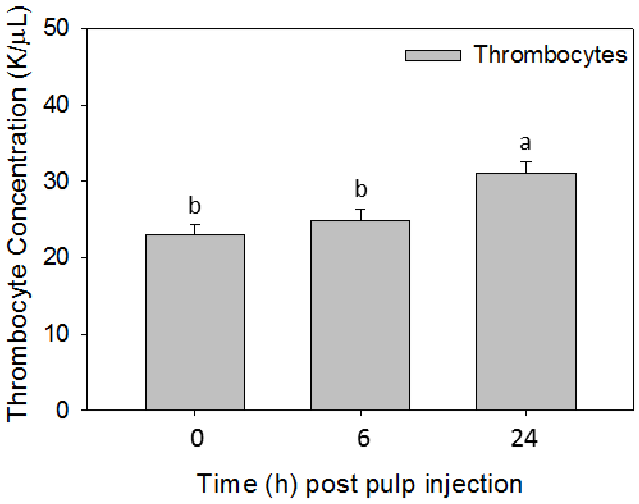

Monocytes
Monocytes (% of leukocytes)
100
80
60
40
20
0
0
6
24
Time (h) post pulp injection

Figure 4. Proportions (%) among peripheral white blood cell populations in blood after injection of lipopolysaccharide (LPS) into the pulp of growing feathers.
Sixteen growing feathers (GF) of 24, 5-wk-old Cobb 500 broilers were injected with 10 μL of LPS (100 μg/mL; 1 μg/GF). Heparinized blood (1 mL) was collected from each chicken before- (0 h) and at 6 and 24 h post-LPS injection for leukocyte population analysis. The proportions (% of total WBC) of heterophils, monocytes and lymphocytes were determined by an automated hematology analyzer (Cell-Dyn). Data shown are time main-effect means ± SEM across dietary treatment; n = 24 birds (6 birds per diet). a, b For each cell-type, means without a common letter are different ($P \leq 0.05$)


White Blood Cell Concentration (K/µL)
50
40
30
20
10
0
WBC
a
b
b
0
6
24
Time (h) post pulp injection


Thrombocyte Concentration (K/µL)
50
40
30
20
10
0
Thrombocytes
b
b
a
0
6
24
Time (h) post pulp injection

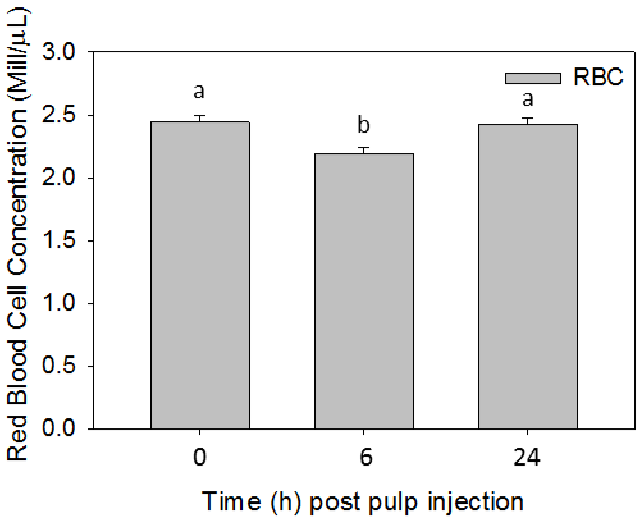

Red Blood Cell Concentration (Mill/µL)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
RBC
a
b
a
0
6
24
Time (h) post pulp injection

Figure 5. Total white blood cell (WBC), thrombocyte and red blood cell (RBC) concentrations in peripheral blood after injection of lipopolysaccharide (LPS) into the pulp of growing feathers.

Sixteen growing feathers (GF) of 24, 5-wk-old Cobb 500 broilers were injected with 10 µL of LPS (100 µg/mL; 1 µg/GF). Heparinized blood (1 mL) was collected from each chicken before- (0 h) and at 6 and 24 h post-LPS injection for cell population analysis. An automated hematology analyzer (Cell-Dyn) was used to determine the total WBC, thrombocyte and RBC concentrations. Data shown are time main-effect means ± SEM across dietary treatment; n = 24 birds (6 birds per diet). a, b For each cell-type, means without a common letter are different ($P \leq 0.05$)

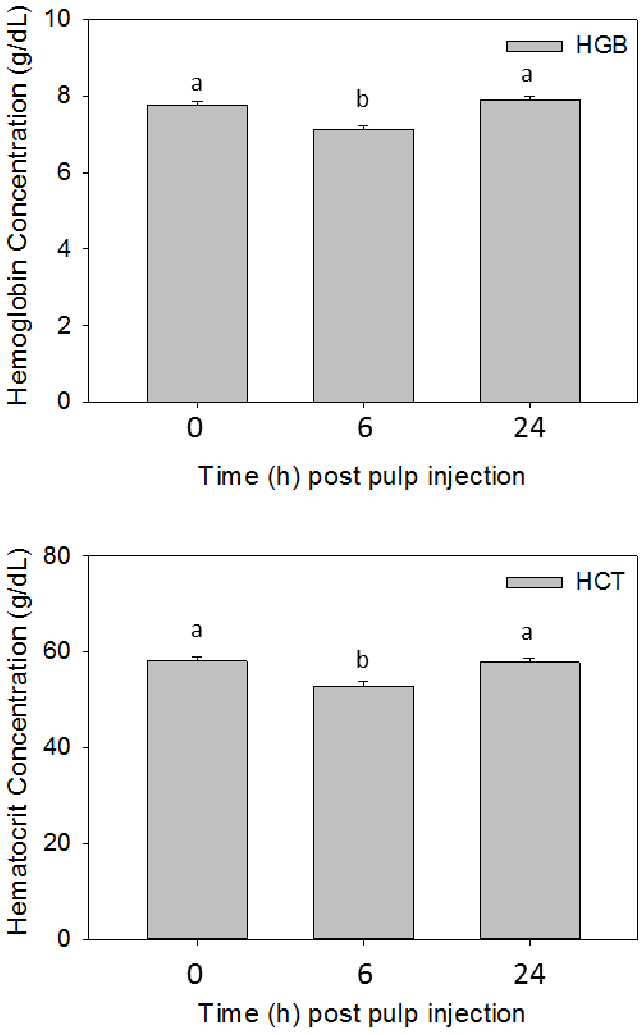


Figure 6. Hemoglobin and hematocrit concentrations in peripheral blood after injection of lipopolysaccharide (LPS) into the pulp of growing feathers.
Sixteen growing feathers (GF) of 24, 5-wk-old Cobb 500 broilers were injected with 10 µL of LPS (100 µg/mL; 1 µg/GF). Heparinized blood (1 mL) was collected from each chicken before- (0 h) and at 6 and 24 h post-LPS injection for analysis. An automated hematology analyzer (Cell-Dyn) was used to determine hemoglobin and hematocrit concentrations. Data shown are time main-effect means ± SEM across dietary treatment; n = 24 birds (6 birds per diet). a, b For each measurement, means without a common letter are different ($P \leq 0.05$)

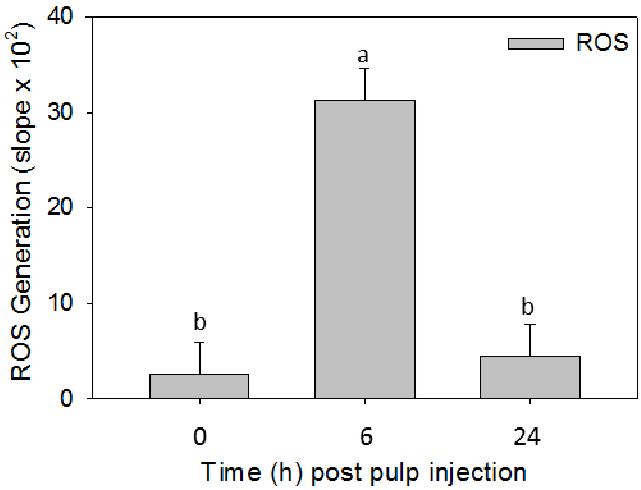


Figure 7. Reactive oxygen species (ROS) generation in the pulp tissue after injection of lipopolysaccharide (LPS) into the pulp of growing feathers.
Sixteen growing feathers (GF) of 24, 5-wk-old Cobb 500 broilers were injected with 10 µL of LPS (100 µg/mL; 1 µg/GF). Two injected growing feathers from each chicken were collected before- (0 h) and at 6 and 24 h post-LPS injection and used to prepare pulp cell suspensions. ROS generation by pulp cell suspensions was determined by kinetic fluorescence assay using 2'7'-dichlorofluorescin-diacetate (DCF-DA, Sigma) reagent. Data shown are time main-effect means ± SEM across dietary treatment; n = 24 birds (6 birds per diet). a, b Means without a common letter are different ($P \leq 0.05$)

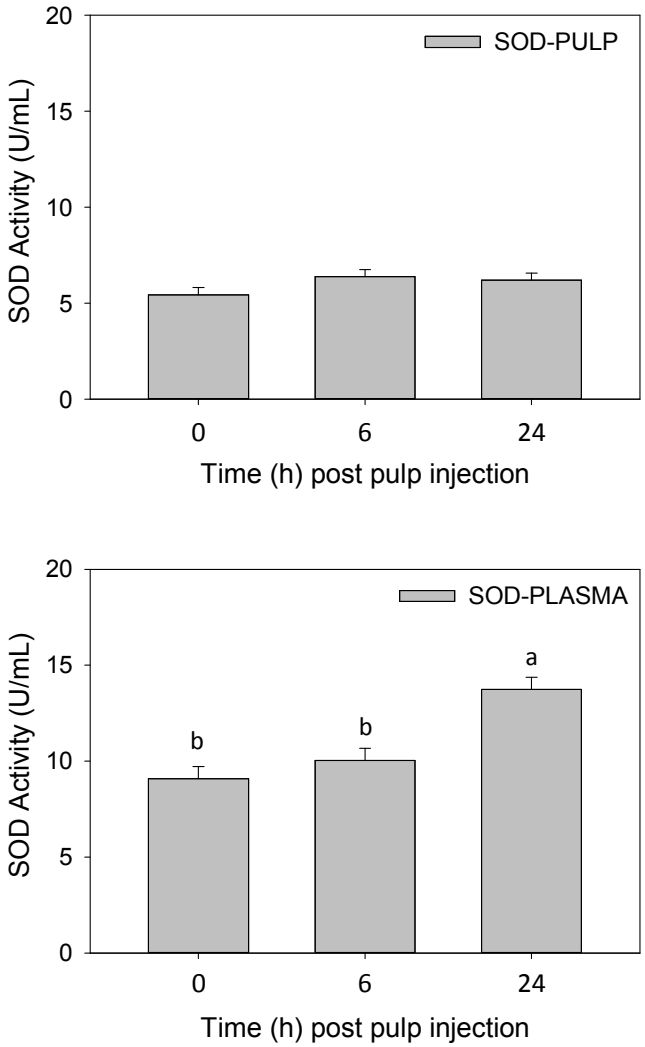


Figure 8. Superoxide dismutase (SOD) activity in the growing feather pulp and plasma after pulp-injection of lipopolysaccharide (LPS).
Sixteen growing feathers (GF) of 24, 5-wk-old Cobb 500 broilers were injected with 10 μL of LPS (100 μg/mL; 1 μg/GF). Heparinized blood (1 mL) and GFs were collected before- (0 h) and at 6 and 24 h post-LPS injection into the pulp. SOD activity levels in homogenized GF pulp and plasma were determined using a SOD assay kit (Cayman Chemicals). Data shown are time main-effect means ± SEM across dietary treatment; n = 24 birds (6 birds per diet). a, b For each measurement, means without a common letter are different ($P \leq 0.05$)

DISCUSSION

This experiment was conducted to determine the effect of diets containing sulfate or hydroxychloride forms of copper, zinc and manganese at two different levels on the local and systemic inflammatory response of broiler chickens following intradermal injection of lipopolysaccharide into the pulps of growing feathers. Specifically, trace minerals were provided in the sulfate form or in the hydroxychloride (Intellibond) form at two levels, either at recommended supplementation levels of 10 mg/kg Cu and 90 mg/kg of Zn and Mn (high-level diet) or at marginally lower levels of 5 mg/kg Cu and 45 mg/kg of Zn and Mn (low-level diet). Only few marginal effects of diet on the various aspects of the local and systemic LPS-induced inflammatory activities examined were observed in this study (i.e. altered pulp lymphocyte levels, blood monocyte concentrations, RBC concentrations and other RBC-related measurements, as well as pulp SOD activity). These will be discussed in the context of the many effects of LPS injection into the dermis of growing feathers examined at 6 and 24 h post-injection. Overall, however, the LPS-induced inflammatory response activities examined here in broilers were not impacted by the source of, or reduction in, trace mineral supplementation. Perez et al. (2017) used similar levels and sources of trace minerals and examined SOD activities of heterophils and monocytes isolated from blood collected from broilers 24 h after 500 μg/kg BW LPS administration. While direct comparison between their and our study is not possible, both studies suggest that conventional dietary supplementation levels of trace minerals appear to be sufficient for broilers to effectively respond to an LPS challenge.

The current study is also the first to describe the early phase (6 and 24 h) of the acute inflammatory response in broilers by monitoring both the inflammatory activities initiated by LPS at the site of injection (i.e. in the GF pulp) and in the peripheral blood circulation, in the

same individual. As inflammatory activities primarily occur in complex tissues, including the skin and its derivatives, assessment of inflammatory activities at the site of infection more clearly reflects local immune system efforts to eliminate the infection. By definition, "inflammation is a complex reaction of vascularized tissue to infection or cell injury that involves extravascular accumulation of plasma proteins and leukocytes" (Abbas et al., 2014). The source of both the accumulating leukocytes and the plasma proteins is the peripheral blood, hence many of the activities occurring at the site of inflammation may also be reflected in the peripheral blood circulation. Therefore, the dual approach used here to examine the LPS-induced inflammatory response at both the affected tissue and the blood is a more comprehensive assessment of the acute inflammatory response than solely examining events taking place in the blood.

As expected, heterophils were the first and most abundant cell type recruited to the pulp following LPS injection (Figure 1). Alterations in heterophil levels following the pulp injection of LPS were also reflected in the blood. However, while heterophils reached their maximum levels in the pulp (% pulp cells) and in the blood (K/μL and % leukocytes) at the 6 h time-point, heterophil levels remained elevated in the pulp but returned to pre-injection levels in the blood (Figure 3) at 24 h. Similarly, as expected monocytes/macrophages started to accumulate in the pulp at 6 h and increased to high levels by 24 h; however in the blood, monocyte concentrations reached their highest levels 6 h post LPS injection and returned to pre-injection levels by 24 h. It should be mentioned that diet did affect blood monocyte levels at 6 h post-LPS injection, with the low-level trace mineral supplementation resulting in higher levels of monocytes than the high-level supplementation, independent of source of trace minerals. The mechanism underlying

the higher levels of monocytes in the blood with lower levels of trace mineral supplementation needs to be further investigated.

Similar alterations in blood heterophil and monocyte levels have been described for chickens following LPS administered via different routes (i.m., s.c., intra-abdominal), as have heterophil and monocyte/macrophage recruitment profiles into tissue and the abdominal cavity in response to local LPS injection. For most of these studies, the dose of LPS administered was much greater than the 16 µg used here with doses reaching 1 mg/kg in several cases (Smith et al., 1975; Qureshi et al., 1986; Qureshi, 2003; Bowen et al., 2009). Chickens are known for their high levels of tolerance to LPS, hence it is surprising that the low dose of LPS (total 16 µg/bird) administered intradermally in the current study resulted in a clearly measurable response with the classical leukocyte profile changes in blood and tissue ascribed to the inflammatory response across various species.

As expected, lymphocytes, which do not tend to contribute to the early phase of the LPS response, were not recruited to the site of LPS injection. In fact, lymphocyte levels in the pulp dropped by 24 h post LPS injection. This drop in pulp lymphocyte levels was preceded by a drop in blood lymphocyte concentrations at 6 h post LPS injection; therefore it is possible that the drop in the pulp lymphocyte levels was due to lower levels in the pulp blood vessels. However, it should be noted that, at 6 h, the pulp T and B lymphocyte analysis only showed a drop in T cells and not B cells. Hence it may be a specific effect of LPS on tissue T lymphocytes that contributed to the overall drop in pulp lymphocytes. In the peripheral blood, lymphocyte concentration (K/µL) dropped at 6 h post LPS injection, however levels increased again to higher than pre-injection levels by 24 h. The observed drop in blood lymphocytes is similar to those seen by both Byrne (2016) and Bowen et al. (2009). The drop in lymphocytes in the blood

samples could be due to lymphocyte recruitment to secondary lymphoid organs, vascular adhesion of lymphocytes, or a direct cytotoxic effect of endotoxin (Bowen et al., 2009). Histological examination of secondary lymphoid organs and lungs in chickens that were injected i.v. with LPS neither supported emigration of lymphocytes from blood to these organs nor their vascular adhesion as an explanation for their drop in blood concentration within hours of LPS administration (Bowen et al., 2009).

Basophils and eosinophils did not seem to be affected by intradermal injection of LPS into the growing feather pulps and there were no differences in the concentrations and proportions of these cells based on dietary treatment. This is most likely due to the fact that these blood leukocytes are not as involved or heavily recruited in the LPS-induced acute inflammatory process.

As expected, total white blood cell concentrations (K/μL) reached maximum levels 6 h post LPS injection (Figure 5) and returned to pre-injection levels by 24 h. The heightened levels in total blood leukocytes at the 6 h time-point appears to be attributed to the increase in heterophils and monocytes, the key players in the LPS-induced inflammatory response. In addition to this, thrombocyte concentrations increased at 24 h post LPS injection. This increase of thrombocytes during the inflammatory response may be attributed to their role in vascular tissue repair as well as other functional capabilities, such as phagocytosis and production of cytokines and chemokines, that are similar to those of macrophages (Ferdous and Scott, 2015; Ferdous et al., 2017).

In contrast to white blood cell concentrations (K/μL), red blood cell concentrations (Mill/μL) dropped at 6 h post LPS injection and returned to pre-injection levels by 24 h. Hemoglobin and hematocrit concentrations (g/dL) followed this same trend seen in in red blood

cells, dropping at 6 h post LPS injection. The drop in RBC levels and other RBC measurements may be due to direct effects of endotoxin on RBCs as well as effects of toxic factors produced during the inflammatory response (Bateman et al., 2017). More research is needed to gain further insights into this phenomenon in broiler chickens.

After examining the distribution and recruitment of leukocytes and other cell populations in response to pulp-injection of LPS, we then focused on the LPS-induced generation of ROS in the injected pulp and on activities of SOD antioxidant enzymes in pulp and plasma. As discussed before, LPS initiates the activation of heterophils, and upon activation causes a respiratory burst. This respiratory burst then leads to production of reactive oxygen species (ROS) (Farnell et al., 2003). As expected, the generation of ROS observed in the pulp cell suspensions followed the trends associated with increased heterophil levels in the pulp, reaching maximum levels at 6 h post LPS injection. During the respiratory burst, SOD antioxidant enzymes are activated to offset the harmful effects of ROS, superoxide anion in particular. Examination of SOD activity levels in pulp homogenates revealed a source by level interaction, whereby pulp SOD activity in broilers fed the low-level trace mineral supplementation in the sulfate form. SOD activity in pulps from broilers fed the high-level trace mineral supplementation was similar, independent of source. This observation is in agreement with the report by Perez et al. (2017) for SOD activity by heterophils and monocytes isolated from blood of broilers fed diets with similar levels and sources of trace minerals and collected 24 h after LPS injection. Unexpected was, however, the lack of a significant time effect on pulp SOD activity, especially considering the increased levels of heterophils in the pulp at the 6 h time-point. However, examining the time course of pulp SOD activity for each diet separately, all diets tended to have higher activity at 6 hours, except

for the high-level sulfate diet, which did not show a change in SOD activity with time. Hence these data are difficult to interpret and warrant further investigation.

An increase in plasma SOD activity was observed at the 24 h time-point after LPS injection into the pulp of growing feathers and was not affected by level and source of dietary trace mineral supplementation. The origin of this extracellular SOD is not clear, but the increase in activity may be a reflection of release of SOD at the site of LPS-induced inflammation (i.e. injected GF pulp) during heterophil degranulation and net formation (Abbas et al., 2014).

In conclusion, this study demonstrated that 1) like in egg-type chickens (Erf and Ramachandran, 2016; Sullivan and Erf, 2017; Erf et al., 2017), the GF can be used as a minimally invasive testing site to monitor the local inflammatory response in broiler chickens; 2) concurrent, periodic sampling of blood and injected GFs at various times post-LPS injection of GF enables simultaneous assessment of inflammatory activities in a complex tissue and in the peripheral blood circulation of the same individual over time; 3) there are temporal, qualitative, and quantitative differences in inflammatory activities observed at the site of inflammation compared to the peripheral blood circulation; and 4) the GF provides an important window into the effects of dietary treatments on the inflammatory response in chickens. Additionally, while only few effects of dietary micronutrient source and level on the broiler's inflammatory responses were observed, this study provides an excellent basis for further studies on the effects of different forms (e.g. hydroxychloride vs. sulfate) and levels of micronutrients on the inflammatory response and other immune system activities in chickens.

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APPENDIX

To: Samuel Rochell
FR: Craig Coon
Date: November 6th, 2017
Subject: IACUC Approval
Expiration Date: March 3rd, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your Modification to protocol # 16054 *Effects of dietary copper, zinc, and manganese source and level on the acute inflammatory response of broilers* extend project dates to 3-2-19.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond March 3rd, 2019 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp