ESTABLISHMENT OF A MODEL TO EVALUATE THE EFFECTS OF ANTIBIOTIC-FREE PROGRAMS ON INTESTINAL DEVELOPMENT AND LOCAL IMMUNE RESPONSE IN THE SMALL INTESTINE OF BROILER CHICKENS

by

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Abstract

In the United States, consumption of chicken has doubled in recent years, making it an important protein source for American families. Recently, the broiler industry has begun to shift away from the use of antibiotic growth promotors, which has introduced some challenges, such as enteric diseases, to broiler production. Broiler gastrointestinal health is an important area of study as the gastrointestinal tract can require significant amounts of amino acids and energy that can then not be directly utilized for muscle protein accretion. To begin studying gastrointestinal health and development, an experiment was conducted to establish a model to evaluate the effects of antibiotic-free programs on intestinal development and local immune response in the small intestine of broiler chickens.

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I. Introduction

The broiler industry is projected to continue growing each year to meet the increasing demands of a growing world population. In 2018, the value of the 9 billion broilers produced in the United States was estimated to be $31.7 billion, with the state of Alabama contributing $3.3 billion to that total (USDA NASS, 2019). Broiler production has grown to this size partially due to the low cost, healthy, and readily available characteristics of broiler meat, making it an attractive protein source for consumers over other animal protein sources. From 1980 to 2018, in the United States, per capita consumption of beef decreased from 76 to 57 pounds, a 25% decrease, and per capita pork consumption has remained relatively stable at 50 pounds. However, per capita consumption of chicken has doubled from 45 to 90 pounds (National Chicken Council, 2019). Therefore, broiler production is an economically important industry that provides a desirable protein source to meet consumer demands.

The primary goal of the broiler industry is to produce salable meat products from broiler muscle protein, but continuous improvements must be made in order for the broiler industry to continue meeting national and global demand. Broiler production can be improved by increasing or refining broiler growth performance and health, and this can be accomplished through different means such as broiler genetics, management strategies, or nutrition. The gastrointestinal (GI) tract is particularly important to focus improvements on as its maintenance requires approximately 25 % of the total basal metabolic needs of an animal (Croom et al., 2000). The GI tract serves as the site of feed digestion and nutrient absorption, and it also plays important roles in metabolism,

endocrinology, and immunology. The majority of digestion and absorption occur within the small intestine. Thus, the GI tract, especially the small intestine, plays a vital role in providing broilers with energy and nutrients necessary to support growth and muscle accretion.

It is of interest to develop a better understanding the effects common industry practices have on broiler GI health, especially with the removal of antibiotic growth promoters (AGP), in order to avoid consequences that may negatively influence growth performance. According to past research, the dietary protein source fed to broilers and the condition of the litter they are reared on can affect the microbial population located in the GI tract, potentially resulting to a shift toward more pathogenic microorganisms (Wilkie et al., 2005; Cressman et al., 2010). A change in the microbial population to more pathogenic microorganisms can affect GI tract morphology and physiology and illicit a local GI immune response (Van Immerseel et al., 2002; Fasina et al., 2010.). Adaptations of GI tract morphology and physiology could modify the rate of proliferation, alter the rate of nutrient absorption, or disrupt gap junction protein expression, while an immune response could increase the infiltration of immune cells and release of immune cell signaling factors. Ultimately, each of these consequences could increase the nutrient and energy requirements of the GI tract, thus lowering the availability of nutrients and energy that could be utilized to support broiler muscle growth.

II. Literature Review

Challenges of Antibiotic-Free Broiler Production

It has been reported that as of 2019, more than 50 % of the broilers produced in the United States were reared under some type of antibiotic-free (ABF) program. This shift has primarily been driven by consumer preferences and concerns that using antibiotics increases the risk of bacteria developing resistance genes (National Chicken Council, 2019). Multiple ABF programs exist, differing in what antibiotics can be used and when the antibiotics can be administered. For example, broilers labeled raised without antibiotics cannot be given antibiotics via feed, water, or injection during the grow-out period while broilers labeled no antibiotic ever cannot be exposed to antibiotics at any point, from incubation in the hatchery to processing. In organic broiler production, broilers may receive antibiotics during incubation or on the first day post hatch, but not after that point. In any case, if broilers receive antibiotics, they must go through a withdrawal period prior to processing in order to ensure antibiotic residues that could be present in the meat products are within safe concentrations (USDA FSIS, 2019).

The move to producing broilers in the United States without antibiotics is likely an inevitable and permanent change rather than a temporary trend. The use of AGP in animal feed was banned in the European Union in 1999, and in 2017 the Veterinary Feed Directive was initiated in the United States, which limits the use of medically important antibiotics to control and treatment of certain diseases under the expertise of a veterinarian (Casewell, 2003; Maurer et al., 2017). ABF production introduces several challenges in terms of broiler production, management, health, and welfare (Cervantes,

2015). Previously, the broiler industry relied on AGP, which are subtherapeutic doses of antibiotics typically administered via the feed, to improve the efficiency of broiler production by improving growth performance and health (Smith, 2011). AGP help control and prevent broilers diseases, especially enteric diseases such as coccidiosis and necrotic enteritis (NE). These two diseases can persist in subclinical forms, thus going undetected and untreated while still negatively impacting growth performance and resulting in production losses (Yegani and Korver, 2008; Timbermont et al., 2011). The decrease in growth performance of the individual broiler may seem like a minimal concern, but when that loss is multiplied among the billions of broilers produced annually, then the losses are much more substantial. ABF broiler production challenges the efficiency, and therefore the sustainability, of the broiler industry. This demonstrates the importance of exploring appropriate alternatives to AGPs in order to avoid losses due to decreased performance.

The AGP alternatives that have been explored currently do not improve boiler growth performance by the same mechanism as AGP, which are thought to improve performance because they reduce the overall microbial load on the GI tract. This contributes to an anti-inflammatory effect and a reduction in the risk of an immune response (Roura et al., 1992; Neiwold, 2007). This would help to minimize the total energy and nutrient requirements of both the immune system and GI tract, thus freeing energy and nutrients for more favorable functions such as muscle protein accretion. However, alternative compounds do not reduce the overall microbial load but can promote broiler performance by altering gut pH, promoting the growth of appropriate microorganisms, inhibiting the growth of pathogens, or increasing nutrient uptake. Proper

use of AGP alternatives requires strategic applications based on the needs of individual producers (Ferket, 2004). With the removal of AGP, the broiler industry will need to develop a better understanding of the GI tract and the immune system's presence in the GI tract in order to prevent the partitioning of energy and nutrients to metabolic functions unfavorable for animal production and to help maintain the efficiency and sustainability of the industry.

Gastrointestinal Tract Anatomy and Physiology

Overview of the Broiler Gastrointestinal Tract

With the primary function of the GI tract being to digest consumed feed into small enough particles to facilitate nutrient absorption, broilers must first ingest feed to begin the process. The dimensions of a broiler's mouth are limited as the mouth is surrounded by hard, keratinized tissue, which form the beak. This, along with the lack of soft tissue lips to form a seal, puts constraints of the size of food broilers can consume efficiently. Food that is too large will be difficult to ingest as broilers cannot chew their food, and food that is too small or fine will fall from the sides of the broiler's beak (Moran, 1982). Upon successful prehension of appropriately sized feed, movement of the tongue coats the feed, at this point of ingestion termed bolus, in saliva to lubricate the bolus as it travels through the esophagus, and the bolus is continually lubricated by mucus glands located along the esophagus. The physical presence of the bolus stimulates

peristaltic contraction, which are wavelike contractions of smooth muscle, of the esophagus (Turk, 1982). These contractions move the bolus to the crop.

The GI tract, from esophagus to large intestine, can be thought of as a tube extending through the body of the animal, and the lumen of the tube is exterior to the body. Four distinct layers form the GI tract: serosa, muscularis mucosae, submucosa, and mucosa. The serosa is the outmost layer of the GI tract and is a continuation of the peritoneum. This layer mainly consists of connective tissue and helps to support the other layers of the GI tract. Moving inward, toward the lumen, the next layer is the muscularis externa. This layer consists of two smooth muscle layers, an outer longitudinal layer and an inner circular layer. Contraction of longitudinal layer shortens the length of the GI tract while contraction of the circular layer decreases the diameter of the lumen. This layer helps to move ingesta through the GI tract. The muscularis externa is also innervated by the myenteric nerve plexus which regulates GI motility. The submucosa is another layer of connective tissue, and it supports larger lymph vessels, blood vessels, and nerves which enter the innermost layer, the mucosa. The mucosa lines the lumen of the GI tract. Each of these layers can change in size or become more specialized in different regions of the GI tract (Silverthorn, 2016).

In mammalian species, secretion of salivary amylase, an enzyme that hydrolyzes starch, plays an important role in initiating carbohydrate digestion during mastication. However, Jerrett and Goodge (1973) found that amylase is not secreted by the salivary glands of chickens. Once the ingested feed reaches the crop, which is an enlarged region of the esophagus, it can be stored and slowly released further along the GI tract if the next region is already occupied. The crop contains mucosal folds that allow it to expand and

contract to accommodate varying amounts of feed that may be consumed. Digestive enzymes are not secreted by the crop, but some digestion does occur via microbial digestion (Moran, 1982). After temporary storage in the crop, ingesta is again propelled by peristalsis, through the lower portion of the esophagus, to the avian stomach.

Monogastric mammals possess a single stomach, capable of chemical digestion and churning activity due to a thick layer of smooth muscle. However, these two functions are spatially separate in chickens as they have a proventriculus specialized for glandular secretion of digestive enzymes and a gizzard specialized for muscular churning. The proventriculus has thick walls due to glands located in the mucosa. The two mains types of glands located in the proventriculus are tubular and gastric glands. The tubular glands secrete mucus, again helping to lubricate the ingesta and protect the mucosa lining the lumen of the proventriculus. The gastric glands contain cells which produce and secrete hydrochloric acid and pepsinogen (Turk, 1982). Pepsinogen is the inactive form of the protease pepsin, and upon secretion into the low pH environment of the proventriculus, it becomes active and breaks down large protein molecules (Baudys and Kostka, 1983). The chemical digestion of ingested feed begins in the proventriculus.

Next, ingesta is transferred to the gizzard which is made up of an asymmetric arrangement of two pairs of smooth muscle. This smooth muscle allows the gizzard to effectively massage, mix, and grind the ingesta, helping to decrease the ingesta's particle size and increase its surface area for exposure to chemical digestion. The hydrochloric acid and pepsinogen secreted by the proventriculus also digest the ingesta within the gizzard. Glands located in the gizzard mucosa produce rod like structures which protrude through the luminal surface of the gizzard lining, providing a more abrasive surface to

grind ingesta (Turk, 1982; Klasing, 1999). Contents of the gizzard next move to the small intestine, however ingesta can be refluxed back to the gizzard to aid in digestion. The majority of digestive and nutrient absorption function occur in the small intestine, and the small intestine will be discussed more thoroughly later. From the small intestine, luminal contents enter the colon, where fine particles, solutes, and fluids may enter the ceca. Broilers have two ceca, which branch from the most proximal region of the colon. Microbial fermentation occurs in the ceca, and water, electrolytes, and volatile fatty acids can be absorbed by the ceca and colon (Moran, 1982). Finally, unabsorbed ingesta are excreted from the bird via the cloaca.

Small Intestine Anatomy and Physiology

The small intestine serves as the primary site of digestion and subsequent nutrient absorption and is the main tissue of interest in this review. It is divided into three separate regions which, from proximal to distal, are the duodenum, jejunum, and ileum (Klasing, 1999). The duodenum begins just distal to the gizzard, forms one large loop around the pancreas, and ends where the pancreatic and bile ducts enter the small intestine. The jejunum extends from the pancreatic and bile ducts to Meckel's diverticulum, which is the point of the small intestine where the yolk sac was attached (Moran, 1982). The ileum is defined as the region from Meckel's diverticulum to the site of cecal attachment (Uni et al., 1999). The points used to describe the regions of the small intestine do not represent strict sites of morphological or physiological changes as these changes occur gradually along the length of the small intestine (Moran, 1982).

The small intestine epithelium is characterized by projections into the lumen known as villi and invaginations of the epithelium which form the intestinal crypts. The villi greatly increase the surface area of the intestinal epithelium and allow more exposure of the epithelium to the digesta within the lumen. In avian species, the villi have a broad, leaflike shape. The small intestine of mammals has a macroscopic folding to further increase the surface area, however avian species lack this characteristic (Turk, 1982). The primary epithelial cell type constituting the intestinal villi are enterocytes, estimated to comprise approximately 90 % of villi cells. Enterocytes facilitate the digestive and absorptive functions of the small intestine (Umar, 2010). Enterocytes; are a columnar shaped cell, whose nucleus is located on the basal end, with microvilli extending from the apical surface. The enterocytes are bound together by tight junctions, which help to form a solid barrier between the intestinal lumen and the *lamina propria* (Karcher and Applegate, 2008). Dispersed among the enterocytes are goblets cells. These cells produce and secrete mucins that form the mucus layer lining the epithelial cells (Turk, 1982).

One important characteristic of the small intestine epithelium is its continuous cell turnover. Intestinal epithelial cells have a short lifespan, generally three to five days, resulting to a rapid rate of apoptosis. However, villus length is maintained by the ongoing mitotic activity of cells within the intestinal crypts (Williams et al., 2015). The quick turnover of intestinal epithelial cells likely helps to maintain health and function of the GI tract as this layer of cells is exposed to a multitude of destructive forces, friction from digesta, digestive enzymes, toxins, pathogens, and antigens, that may quickly lead to senescence of the epithelial cells. It has been estimated that 2×10^8 cells are shed from

the small intestine of mice every 24 hours (Potten, 1990; Williams et al., 2015). So, by rapidly replacing cells that may be damaged, the small intestine can properly function as a site of nutrient absorption and as a barrier to potential pathogens.

Epithelial Cell Migration and Shedding

Several models of intestinal cell migration were suggested by Wright and Alison (1984) and Kaur and Potten (1986). However, this is likely a complex mechanism as the cytoskeleton structure of enterocytes does not reveal any obvious means of movement and enterocytes lack typical cellular features of locomotion such as pseudopodia or lamellipoda. Therefore, Heath (1996) proposed a co-operative model which stated that several of the forces described by Wright and Alison and Kaur and Potten, which alone do not seem robust enough to induce the dorsal movement of villi cells, work in concert to produce enterocyte migration. Four mechanisms comprise Heath's co-operative model, and these are: cell proliferation, cell cohesiveness, actions of the cytoskeleton, and actions of the basement membrane.

In the intestine, cell proliferation, which will be discussed later in greater detail, occurs in intestinal crypts. The intestinal crypts are surrounded by a pericryptal sheath, consisting of specialized fibroblasts that are tightly bound together and follow the contours of the crypts (Mutoh et al., 2005). The pericryptal sheath may fix crypt size and help to direct mitotic pressure dorsally along the villi rather than toward crypt enlargement. The epithelial cells act as one cohesive sheet due to cell linkage via lateral adhesive complexes, which may aid in cell migration by applying forces acting on one cell to nearby cells (Garcia et al., 2018). This would help to push or pull epithelial cells

together as a whole rather than individual cells migrating independent of others. Due to the tall, narrow shape of intestinal epithelial cells, the locomotory function of the cytoskeleton of these cells is likely different than the cytoskeleton mechanism producing movement in other cell types. The cytoskeleton of intestinal epithelial cells consists of apical rings of actin filaments, linked to *zonulae adherens,* which binds the actin cytoskeleton to cell junctions. Contraction of actin rings narrows the intestinal epithelial cells apical region and elongates the cells, thus generating tension. If this occurs in a gradient of tension along a villus, it could move cells along the villus. Finally, upward movement of the basement membrane may passively move overlying epithelial cells. Again, individually, each of these forces may be too weak to illicit epithelial cell migration, but working together, these forces may generate cell migration (Heath, 1996).

Under normal, homeostatic conditions, cells of the intestinal epithelium are ready to be shed by the time they migrate to the tip of the villi. Currently, little evidence exists to explain whether the shedding events induce apoptosis or apoptosis induces shedding events. There is some variance in the mechanism of extrusion used among different species, such as shedding of cell fragments or via phagocytosis by macrophages (Han et al., 1993; Mayhew et al., 1999). However, enterocyte shedding has been more thoroughly characterized in humans, mice, rats, and hamsters which shed enterocytes via whole cell extrusion. Bullen et al. (2006) described the role of the tight junction protein zona occluden (ZO)-1 and myosin light chain kinase (MLCK), an enzyme that regulates myosin-actin crossbridge formation. These two proteins were generally observed at the apical end of the epithelial cells lining a shedding cell, suggesting they may participate in a mechanism to close the gap left by the shed cell. Further study of this process by Guan

et al. (2011) helped to reveal the progression of cell shedding in the mouse jejunum by use of *in vivo* immunocytochemistry. Guan et al. observed that initially, ZO-1 concentrates at the apical end of an enterocyte. Then, the nucleus of the shedding cell begins to shift apically while ZO-1 migrates around the basal end of the enterocyte and contracts, forming a funnel shape and helping extrude the shedding cell. This identified ZO-1 redistribution as one of the earliest markers of cell extrusion. However, it is likely that more proteins are yet to be identified that play a role in this complex process.

In the case of production animals such as broilers, where efficient growth is a major concern, the energy, amino acids, and other molecular components required to create new intestinal epithelial cells to replace those that are shed can be viewed as a cost that does not directly support muscle growth and muscle protein accretion, which is the primary goal of animal production. Alternatively, high rates of proliferation in the GI tract may be necessary to maintain GI and animal health and nutrient uptake, and without these important processes, muscle growth would be impossible. Also, rapid closure of gaps left by shed cells is vital in preventing pathogens and toxins from entering host tissues. Therefore, it will be important to better understand these physiological processes in production animals in terms of factors that may affect the rate of proliferation and cell shedding, how gaps left by shedding cells are closed, and what effects different rates of proliferation and cell shedding have on GI health and overall growth performance.

Intestinal Stem Cells

The proliferative cells of the small intestine are located in the intestinal crypts that surround each villus, and the intestinal stem cells (ISC) are found near the base of the

crypts. Although the location of ISC have been better characterized in mammalian species, their exact location is still a debated topic. Two models have been proposed to describe ISC location, the stem cell zone model by Cheng and Leblond et al. (1974) and the +4 model by Potten (1977). In the stem cell zone model, ISC are thought to be the crypt base columnar cells dispersed among Paneth cells at the base of the crypt. While in the +4 model, ISCs are thought to be located in a ring of 16 cells just above the Paneth cells, which comprise the base of the crypt. More recently, a new model has been proposed that incorporates both of these models. This model describes the crypt base columnar cells as the primary proliferative cells. These maintain daily homeostasis of the intestinal epithelium, and the +4 stem cells are a group of quiescent, reserve stem cells which become active to perform tissue repair after injury (Barker, 2013). It is important to note that this model was proposed based on information gathered from murine ISC models, and it may need to be adapted to appropriately describe the ISC location in broiler intestinal crypts.

Regardless of their location within the crypt, ISC have been described by Marshman et al. (2002) as having three defining characteristics. First, ISC are self-maintaining. This means they are able to divide asymmetrically, producing both a more differentiated daughter cell and an ISC, allowing their population numbers to be maintained. Second, ISC are multipotent, meaning that the daughter cells they produce are able to differentiate down different cell lineages to produce different intestinal cell types. Third, the ISCs have a clonogenic capacity. This allows nearby ISC to detect damaged or dying ISC and divide symmetrically to produce an ISC to replace dead ISC. There is a separate region of proliferative cells located above the ISC, known as the

transit amplifying (TA) cells. Although these cells still possess mitotic capabilities, they are more differentiated than the ISC. This means they no longer possess the multipotent capacity of their precursor ISC, but TA cells are important to exponentially increase the total number of cells that can be produced from a single ISC as TA cells are able to divide approximately 6 times. However, TA cells are fully differentiated by the third division (Marshmen, 2002). The TA cells produce the mature cell types which comprise the intestinal epithelium, and these mainly include enterocytes, goblet cells, Paneth cells, and enteroendocrine cells.

Enteric Diseases

To understand the functions of immune system within the GI tract, it is important to be aware of different agents which may elicit an immune response, such as dietary antigens, physical injury to the mucosa, or more typically, pathogenic organisms. The GI tract harbors a diverse and complex community of microorganisms, known as the microbiome, that primarily consists of bacteria but also includes fungi, protozoa, and viruses (Shang et al., 2018). Previously, study of microorganisms was limited to those which could be cultivated. However, some microbes are difficult to grow in culture. Of the bacteria comprising the human intestinal microbiome, approximately 20 % are cultivable compared to those identified by 16S ribosomal RNA (Eckburg et al., 2005). Fortunately, advances in microbe identification techniques, such as sequencing of 16S

rRNA and metagenomics, have provided methods to more accurately study the intestinal microbiome in the absence of a cultivability bias (Oakley et al., 2014).

The microbiome includes both commensal and pathogenic microorganisms. Commensal microbes can be beneficial to the overall health of broilers by playing a role in the development of the GI tract and immune system, producing nutritional compounds, and inhibiting pathogen growth. Although these functions are beneficial, commensals do have some negative effects, such as reducing the energy and amino acids available to the host, producing toxic metabolites, and increasing mucin secretion and epithelial cell turnover rate (Shang et al., 2018). The benefits of commensals may outweigh any costs the host incurs. The line between commensal and pathogenic microbe can be blurred, as in some circumstances a microorganism that is typically commensal may overgrow and cause disease in the host. These include opportunistic pathogens such as *Clostridium perfringens*. *C. perfringens* is typically found in healthy broilers, but in the presence of certain predisposing factors, it can cause necrotic enteritis (Wigley, 2015). There are several enteric microorganisms typically studied because they are important to broiler health and performance or potentially cause disease in humans. Two enteric diseases that are prominent in the broiler industry will be discussed here.

Coccidiosis

Prevention, treatment, and production losses due to coccidiosis have been estimated to have a global economic impact of $3 billion USD (Blake and Tomley, 2014). Therefore, coccidiosis is an economically important disease to the broiler industry. The primary causative agent of coccidiosis is a protozoan parasite of the genus *Eimeria.*

Seven species of *Eimeria* are known to infect chickens, each of these affecting different regions of the small intestine, and often multiple species co-infect the host. (Huang et al., 2008; Quiroz-Castañeda and Dantán-González, 2015). However, *E. acervulina*, *E. maxima*, and *E. tenella* are typically the most prominent species. Clinical symptoms include lethargy, diarrhea, muscle wastage, and sudden death (Bould et al., 2009). Ultimately, these result in decreased growth performance. Research has indicated that coccidia-infected broilers have a reduced feed intake and lower gene expression of brush border amino-acid transporters, which may contribute to reduced growth performance (Miska and Fetterer, 2018). Coccidiosis is often diagnosed by the presence of intestinal lesions identified in a necropsied broiler, which upon direct smearing of the lesion and microscopy analysis reveal the presence of *Eimeria* oocysts. Molecular techniques, such as PCR, are necessary to determine the species of *Eimeria* (Fatoba and Adeleke, 2018). Even in the absence of clinical symptoms, coccidiosis can persist in a subclinical form and have deleterious effects on growth performance of broilers (Vermeulen, 2001).

Infection by *Eimeria* begins upon the host ingesting *Eimeria* oocysts. These oocysts are resilient to environmental and chemical challenges due to a robust, protein-rich, oocyst wall, making it challenging to kill the oocysts through typical disinfection measures (Mai et al., 2009). Initially, the oocyst contains a single-celled zygote that undergoes division to form sporozoites. Once ingested, the sporulated oocyst is digested by mechanical and chemical means, releasing motile sporozoites from the oocysts, and the sporozoites are then able to penetrate the cells of the intestinal epithelium, becoming trophozoites (Jeurissen et al., 1996). The trophozoites undergo asexual reproduction, called schizogony, which produces hundreds of new daughter cells, called merozoites. As

merozoites mature, tissue destruction occurs, forming clinical lesions (McDougald, 1998). Merozoites can break free, enter more epithelial cells, and produce several more generations, which will terminally develop into male microgametes and female macrogametes. The microgametes are motile and will sexually fuse with macrogametes to form new zygotes that develop into oocysts that will be shed with the feces. Oocysts are present in the feces 4 to 5 days post-infection (Allen and Fetterer, 2002).

It is challenging to rear broilers free of *Eimeria*, especially without a thorough cleanout between flocks, as they are a ubiquitous organism, reproduce rapidly, and oocysts readily sporulate in chicken litter. In the past, producers have relied on vaccination programs or inclusion of coccidiostats (particularly ionophores) in feed to control coccidiosis (Allen and Fetterer, 2002). However, with the risk of resistant *Eimeria* strains developing and restriction of coccidiostats, increasing importance will be placed on developing alternative methods to control coccidiosis in broilers. Wang et al. (2019) performed a 2 × 5 factorial treatment experiment to evaluate broilers receiving coccidial vaccination and antimicrobial alternatives (control, antimicrobials, prebiotics, probiotics, and probiotics + prebiotics) during an *Eimeria* challenge. Broilers vaccinated against coccidia had a lower feed intake and body weight gain for the overall period (d 0-56), and broilers fed antimicrobials (bacitracin and salinomycin) had an improved FCR post-challenge but an FCR similar to control, prebiotic, and prebiotic + probiotic broilers for the overall period. It was also observed that broilers fed antimicrobials had lower small intestine and proventriculus relative weights but not lower body weight, possibly indicating a lower partitioning of energy and nutrients toward organ maintenance and freeing it for muscle growth. The complex life cycle of *Eimeria* requires a complex

immune response from the host. This warrants future investigation of immune response parameters, such as cytokine expression or immune cell activity, of broilers during *Eimeria* challenge under administration of antimicrobials and antimicrobial alternatives to better understand methods to control coccidiosis.

Necrotic Enteritis

NE is another economically important enteric disease, estimated to cost global broiler production $5-6 billion, whose etiological agent is *Clostridium perfringens* (Wade and Keyburn, 2015; Fasina and Lillehoj, 2019). NE can occur in clinical or subclinical forms, and it is clinically characterized by abrupt flock mortality, with large necrotic loci on the intestinal mucosa observed upon necropsy. Key economic losses occur with subclinical forms of NE as it often goes undetected and untreated but still causes chronic damage to the intestinal mucosa and reduces growth performance of broilers (Van Immerseel et al., 2009). NE was first described in 1961, and until recently it was primarily managed by AGPs. Removal of AGPs from broiler production has increased the incidence of NE (Timbermont et al., 2011).

C. perfringens is a gram-positive, rod shaped, spore-forming bacteria that is typically present in the small intestine of broilers, but toxin-producing strains can act as an opportunistic pathogen. Initially, alpha toxin was thought to be the primary agent of *C. perfringens* that induced NE. However, more recent research has placed this role on NE B-like toxin (NetB), a member of the β-barrel pore-forming toxin family, as studies using NetB-negative *C. perfringens* are unable to induce NE (Timbermont et al., 2011). Several factors have been shown to predispose to broilers to developing NE, such as

dietary protein source, non-starch polysaccharides, stress, and coccidiosis (Shojadoost et al., 2012). Dietary protein source and coccidiosis have been shown to promote *C. perfringens* growth through a similar mechanism. It has been demonstrated that glycine supports *C. perfringens* growth whether *in vivo* or *in vitro* (Dahiya et al., 2005; Wilkie et al., 2005; Dahiya et al., 2007). In the case of over formulation of dietary amino acids or inclusion of glycine rich dietary protein sources, glycine may be overfed, not capable of being fully absorbed by the broiler, and pass to the ileum where *C. perfringens* is typically located. As previously discussed, during its intracellular phase, *Eimeria* kills host epithelial cells, and this results in leakage of plasma proteins into the intestinal lumen, providing a rich protein source for *C. perfringens* (Van Immerseel, 2004). Also, *Eimeria* stimulates intestinal mucogenesis due to an inflammatory immune response mediated by the cytokine interleukin (IL)-4 and possibly IL-9 and IL-13, and *C. perfringens* is capable of using mucus as a substrate (Collier et al., 2008). Interestingly, glycine is a predominate amino acid in mucin glycoproteins (Strous and Dekker, 1992). Ultimately, *C. perfringens* induced NE is an important broiler pathogen that becomes problematic after exposure to some type of predisposing factor.

Multiple studies have been performed to evaluate antibiotic alternatives and methods to control NE. Vaccination offers an ABF method of controlling bacterial diseases. However, research by Mot et al. (2012) indicated that vaccinating broilers against *C. perfringens* once at day-of-hatch was not sufficient to illicit immunity to NE, rather vaccination on d 3 or double vaccination on d 3 and 12 provided effective results but may be too labor intensive to be practical in broiler production. Tsiouris et al. (2013) were able to use feed restriction to lower NE lesion scores in *C. perfringens* challenged

broilers (inoculated 3 times daily for 4 d) when feed was removed 12 h prior to the first oral inoculation each day. Emami et al. (2019) evaluated the effect of an AGP (virginiamycin) and probiotics (PrimaLac®) on the gene expression of tight junction proteins and cytokines in the jejunum of broilers under an NE challenge. Probiotic broilers had higher claudin-3 and ZO-2 mRNA expression than control broilers but not AGP broilers, whose claudin-3 and ZO-2 mRNA expression was similar to both probiotic and control broilers. This may have contributed to GI integrity and the lower lesion score observed in the broilers receiving probiotics. Broilers administered the AGP had decreased expression of IFN-γ, a proinflammatory cytokine, and similar results were observed by Fasina and Lillehoj (2019) using a different AGP (bacitracin methylene disalicylate). However, probiotic broilers had increased expression of IL-10, an anti-inflammatory cytokine, and these results may explain the positive health and performance effects AGP and probiotics have but suggest that AGP and probiotics produce this effect via different mechanisms.

One study by Lee et al. (2014) compared the effects of the probiotic *Bacillus subtilis* to salinomycin, used as an AGP, on growth performance, and serum antibody concentrations against *Eimeria* spp. and *Clostridium* spp. Although the broilers used in this study were not directly challenged, they were reared on litter that was reused by ten flocks and contained *Eimeria* spp. and *Clostridium* spp. This exposed the broilers to these two pathogens in a manner similar to a commercial setting. On d 28, broilers fed *B. subtilis* had a higher body weight than those fed salinomycin, and salinomycin broilers had a lower body weight than the control group. Salinomycin and *B. subtilis* broilers had lower concentrations of *Eimeria*-specific serum antibodies compared to the control, but

C. perfringens serum antibody concentrations were similar among the three groups, which may indicate the broilers were not exposed to enough *C. perfringens* in the used litter to illicit an immune response or the response had decreased by the time of sampling on d 28. *B. subtilis* increased serum nitric oxide concentrations, but salinomycin lowered the expression of proinflammatory and increased the expression of anti-inflammatory cytokine mRNA. These results may indicate that although probiotics and AGP have a beneficial effect on broiler health, they work through different mechanisms.

Broiler Immune System

Overview of the Immune System

The immune system, regardless of which vertebrate species, is generally divided into the innate and the adaptive immune systems. These two branches of the immune system differ in many characteristics, such as their mechanism of defense, response time, and primary cell type (Katz, 1982). The innate immune system is designed to respond quickly and in a generalized manner to pathogen invasion. This allows the innate immune system to deal with a vast variety of pathogenic microorganisms early in the infection process before the microbes can spread from the site of infection to other tissues (Tizard, 2004). However, the innate immune system may also cause damage to host tissue (Beutler, 2004). In the case of challenging infections, where the innate immune system is unable to fully restrain the pathogens, the adaptive immune system begins to mount an attack. While the adaptive immune system may take up to a week to become active, its

attack is targeted to specific microorganisms (Tizard, 2004). Thus, the adaptive immune system can direct its attack on the pathogen infecting the host, and it retains some immunological memory to mount faster attacks upon subsequent exposure (Parkin and Cohen, 2001). Although the two branches of the immune system are distinct in the timing and specificity of their attack, both play an important role in maintaining animal health.

The innate and adaptive immune systems are further subdivided into humoral and cellular components. Humoral components consist of the macromolecules involved in an immune response rather than the cells involved, which comprise the cellular components. The humoral components of the innate immune system include cytokines, antimicrobial peptides, and microbial antigen binding proteins, while the cellular components include cells such as mononuclear and polymorphonuclear phagocytes, natural killer cells, and mast cells (Beutler, 2004). The humoral adaptive immune system consists of antibodies, while the cellular adaptive immune system consists of T- and B-lymphocytes (Katz, 1982). Although the immune system can be subdivided into several distinct categories, the components of the innate immune system communicate and aid components of adaptive immune system and vice versa (Tizard, 2004). However, significant focus will be given here to the cellular and humoral components of the innate immune system, specifically macrophages and their relevant cytokines.

Inflammatory Immune Response

In the case of pathogen invasion, the first response of the host is to induce inflammation at the site of infection. Inflammation is characterized by redness, swelling, heat, pain, and loss of function. Invading pathogens are first detected by sentinel cells,

which include macrophages, mast cells, and dendritic cells (Tizard, 2004). In the small intestine, these cells are located just deep to the epithelial cell layer, within the *lamina propria*, which optimally positions these cells to detect invading pathogens (Zigmond and Jung, 2013). Sentinel cells are capable of detecting pathogens because they possess toll-like receptors (TLR) on their surface. There are many TLR, each designed to recognize specific pathogen-associated molecular patterns (PAMP), such as peptidoglycans, lipopolysaccharides (LPS), and glycolipids. Some TLR can also detect host cell components released by cells that were damaged, possibly by pathogens (Davidson et al., 2008). When a PAMP binds to the proper TLR, it induces a signaling cascade to appropriately respond to the PAMP, and this typically leads to activation of the innate immune system and a local inflammatory response via release of cytokine messengers from the sentinel cell (Tizard, 2004).

The inflammation process occurs quickly after pathogens are detected. Initially, blood flow to the affected area is decreased to facilitate leukocyte binding at the site of infection. Then, blood vessels dilate to increase blood flow and induce swelling (Tizard, 2004). The activated sentinel cells will release the cytokine tumor necrosis factor-α (TNF-α), which induces local symptoms of inflammation and recruits neutrophils, a polymorphonuclear leukocyte (homologous to heterophils in avian species), to the site of infection (Genovese et al., 2013). These are a phagocytic cell, which engulf and destroy any pathogens, and are always present in systemic circulation. Although neutrophils are quick to respond, they are unable to sustain phagocytosis for long periods because of a limited, non-replenishable energy reserve. So, a second wave of mononuclear leukocytes, the macrophages, are recruited to the area by signals released from the neutrophils.

Macrophages are slower to respond than the neutrophils, not arriving to the site until hours after the neutrophils, but they are able to repeatedly phagocytize pathogens (Tizard, 2004). Thus, the recruited macrophages act as a backup to ensure all of the pathogens have been destroyed.

Macrophages play a key role in the inflammatory immune response due to their high capacity for phagocytic activity, which allows macrophages to uptake particulates within a plasma membrane envelop known as a phagosome (Gordon, 2016). This is in part due to their expression of TLR, which allow macrophages to detect a multitude of pathogens, but macrophages are also capable of respiratory burst activity. Upon engulfing pathogens within a phagosome, macrophages are able to manipulate the environment within the phagosome to kill the contained pathogen. The phagosome matures into a phagolysosome as proteases, antimicrobial peptides, and lysozyme are delivered to the phagosome and the pH of the phagosome lowers (Slauch, 2011). Also, NADPH-dependent phagocytic oxidase can be embedded in the phagolysosome membrane, which pumps electrons into the phagolysosome to reduce oxygen to superoxide anion, and an embedded nitric oxide synthase produces nitric oxide (Fang, 2004). Reactive oxygen species (ROS), such as superoxide anion and nitric oxide synthase, produced by macrophages have potent antimicrobial properties, however the exact mechanism by which ROS kill pathogens has yet to be fully elucidated (Fang, 2011; Slauch, 2011).

Near the end of an inflammatory immune response, there must be a switch from killing pathogens to repairing tissue that was damaged by the pathogen or by the immune response itself. It is also important that this switch not occur too early as not killing all the pathogens present would be problematic. Again, neutrophils have a short lifespan, so

they typically die during the inflammatory response and must also be cleared from the site. Macrophages play an important role during the repair process by phagocytizing any dead or dying neutrophil, releasing tissue growth factor-β (TGF- β), recruiting fibroblasts to deposit collagen at the site of injury, and promoting angiogenesis if needed (Tizard, 2004). Eventually, tissue homeostasis will be restored. This process has been well described, however some of the underlying signaling mechanisms that induce changes in macrophage phenotype require further study.

Innate Immune System Receptors

Immune cells of the innate immune system possess specific pathogen-recognition receptors (PRR) designed to detect various, generalized immunostimulatory compounds. In contrast to adaptive immune cell receptors, PRR are genetically inherited, trigger an immediate response, recognize broad classes of microorganisms, and are expressed similarly among cells of the same type. Once PRR are triggered, they can promote phagocytosis, help guide immune cells to the site of infection, or induce the release of cytokine signaling molecules (Janeway, 2001). Pathogen-recognition receptors can recognize PAMP, which are typically highly conserved pathogen molecules, as well as damage-associated molecular patterns (DAMP), in order to identify tissue damage possibly due to pathogen infection (Kawasaki and Kawai, 2014). There are four PRR families, which are TLR, nucleotide oligomerization domain-like receptors (NLR), C-type lectin receptors (CLR), and retinoic acid-inducible gene I-like receptors (RLR). More thorough review of NLR, CLR, and RLR has been accomplished by Neerukonda and Katneni (2020); however, avian TLR will be described here.

Toll-like Receptors. Of the PRRs, TLR are the most well studied, and they are a family of type I transmembrane proteins. The general structure of TLR consists of an extracellular N-terminal domain, which contains of 19-25 leucine-rich repeats (LRR) that form a horseshoe shape, a transmembrane domain, and a cytosolic signaling domain. Modification of the LRR of the extracellular domain provide variability necessary for TLR to recognize different PAMP (Botos et al., 2011). Also, TLR can be subdivided into two main groups based on their location, either on the cell membrane, which detect pathogen membrane molecules, or intracellular, which span the membrane of an endosome and detect pathogen nucleic acids. Although TLR are primarily associated with immune cells, they can be expressed by other cell types.

Mammalian TLR have been more thoroughly studied, but some research has investigated avian TLR. One study found that chicken TLR (chTLR) 2 responded to lipoproteins and LPS, a role accomplished by TLR2 and TLR4 in mammalian species (Fukui et al., 2001). While, chTLR3 detects double stranded RNA, which is characteristic of many pathogens, and induces the expression of interferons (IFN) and IL-10 (Karpala et al., 2008; He et al., 2011). Avian TLR4 forms a receptor complex with myeloid differentiation protein-2 (TLR4/MD-2), and this receptor responds to LPS. However, TLR4/MD-2 may activate signaling pathways differently than its mammalian counterpart (Keestra and Putten, 2008). Bacterial flagellin, the structural monomer of flagella, is detected by chTLR5 (Keestra et al., 2008). Detection of single stranded RNA is accomplished by chTLR7. In mammals, this role is played by both TLR7 and TLR8, but chTLR8 is nonfunctional (Philbin et al., 2005). Avian species uniquely express TLR15. Research has suggested that it plays an important role in bacterial detection, but the

specific TLR15 ligand has yet to be identified (Higs et al., 2006; Nerran et al., 2010). The functional ortholog of mammalian TLR9 is chTLR21, and it recognizes DNA (Keestra et al., 2010). The response of ligand binding to TLR is specific to each TLR to provide an appropriate response to the recognized pathogen, but possible results to TLR activation include stimulating production of anti-microbial peptides, proinflammatory cytokines, IFNs, induction and activation of enzymes associated with innate immunity, and up-regulation of both MHC class I and class II costimulatory molecules (Brownlie and Allan, 2010).

Innate Immune System Signaling Molecules

Upon detection of pathogens via PRR, immune cells must quickly respond in order to terminate pathogen invasion before significant consequences occur. The cells of the immune system express several signaling molecules known as cytokines and chemokines. Similarly, to PRR, non-immune cells can also express these signaling molecules. Cytokines help regulate the character and duration of an immune response from initiation to resolution and can act on target cells in an autocrine, paracrine, or endocrine fashion (Altan-Bonnet and Mukherjee, 2019). Major cytokines include the interleukins (IL), IFN, transforming growth factor-β (TGF-β), tumor necrosis factor (TNF), and colony stimulating factor (CSF), and these are classified as either proinflammatory or anti-inflammatory based on the response they induce (Davidson et al., 2008). Chemokines help attract immune cells to the site of infection, and can be divided into two groups, homeostatic or inducible. Chemokine nomenclature is based on the spacing of cysteine residues at the NH_2 terminus. Chemokines are placed into one of

four groups XC, CC, CXC, or CX3C groups, where X represents a separating amino acid. The suffix L is added to designate ligands, and the suffix R is added to designate receptors (Tizard, 2004). Some commonly studied cytokines relevant to macrophages will be discussed here.

Proinflammatory cytokines. Interleukin-1β is a potent inducer of inflammation, that is expressed early during an immune response. Although monocytes and macrophages are the primary producers of IL-1β, it can be expressed from multiple cell types, including neutrophils, natural killer cells, hepatocytes, and epithelial cells. Interleukin-1β is synthesized as pro-IL-1β, which is biologically inactive until it the pro-domain is cleaved by caspase-1 (Gyorfy et al., 2003; Arend et al., 2008). The chicken homologue of mammalian IL-1β was identified and cloned by Weining et al. (1998), and it was demonstrated that chicken macrophages have high transcript levels of chIL-1β mRNA for up to 12 hours after exposure to LPS. Similarly, a study by Kogut et al. (2005) showed that IL-1β mRNA expression was elevated in IFN-γ-primed heterophils (avian equivalent to neutrophils) stimulated by *Salmonella enteriditis*, a gram-negative, LPS producing pathogen. Interleukin-1β mRNA has also been shown to be elevated in chicken jejunum lymphocytes four days post *E. maxima* challenge (Hong et al., 2006). Upon reaching the liver, IL-1β stimulates the release of acute phase proteins, which play a role in the acute phase response, and IL-1β induces mast cells to release histamine, which promotes local vasodilation (Gruys et al., 2005; Duque and Descoteaux, 2014). Although initially an inflammatory immune response is beneficial to combat pathogen invasion, the inflammatory response must terminate to avoid detrimental effects to host tissue. Induction of inflammation by IL-1β is regulated by another member of the IL family, IL-

1 receptor antagonist (IL-1Ra), which binds to the IL-1 receptor with a similar affinity as IL-1β but fails to activate cells (Arend, 2002). Thus, IL-1Ra can be used to block IL-1β from binding its receptor.

An important, pleiotropic proinflammatory cytokine is TNF-α, which is a member of the TNF superfamily and was first described in the 1970s (Carswell, 1975). Since that time, TNF-α has been characterized as a key component in many processes such as cell survival, apoptosis, necroptosis, and intercellular communication (Blaser et al., 2016). Many similarities are shared between TNF-α and IL-1β, such as being primarily produced by macrophages, stimulating the acute phase response, and inducing fever. After being synthesized, TNF-α precursors can be found in the Golgi complex and are then transported to the cell surface via specific LPS-regulated tubulovesicular carriers. Upon delivery to the cell surface, the precursors are quickly cleaved by TNF converting enzyme, in order to release soluble TNF-α (Stow et al., 2009). The soluble TNF-α can bind to two receptors, TNFR1 and TNFR2, which are differentially expressed in different cell types. Nearly all cell types express TNFR1, and its activation elicits cellular responses while TNFR2 is expressed only on immune cells and plays a role in lymphoid cell proliferation (Parameswaran and Patial, 2010; Yang et al., 2019). One important role of TNF-α is to activate macrophages which then migrate to the site of inflammation to phagocytize and lyse pathogens (Parameswaran and Patial, 2010). There is some debate that recent research involving chicken TNF-α was actually analyzing LPS-induced TNF-α (LITAF), which are unrelated proteins (Ellender and Kaspers, 2019). Also, there was speculation that chickens lacked TNF-α, but the gene has now been identified (Bornelöv et al., 2017; Rohde et al., 2018).

Anti-inflammatory Cytokines. Although proinflammatory cytokines play an important role in initiating responses to defend against infection, their counterpart, the anti-inflammatory cytokines, have an equally important role in resolving the inflammatory immune response and preventing an inflammatory immune response at inappropriate times. Without anti-inflammatory cytokines, an inflammatory immune response could persist, resulting in excessive damage to host tissue, and IL-10 is one cytokine that has an important function in this role. Some of the cell types which produce IL-10 include monocytes, macrophages, T cells, B cells, dendritic cells, keratinocytes, and epithelial cells (Mosser and Zhang, 2009). Cell which produce IL-10 may regulate each other because production by one cell type can affect the ability of another cell type to produce IL-10. This is important as the effect of IL-10 can depend on the timing of its production and release, which is dependent upon the cell that produced it. Production of IL-10 too early can lead to an ineffective immune response while late production of IL-10 can lead to excessive tissue damage (Couper et al., 2008). Interleukin-10 is detected by the IL-10 receptor (IL-10R), which is a tetramer consisting of two IL-10R1 polypeptides and two IL-10R2 polypeptides (Donnelly et al., 1999). Interleukin-10R1 is the ligand binding subunit of the IL-10R complex and is expressed in immune type cells, and IL-10R2 is the signaling subunit, which is expressed in many cell types and can function with other cytokine receptors (Shouval et al., 2014). Activation of IL-10R initiates the JAK/STAT signaling pathway, which terminates in gene transcription regulation. One particular gene IL-10 can upregulate is Suppressor of Cytokine Signaling-3 (SOCS-3), which contributes to IL-10's ability to inhibit monocyte production of TNF-α and IL-1 (Donnelly et al., 1999). Interleukin-10 also inhibits macrophage antigen presentation and

microbicidal activity, and low expression of IL-10 favors the development of chronic inflammatory GI diseases (Duque and Descoteaux, 2014).

Transforming growth factor β (TGF-β) is another anti-inflammatory cytokine that has a role in development, tissue repair, cell generation and differentiation, and immune tolerance and regulation (Cameron and Kelvin, 2013). Five glycoproteins comprise the TGF-β family. TGF-β1, TGF-β2, and TGF-β3 are expressed in mammals, while TGF-β4 and TGF-β5 are expressed in chickens, and TGF-β can be expressed by platelets, activated macrophages, neutrophils, B cells, and T cells (Tizard, 2004). Transforming growth factor β is translated as a precursor protein, which contains a signal peptide that directs it to the endoplasmic reticulum. The precursor is inactive until it is cleaved by endoprotease furin in the endoplasmic reticulum or extracellular matrix (Gleizes et al., 1997). Active TGF-β can induce an anti-inflammatory macrophage profile by downregulating proinflammatory cytokine expression, which is regulated by the SNAIL pathway, and upregulating anti-inflammatory cytokine expression (Zhang et al., 2016). One study indicated that broiler-type chickens have reduced inflammatory response to LPS challenge when compared to layer-type chickens, and this may be due to increased expression of TGF-β mRNA from splenocytes of the broiler-type chickens (Leshchinsky and Klasing, 2001). Similar results were observed in an early feeding study by Simon et al. (2014), where the broiler-type chickens had up to three times lower mRNA expression of TGF-β compared to layer-type chickens.

Chemokines**.** Chemokines can be classified as homeostatic or inducible. Homeostatic chemokines mainly function in trafficking of lymphocytes through lymphoid organs, whereas inducible chemokines are important for attracting

inflammatory lymphocytes to the site of infection (Shachar and Karin, 2012). One particularly important chemokine to the inflammatory process is CXCL-8. The chemokine is a potent attractor of neutrophils by binding to CXCR-1 and CXCR-2. Early in the infection process CXCL-8 is released, lending to neutrophils being the first cell type recruited to the site of infection (de Oliveira et al., 2013). Activation of receptors on neutrophils by CXCL-8 initiates a signaling cascade that terminates with cytoskeletal changes which induce movement along the concentration gradient (Petri and Sanz, 2018). Chickens may have two CXCL-8 like chemokines, K60 and CAF. The expression of K60 is primarily limited to lymphoid tissue, and it is most efficient at attracting heterophils. However, CAF is expressed in both lymphoid and non-lymphoid tissue and is most efficient at attracting monocytes (Davidson et al., 2008). Research has indicated that K60 mRNA is elevated in the intestine of chickens following *Eimeria* spp. infection, and similar results were observed in chicks infected with *Salmonella enterica* serovar Typhimurium (Laurent, 2001; Withanage et al., 2004).

Macrophages in the Gastrointestinal Tract

Macrophages are a type of mononuclear leukocyte that can be found in many body tissues but also exist as a resident cell type in tissues such as the central nervous system, skin, and spleen. However, the largest pool of resident macrophages is found in the GI tract (Smith et al., 2011). Tissue-resident macrophages play an important role in defending against pathogens by functioning as sentinel cells, pathogen killers, activators of the immune system, and primers for antibody production (Davies et al., 2013). In the small intestine, resident macrophages reside within the *lamina propria*, along with other

immune cells (Bain and Mowat, 2014). In some tissues, resident macrophages are maintained by *in situ* proliferation, however macrophages resident to the GI tract are thought to be replenished by blood monocytes (Zigmond and Jung, 2013). Monocytes originate from hematopoietic stem cells located in bone marrow, mature in circulation, and then enter GI tissue to differentiate into resident macrophages (Qureshi et al., 2000). Macrophages can express different phenotypic profiles, depending on the local environment they reside in.

Classically, macrophages can be classified as M1 or M2, proinflammatory or anti-inflammatory respectively. However, classifying macrophages as M1 or M2 oversimplifies their function. Rather, macrophages can express a spectrum of phenotypes between M1 and M2 and may be able to alter their phenotype based on environmental signals, further complicating the characterization of macrophages (Martinez and Gordon, 2014). Resident macrophages of the GI tract provide an example of the complexity of macrophage typing. GI resident macrophages have a high capability of antigen presentation and continuously produce TNF-α, and these properties are characteristic of M1 macrophages. However, they have also been shown to continually express low amounts of IL-10, like an M2 macrophage but do not produce arginase, which is a primary feature of M2 macrophages (Bain and Mowat, 2014). GI resident macrophages are also less likely to trigger an inflammatory immune response upon detection of pathogens (Smith et al., 2010). This is likely to avoid chronic inflammation of GI tissue, as a multitude of microorganisms are constantly present within the lumen.

In response to PAMP, GI resident macrophages display strong phagocytic and bactericidal activity, more so than blood monocytes, but lack a proinflammatory

cytokine release response. Also, it has been suggested that GI resident macrophages kill phagocytized bacteria independent of reactive oxygen intermediates, which is the typical mechanism used by monocytes and macrophages (Rugtveit, 1995). Even though GI resident macrophages express an anti-inflammatory profile, inflammation does occur in the GI tract, but this is propagated by newly recruited monocytes that will express a classical M1 phenotype upon differentiating into macrophages in the GI tissue. The exact trigger of inflammation in the GI tract is not well described, but pathogen invasion of GI tissue may need to reach a threshold value before changes occur in the microenvironment and an inflammatory response begins. Similarly, the exact mechanism of post-inflammation resolution in the GI tract is not well understood, but some change in local signaling by the immune cells or mucosal cells induce clearing of the newly recruited proinflammatory macrophages likely occurs in an effort to reestablish tissue homeostasis (Smith et al., 2010; Zigmond and Jung, 2013; Bain and Mowat, 2014).

Depressed Immunity in Modern-Day Broilers

The modern-day broiler chicken has been highly selected for lean muscle accretion, producing a rapidly growing bird that, in terms of muscle growth, is very efficient at feed utilization. However, heavy selection for a single trait, without consideration of other traits, can lead to inadvertent selection for deleterious traits. For example, heavy selection for high growth rate and breast yield in broilers may be the origin of the myopathy known as wooden breast (Petracci et al., 2019). Similarly, it has been reported that broilers, when compared to slow growing layers, do not exhibit a fever response and have a decreased expression of mRNA coding for proinflammatory

cytokines when presented with an immune challenge (Leshchinsky and Klasing, 2001). This may have originated from genetic selection for high growth rate because this selection would require increased appetite, and the expression of proinflammatory cytokines results in suppressed appetite. So, selection for high growth rate may also select for a less reactive proinflammatory response. Also, Qureshi and Havenstein (1994) observed that high growth rate broilers had a less reactive adaptive immune system than slow growing broilers, but they observed little to no effect on the innate immune system in terms of the phagocytic capacity of phagocytic immune cells. High yielding broilers may have a less robust immune response when faced with infectious pathogens compared to their slow growing counterparts due to lowered inflammatory and antibody responses, and this could prevent broilers from overcoming pathogenic diseases.

Inflammatory responses in broilers are detrimental to the goals of the broiler industry as inflammation results in worsened growth performance by reducing feed intake and muscle protein accretion while increasing the rate of the metabolism, synthesis of immune related proteins, and liver mass (Klasing, 2007). This results in a shift of broiler energetics and metabolism from muscle growth to immune response development, potentially requiring muscle protein degradation to provide amino acids for the immune system. The limiting amino acids for immune system protein synthesis may be different than the limiting amino acids for muscle protein synthesis (Le Floc'h, 2004). Broilers diets are formulated to provide the optimal nutrients to promote muscle growth, and when under an immune challenge, these diets may not be optimally formulated for synthesizing immune proteins.

Also, during an immune response, appetite can be suppressed, leading to a reduction in the intake of amino acids from the feed, and again, these may not be the optimal amino acids for immune proteins. Thus, a catabolic state is initiated in an effort to supply the necessary amino acids for immune function. As skeletal muscle represents the largest pool of expendable amino acids, it can be degraded to provide a source of amino acids for the immune system (Sirimongkolkasem, 2007). Since the amino acid content of skeletal muscle and immune proteins may contain different relative amounts of specific amino acids, it may be necessary that more muscle is degraded to free amino acids that are less prevalent in skeletal muscle but needed in high amounts for the immune system. As such, excess mobilization of skeletal muscle amino acids may be needed to support the immune system at times of challenge. Although an immune response decreases broiler growth performance, proper function of the immune system is needed to combat pathogen invasions, which if allowed to persist could lead to decreased growth performance or even death. Therefore, it is important that any potential immune response is prevented or in the inevitable event of disease, the response must be monitored in order to minimize the length of the response and prevent excessive reductions in growth performance.

As AGP are removed from broiler production strategies, it will become increasingly important to better understand the broiler GI tract in terms of epithelial cell renewal and immune response. The GI tract is rapidly renewed by the intestinal stem cells, and an immune response can shift the metabolic and energetic processes of an animal. Without AGP, more stress is placed on the immune system within the broiler GI

tract. GI mitotic activity and immune response represent two normal processes that can place a substantial drain on nutrient and energy partitioning in a rapidly growing broiler. Further research is necessary to better understand these functions, their importance, underlying mechanisms, relationship to growth performance in the absence of AGP, and appropriate AGP alternatives in order to maintain and improve the efficiency and sustainability of broiler production.

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III. Effect of dietary protein source and litter condition on macrophage and mitotically active cell densities in the small intestine of young broilers

Abstract

As the broiler industry continues to shift to antibiotic-free broiler production, it has become increasingly important to develop a better understanding of the immune system and health of the small intestine to minimize reductions in growth performance in broilers not fed antibiotic growth promoters. Mitotically active cells, the majority of which will become enterocytes, help maintain the cell populations that make up the intestinal epithelial barrier. Macrophages help prevent pathogen invasion by their phagocytic activity, by functioning as immune response amplifying cells to aid in the recruitment of additional immune cells, and by stimulating cytokine production in other adjacent cells. The objective of this experiment was to evaluate commonly used practical production practices on intestinal cell mitotic activity and local intestinal immunological responses. A randomized complete block design experiment with a 3 × 2 factorial treatment structure was conducted. The 3 dietary protein sources were: soybean meal (SBM), a mix of 50% poultry by-product meal and 50% feather meal (PFM), and porcine meat and bone meal (MBM) and broilers were reared on either new litter (NL) or used litter (UL). On d 3, 8, 11, 15, and 21, 6 birds per treatment from 6 different blocks (total n = 36 per d) were randomly selected for sampling. Broilers were injected intraperitoneally with 5’-bromo-2’-deoxyuridine (BrdU) 1 h prior to sampling to label mitotically active cells. Samples were analyzed using cryohistology, immunofluorescence staining, and digital fluorescence microscopy to determine the density of mitotically active cells and

macrophages. Data were analyzed using SAS V9.4 PROC GLIMMIX and treatment means were separated using PDIFF at $P \leq 0.05$. The broilers which received PFM had the lowest FCR ($P \leq 0.0002$). Mitotically active cell and macrophage densities changed in both the duodenum and ileum over time. No consistent effect of dietary protein source or litter condition was observed on either macrophage or mitotically active cell densities. However, on d 3 and 15 in the duodenum ($P \leq 0.0126$) and d 21 in the ileum ($P \leq 0.0009$), broilers reared on UL had a greater mitotically active cell density than those reared on NL. On d 8 in the duodenum, broilers fed MBM had an increased macrophage density compared to those fed PFM and SBM ($P \leq 0.0401$). These results indicate dietary protein source and litter condition may impact the physiology of the broiler small intestine, though additional work with this model is necessary to understand the underlying mechanisms.

Introduction

The broiler industry is primarily concerned with producing broiler growth via lean muscle accretion; however, several different physiological systems support the complex process of muscle growth and development. Although necessary, these systems can place a burden on broiler metabolism and reduce the rate of muscle protein accretion by partitioning energy and nutrients that could be utilized for lean muscle growth in support of other functions. It has been estimated that maintenance of the gastrointestinal (GI) tract alone requires 25 % of the total basal metabolic needs of an animal, while this tissue does

not contribute as significantly as lean muscle tissue to the profit gained from broiler processing (Croom et al., 2000). However, growth and maintenance of the GI tract are important for digestion and nutrient absorption, without which muscle growth would be impossible. Intestinal growth and maintenance are supported by two groups of proliferative cells, intestinal stem cells (ISC) and transit amplifying (TA) cells. As ISC divide, the daughter cells enter the TA cell pool, which divide and differentiate into the mature cell types of the intestinal epithelium (Barker, 2013). Intestinal epithelial cells continuously migrate up the villi and are shed upon reaching the tip. Also, the intestinal epithelium provides some immune protection as its integrity must be maintained to prevent translocation of microorganisms into host tissue. Furthermore, there is a strong presence of immune cells within the GI tract, and it contains the largest pool of tissue-resident macrophages found in the body (Smith et al., 2011). Therefore, in order to continue improving the sustainability and efficiency of broiler production, it will be important to develop a better understanding of the broiler GI tract in terms of its development, maintenance, and immune functions.

Currently, the broiler industry is experiencing a shift away from the use of antibiotic growth promoters (AGP). As of 2019, more than 50 % of broilers produced in the United States were reared under some type of antibiotic-free (ABF) program (nationalchickencouncil.org, 2019). In the past, the broiler industry has relied on AGP to improve the efficiency of broiler production as research has indicated that AGP reduce intestinal length and weight and produce an anti-inflammatory effect (Visek, 1978; Ferket, 2004; Neiwold, 2007). Thus, these two benefits of AGP help to shift the partitioning of energy and nutrients away from metabolic functions, such as GI tract

maintenance and immunology, that can be unfavorable to broiler production. Overall, this contributes to improved growth performance, health, and mortality of broilers receiving AGP (Smith, 2011).

ABF broiler production has increased the incidence of enteric diseases like coccidiosis and necrotic enteritis (NE), both of which can induce costly production loses (Blake and Tomley, 2014; Cervantes, 2015; Wade and Keyburn, 2015). Certain controllable elements of broiler management, such as dietary protein source and litter condition, may influence the occurrence of enteric disease (Wilkie et al., 2005; Cressman et al., 2010). The control of coccidiosis and NE, whether through exogenous treatments or by the broiler immune system itself, is important as some research has suggested that high growth rate broilers may be less adept at quickly controlling and resolving an immune challenge (Qureshi and Havenstein, 1994; Leshchinsky and Klasing, 2001). A prolonged immune response can lead to worsened feed conversion ratio (FCR), increased immune protein synthesis, and increased liver mass (Klasing, 2007). Immune proteins differ in amino acid ratios compared to skeletal muscle proteins, and diets are formulated to meet the requirements of skeletal muscle, which may mean that during an immune response the appropriate amino acids are not provided in the diet. Thus, skeletal muscle may act as an amino acid reservoir and be catabolized to free amino acids needed for sufficient immune function (Sirimongkolkasem, 2007). In the face of present challenges of ABF broiler production, it is aware that knowledge is lacking in the areas of broiler GI development and immunology that may aid in revealing and evaluating appropriate AGP alternatives and improving broiler performance in an ABF environment. Therefore, this study sought to evaluate the effects of common industry practices, such as different

dietary protein sources and litter conditions, on local GI immune responses and proliferative activity as a means to begin developing a model to study GI health in ABF broiler production.

Materials and Methods

All procedures involving the use of live birds in this work were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2017-3146).

Dietary and Litter Treatments

This experiment had a randomized complete block design (n = 50 blocks, each comprised of 6 pens, with each pen representing a different treatment) with a 3 × 2 factorial treatment arrangement. Three diets were fed, differing primarily in the dietary protein source included. The dietary protein sources were soybean meal (SBM), porcine meat and bone meal (MBM), and a 50/50 poultry by-product and feather meal mix (PFM). These diets were formulated based on diets typically fed in the broiler industry, therefore each of the diets contained some SBM, but the MBM and PFM diets included 4 % MBM or PFM, respectively (Table 1). Two different litter conditions were also used to imitate common industry practices. Broilers were either reared on new litter (NL; fresh pine shavings) or used litter (UL: pine shavings previously used by 3 flocks). The UL was obtained from the Auburn University Poultry Research Unit and was previously used in nutrition experiments, not disease trials, making it a suitable mimic of reused industry litter.

Bird Husbandry

On d of hatch, 1500 Yield Plus × Ross 708 female broilers (ROSS, Aviagen Group, Huntsville AL) were received from a commercial hatchery, where they were vaccinated for Marek's disease, Newcastle disease, infectious bronchitis, and coccidiosis. Each bird was tagged with a wing tag for identification, individually weighed to determine d 0 bodyweight, and randomly distributed to 300 pens (n = 5 birds per pen at placement; 0.04 m^2/bird). On d 8, each pen was culled to 3 birds per pen (0.07 m^2/bird). Each pen was equipped with an individual feeder and 2 water nipples, providing *ad libitum* access to feed and water. A starter, crumble diet was suppled throughout the experiment (1 to 21 d). At placement, ambient temperature was set at 30.5°C and reduced to maintain bird comfort as the birds aged, with a final set point of 24.5°C on d 21. From d 0 to 7, birds were exposed to a photoperiod of 23L:1D with a light intensity of 30 lux, and from d 8 to 21, the photoperiod was adjusted to 18L:6D at 10 lux. Light intensity settings were verified using a photometric sensor, placed at bird level, with National Institute of Standards and Technology-traceable calibration (403125, Extech Instruments) for each adjustment of light intensity. Neither NL nor UL were changed or replaced during the experiment.

Growth Performance and Bromodeoxyuridine Injection

Individual bird weights and pen feeder weights were recorded on d 0 and 21 to calculate individual bodyweight gain (BWG) and mortality corrected pen feed intake (MCFI). Mortality was recorded daily, and FCR was mortality corrected. Sample

collection was performed on d 3, 8, 11, 15, and 21. On each sampling day, 6 blocks were randomly selected, and 1 bird was randomly selected from each of the pens in those blocks for sample collection (total n = 36 sampled per sampling day). The sampled birds were injected intraperitoneally with an aqueous solution (25 mg/ML) of 5'-bromo-2'-deoxyuridine (BrdU; Alfa Aesar, Haverhill, MA; pH of 8.0; 100 µg of BrdU per g of body weight) to label mitotically active cells, as described by Hutton et al. (2014). Following BrdU injection, birds were placed in disposable containers for 1 h to allow BrdU to be incorporated into mitotically active cells. BrdU is incorporated into the DNA of proliferating cells, providing a label that can be detected during later immunohistochemical and fluorescence microscopy analysis. After the 1 h cell-labeling period, birds were euthanized by CO_2 asphyxiation, followed by immediate cervical dislocation.

Tissue Sample Collection and Analysis

Immediately following euthanasia, tissue samples were collected from the proximal and distal small intestine, duodenum and ileum, respectively. The duodenal samples were collected just distal to the duodenal loop to avoid accidental inclusion of pancreatic tissue, and the ileal samples were collected proximal to the ileocecal junction. After being excised, the small intestine samples were flushed with phosphate buffered saline (PBS; pH 7.4; Invitrogen, Carlsbad, CA) to remove intestinal contents. The samples were then flash frozen in liquid nitrogen and stored at -80°C. Before freezing, the cylindrical samples were cut lengthwise to better expose the mucosal surface to the

liquid nitrogen and promote rapid freezing, rather than slow freezing which would damage the morphology of the tissue.

***Cryohistological Analysis*.** Samples were held at -20°C for 24 h to bring samples to an appropriate temperature for cryosectioning. After being embedded in frozen section compound (VWR International, Westchester, PA), 5-µm thick cryosections were collected from the intestinal samples, transverse to the length of the intestine, using a Leica CM 1950 cryomicrotome. The cryosections were mounted on positively charged glass slides (VWR International; 5 cryosections per slide) and stored at 4°C for no more than 48 h before immunofluorescence staining.

Immunofluorescence Staining. Cryosections were fixed and stained following methods similar to Day et al. (2009) and Tejeda et al. (2019), with some modifications as described below. All procedures were conducted at room temperature. Slides were first rehydrated in PBS for 10 min and then fixed in paraformaldehyde (4 % in PBS; VWR International) for 10 min, followed by 2 rinses in PBS. Next, slides were treated with 0.5 % Triton X-100 (VWR International) for 10 min to permeabilize the cell membranes. Following permeabilization, slides were exposed to 1N hydrochloric acid for 10 min to denature the DNA and improve BrdU detection, followed by a single rinse in PBS. The slides were then incubated for 30 min in a blocking solution containing 10 % horse serum (Sigma-Aldrich), 2 % bovine serum albumin (VWR International), and 0.2 % Triton X-100 in PBS to prevent non-specific antibody binding. Antibody reactions were conducted in a dark, humidified box. Primary antibodies were diluted in blocking solution and allowed to react with tissue cryosections for 1 h, followed by rinsing 3 times for 5 min per rinse in PBS. Similarly, secondary antibodies were also diluted in blocking solution

and allowed to react with tissue cryosections for 30 min, followed by rinsing 3 times for 5 min per rinse in PBS. Then, the cryosections were exposed to 4',6-diamidino-phenylindol (DAPI; 1 μg/mL; VWR International) to label all nuclei within the tissue and immediately rinsed twice in PBS. Slides were mounted using florescence mounting media with Tris buffer (Fluoro-gel, Electron Microscopy Sciences, Hatfield, PA) and thin glass coverslips (VWR International), then permanently sealed with clear fingernail polish (Sally Hansen, New York, NY) and allowed to dry at room temperature. All cryosections were digitally imaged within 72 h of immunofluorescence staining.

Primary and Secondary Antibodies. Primary antibodies were as follows: mouse monoclonal IgG1 anti-chicken monocyte/macrophage (KUL01, 1:500 dilution, Cat. No. SC52603, Santa Cruz Biotechnology, Santa Cruz, CA, validated by Mast et al. (1998)) and mouse monoclonal IgG2a anti-BrdU (1:500 dilution, Cat. No. MA3-071, Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA). Secondary antibodies (Invitrogen; 1:1000 dilution) were: Alexa-Fluor 546 goat anti-mouse IgG1 (Cat. No. A21123) and Alexa-Fluor 633 goat anti-mouse IgG2a (Cat. No. A21136).

Fluorescence Photomicroscopy and Digital Image Analysis. After immunofluorescence staining, cryosections were imaged at 200X magnification using a semi-automated inverted fluorescence microscope (Nikon Eclipse Ti-U; Nikon Instruments, Inc., Melville, NY) equipped with a solid-state LED light source (Lumencor SOLA Sm II 365 light engine). Images were captured and analyzed using an Evolve 512 EMCCF camera (Photometrics, Tuscon, AZ) and the Basic Research version of Elements software (Nikon Instruments, Inc.). Two representative images were captured from each slide (1 slide per bird), focusing on the crypt region of the tissue section. The area of

mucosa in each image was measured, and BrdU+ mucosal nuclei and macrophages located within the *lamina propria* were counted to calculate mitotically active cell and macrophage density, which are reported as the number of positive cells per square millimeter. All nuclei determined to be positive for BrdU must have also been DAPI-stained to confirm it was a true nucleus.

Statistical Analysis

Performance and cell density data were analyzed as a randomized complete block design, using pen location as the blocking factor and bird as the experimental unit. Dietary protein source and litter condition served as the fixed effects. Cell density data were also analyzed including day as a fixed effect to analyze changes over time. There were a total of 6 replicates per treatment on each sampling day. All data were subjected to analysis of variance using the GLIMMIX procedure of SAS version 9.4 (SAS Institute, Cary, NC). Satterthwaite adjustment was used to correct the degrees of freedom. For all hypothesis tests, treatments means were separated using the PDIFF option and declared different with a significance level of $P \leq 0.05$.

Results and Discussion

Growth Performance

No interactions among the main effects were observed for d 0 to 21 growth performance data ($P > 0.3659$; Table 2). Similar BWG and FI were observed for each of

the treatments ($P > 0.3427$ and $P > 0.6333$, respectively). However, broilers fed PFM had reduced FCR (1.2392) compared to those fed MBM or SBM (1.3008 and 1.2874, respectively; $P \leq 0.0002$). As the diets were formulated based on typical industry diets, they were not necessarily isocaloric or isonitrogenous. Therefore, the diets may have differed in energy content or amino acid composition, leading to the lower FCR in broilers fed PFM.

Mitotically Active Cell and Macrophage Density

Mitotically active cell density changed in both the duodenum and ileum over time. Mitotically active cell density of the duodenum increased on d 8 to 1,694 mitotically active cells per mm^2, decreased on d 15 to 1,444 mitotically active cells per mm^2, and increased again on d 21 to 1,746 mitotically active cells per mm^2 ($P \leq 0.0001$; Figure 11), and in the ileum, mitotically active cell density increased to its highest value on d 11 at 2,016 mitotically active cells per mm^2, decreased on d 15 to 1,755 mitotically active cells per mm^2 and decreased again, to its lowest value of 1,364 mitotically active cells per mm^2, on d 21 ($P \leq 0.0001$; Figure 12). The GI tract of chicks quickly develops, both in maturity and overall size, post-hatch in order to facilitate rapid ability to digest and absorb nutrients from ingested feed, and this must be accomplished by mitotic activity. Geyra et al. (2001) described the development of chick enterocytes as occurring in two periods. During the first 24 h post-hatch, enterocytes differentiated from small, round cells lacking a well-defined brush border to the polar shape of mature enterocytes with a distinct brush border. During the second period, from d 3 to d 6, the enterocytes in the duodenum increased in cell length; however ileal enterocytes did not undergo

significant morphological change post-hatch. In a study by Iji et al. (2010), it was observed that duodenum and ileum length increased until d 7 but not after d 14, with similar results of villus surface area. Therefore, the timepoints selected in this study may not have captured the early development of the small intestine. Mitotically active cell density of a certain magnitude is required for maintenance and growth of villus size, but increases in mitotically active cell density may also be needed to replace villus cells that were damaged due to some type of insult or in response to changes in enterocyte migration rate. Parker et al. (2019) suggested that under the influence of TNF-induced intestinal inflammation, murine villi decrease in size due to apoptosis of enterocytes of the villus body but a change in proliferation rate was not observed. However, changes in proliferation rate during the resolution of inflammation were not studied, and changes may occur during this time to return the tissue to its homeostatic state. Again, as this study did not capture early GI development, changes observed as the birds aged may be in response to some type of environmental stimuli rather than efforts to increase the overall volume of intestinal mucosa.

No interactions among the main effects, dietary protein source, litter condition and day, were observed for mitotically active cell and macrophage densities, and no treatment effects were observed for mitotically active cell or macrophage density on d 11 and 21 in the duodenum ($P \geq 0.1016$; Figures 3 and 5, respectively) or on d 3, 8, 11, and 15 in the ileum ($P \geq 0.059$; Figures 6, 7, 8, and 9, respectively). However, on d 3 and 15 in the duodenum ($P \leq 0.0126$; Figures 1 and 4, respectively) and d 21 in the ileum ($P \leq 0.0009$; Figure 10), broilers reared on UL had a higher mitotically active cell density than those reared on new litter (1,481 vs. 1,202 on d 3 and 1,540 vs. 1,298 on d 15 in the

duodenum; 1,484 vs. 1,244 on d 21 in the ileum) . Chickens are often observed scratching, pecking, and consuming litter, and in the case of used litter, which can contain microorganisms shed by past flocks, litter consumption could expose chicks to pathogens or other microorganisms that they are not immunologically mature enough to manage or defend against. Previous research has indicated that UL can alter the profile of the intestinal microbiota toward more intestinal derived microbes (Cressman et al., 2010). The microbiota may play a role in regulating intestinal epithelial proliferation (Rinttilä and Apajalahti, 2013). Although it cannot be definitively stated based on the parameters measured in this study, the higher density of mitotically active cells may be due to an altered intestinal microbiota in broilers reared on UL.

Macrophage density was lowest on d 3 in the duodenum (453 macrophages per mm^2), and it increased on d 8, decreased on d 11, increased on d 15, and decreased on d 21 ($P \leq 0.0001$; Figure 11). In the ileum, macrophage density was also lowest on d 3 at 411 macrophages per mm^2 but increased until d 15 to 610 macrophages per mm^2 and decreased on d 21 to 558 macrophages per mm^2 ($P \leq 0.0001$; Figure 12). There is a lack of literature discussing changes in macrophage density in the intestines of a growing and developing animal. Therefore, it is possible that the changes in macrophage density observed over time represent changes that typically occur rather than an influx of macrophages in response to some type of enteric insult. In this study, macrophage density includes both pro and anti-inflammatory macrophages, as the marker used was a general macrophage marker. As this does not elucidate the nature of the immune response occurring, this could be further studied by differentiating pro and anti-inflammatory macrophages as intestinal resident macrophages express an anti-inflammatory profile

while macrophages recruited to the intestines during an immune response express a proinflammatory profile (Zigmond and Jung, 2013). So, knowing whether the changes in macrophage density is due to pro or anti-inflammatory macrophages would better describe whether a pro or anti-inflammatory immune response is occurring.

It was observed that macrophage density was greater in the duodenum of broilers fed MBM on d 8 (781 vs. 682 and 686 macrophages per mm^2, MBM, PFM, and SBM, respectively; $P \leq 0.0401$; Figure 2). The chick yolk sac is typically absorbed before d 8, meaning the chicks are no longer receiving maternal antibodies from the yolk. The increase in local macrophage density on d 8 could be the result of a local, mild immune response which would recruit proinflammatory macrophages to the area. There is some evidence suggesting that diets containing high levels of glycine, such as those including MBM, support the proliferation of *Clostridium perfringens*, which is the primary causative agent of NE. Wilkie et al. (2005) established a positive correlation between dietary and ileal glycine content with *C. perfringens* numbers in the ileum and ceca in broilers orally inoculated with *C. perfringens* and fed a variety of protein sources. Necrotic enteritis lesions typically affect the lower portions of the small intestine, but lesions can occur in the duodenum (Shojadoost et al., 2012). Dahiya et al. (2005) found that in broilers orally challenged with *C. perfringens* higher inclusion of glycine in the diet increased the *C. perfringens* populations in the ceca, and they also noted the occurrence of duodenal lesions. Higher glycine content in the MBM diet could predispose broilers to NE and be the cause of the higher macrophage density observed in this study.

Ultimately, increased mitotically active cell density observed in broilers reared on UL and increased macrophage density in broiler fed MBM represent a use of energy and amino acids, which must be supplied through the diet, not directly utilized to support lean muscle growth. As producing lean meat products is the primary goal of the broiler industry, the underlying mechanisms producing these results warrants future study. Despite the limitations of this study, the results and knowledge gained from it will help in the development of a model to evaluate challenges affecting ABF broilers in an effort to continue improving the efficiency and sustainability of animal agriculture.

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Table 1. Ingredient and calculated nutrient composition of diets fed to broilers reared to 21 d of age

Ingredient, %	MBM[1]	PFM[2]	SBM[3]
Corn	60.34	64.59	59.69
MBM[1]	4.00	-	-
PFM[2]	-	4.00	-
SBM[3]	32.29	27.79	35.54
Soybean oil	0.94	0.50	1.47
Dicalcium phosphate	0.55	0.95	1.34
Calcium carbonate	0.57	0.67	0.69
Salt	0.37	0.36	0.33
Phytase	0.01	0.01	0.01
Vitamin and Mineral Premix[5,6]	0.10	0.10	0.20

[1]MBM = porcine meat and bone meal
[2]PFM = 50% poultry by-product and 50% feather meal
[3]SBM = soybean meal
[4]Poultry oil was added with 1% placed in the mixer and 2% spray-applied post-pelleting.
[5]Vitamin premix provided the following per kilogram of diet: Vitamin A (Vitamin A acetate), 9,370 IU; Vitamin D (cholecalciferol), 3,300 IU; Vitamin E (DL-alpha tocopheryl acetate), 33 IU; menadione (menadione sodium bisulfate complex), 2 mg; Vitamin B12 (cyanocobalamin), 0.02 mg; folacin (folic acid), 1.3 mg: D-pantothenic acid (calcium pantothenate), 15 mg; riboflavin (riboflavin), 11 mg; niacin (niacinamide), 44 mg; thiamin (thiamin mononitrate), 2.7 mg; D-biotin (biotin), 0.09 mg; and pyridoxine (pyridoxine hydrochloride), 3.8 mg.
[6]Mineral premix includes per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (iron sulfate monohydrate), 30 mg; Cu (tri-basic copper chloride), 8 mg; I (stabilized ethylenediamine dihydriodide), 1.4 mg; Se (sodium selenite), 0.3 mg.

Table 2. Effect of dietary protein source and litter condition on growth performance of broilers reared to 21 d.

	Protein Source					Litter Condition				
Item	MBM[4]	PFM[5]	SBM[6]	SEM	PROT *P*-value	NL[7]	UL[8]	SEM	LITTER *P*-value	PROT x LITTER *P*-value
BWG[1], g	845	869	821	23	0.3427	848	842	18	0.8341	0.4994
FI[2], g	1,100	1,077	1,057	31	0.6333	1,079	1,077	25	0.9524	0.6241
FCR[3]	1.3008[a]	1.2392[b]	1.2874[a]	0.01	0.0002	1.2735	1.2781	0.01	0.6725	0.3659

[a, b] means with different superscripts differ $P \leq 0.05$
[1] BWG = body weight gain
[2] FI = feed intake
[3] FCR = feed conversion ratio (FI:BWG)
[4] MBM = meat and bone meal
[5] PFM = poultry by-product and feather meal
[6] SBM = soybean meal
[7] NL = new litter
[8] UL = used litter

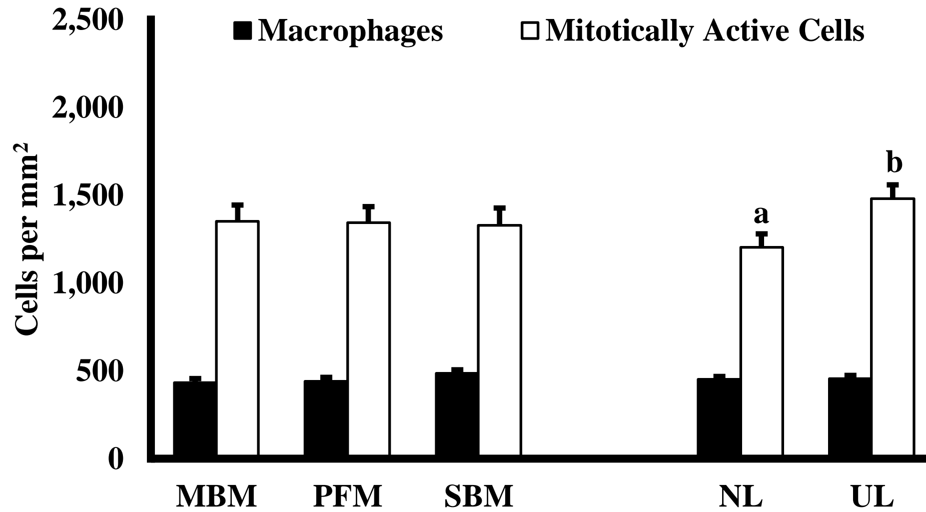

Figure 1. Effect of dietary protein source and litter condition on macrophage and mitotically active cell densities in the duodenum of 3 d old broilers. (n = 6)
MBM = meat and bone meal, PFM = poultry by-product and feather meal, SBM = soybean meal; NL = new litter, UL = used litter. [a-b]Means within either main effect with different superscripts, differ $P < 0.05$.

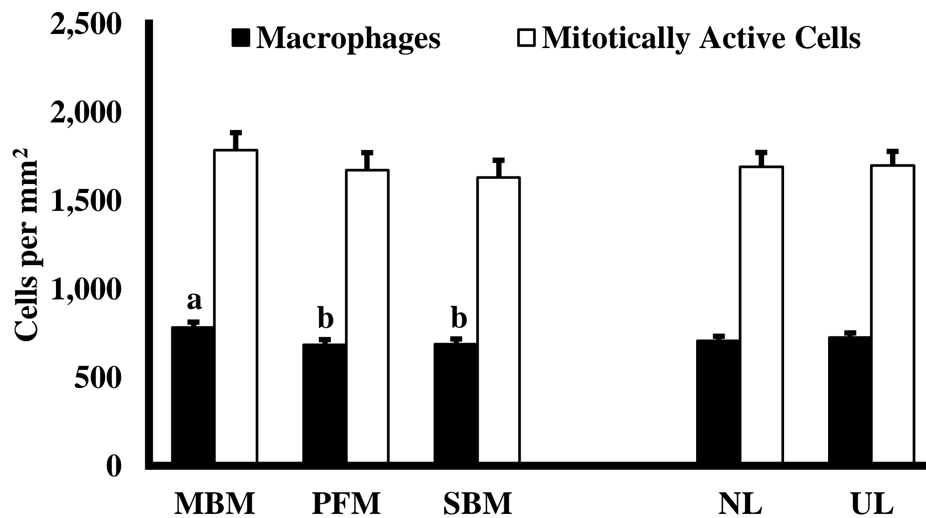

Figure 2. Effect of dietary protein source and litter condition on macrophage and mitotically active cell densities in the duodenum of 8 d old broilers. (n = 6)
MBM = meat and bone meal, PFM = poultry by-product and feather meal, SBM = soybean meal; NL = new litter, UL = used litter. [a-b]Means within either main effect with different superscripts, differ $P < 0.05$.

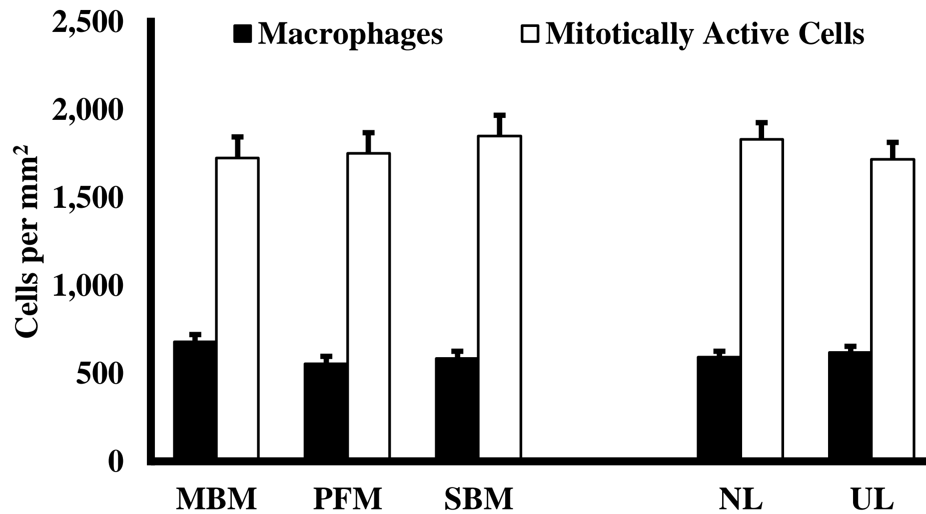

Figure 3. Effect of dietary protein source and litter condition on macrophage and mitotically active cell densities in the duodenum of 11 d old broilers. (n = 6)
MBM = meat and bone meal, PFM = poultry by-product and feather meal, SBM = soybean meal; NL = new litter, UL = used litter.

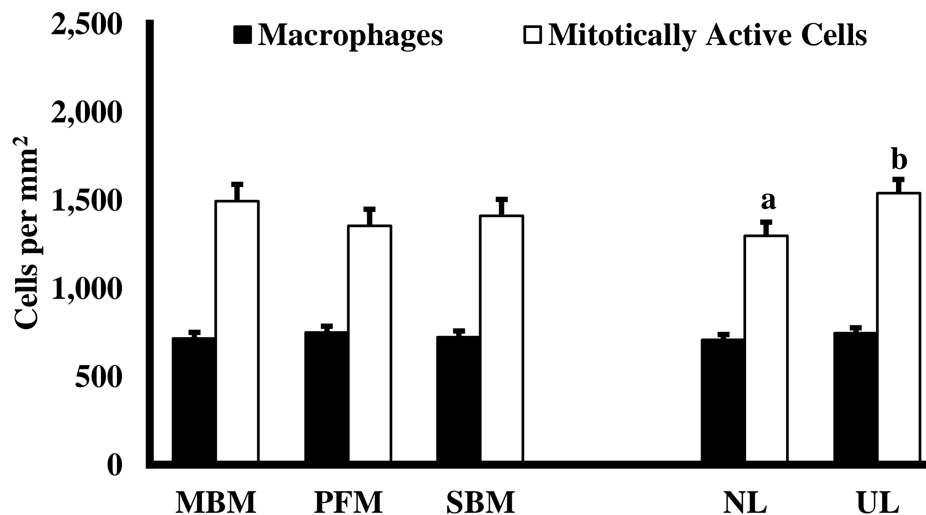

Figure 4. Effect of dietary protein source and litter condition on macrophage and mitotically active cell densities in the duodenum of 15 d old broilers. (n = 6)
MBM = meat and bone meal, PFM = poultry by-product and feather meal, SBM = soybean meal; NL = new litter, UL = used litter. [a-b]Means within either main effect with different superscripts, differ $P < 0.05$.

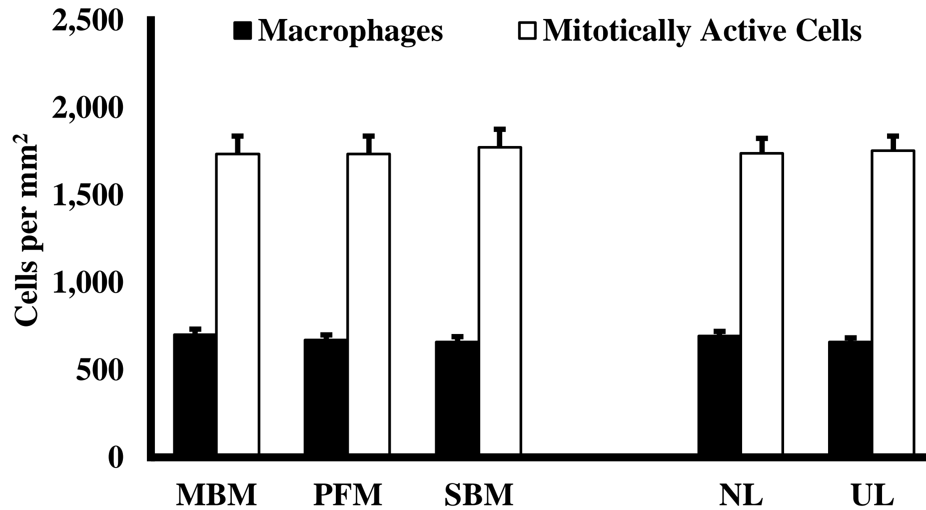

Figure 5. Effect of dietary protein source and litter condition on macrophage and mitotically active cell densities in the duodenum of 21 d old broilers. (n = 6)
MBM = meat and bone meal, PFM = poultry by-product and feather meal, SBM = soybean meal; NL = new litter, UL = used litter.

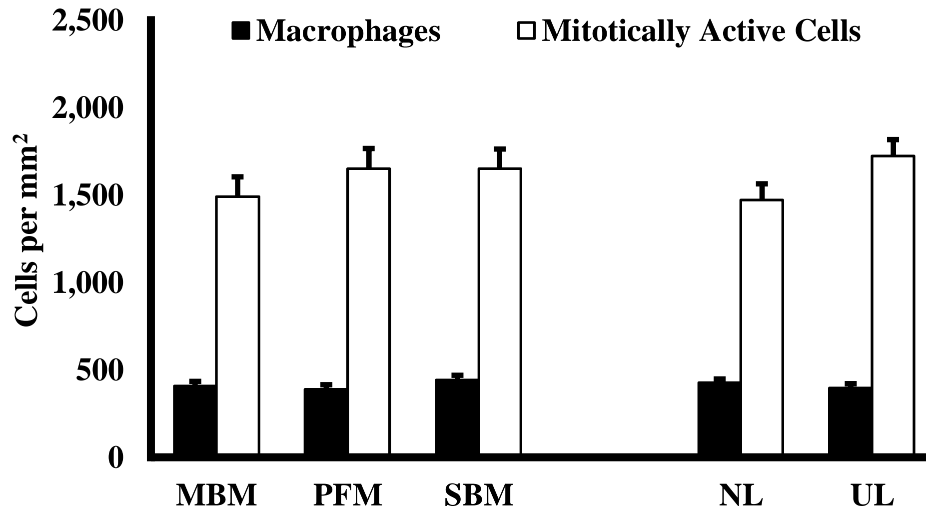

Figure 6. Effect of dietary protein source and litter condition on macrophage and mitotically active cell densities in the ileum of 3 d old broilers. (n = 6)
MBM = meat and bone meal, PFM = poultry by-product and feather meal, SBM = soybean meal; NL = new litter, UL = used litter.

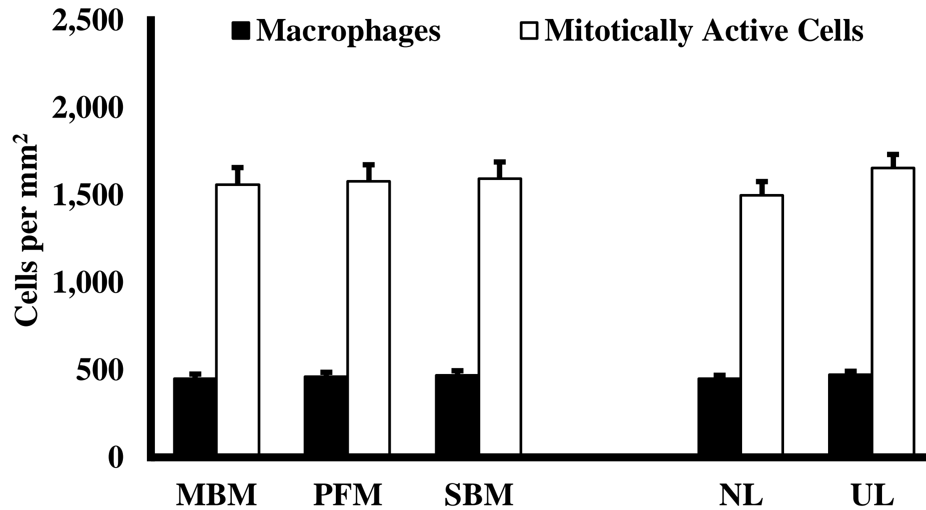

Figure 7. Effect of dietary protein source and litter condition on macrophage and mitotically active cell densities in the ileum of 8 d old broilers. (n = 6)
MBM = meat and bone meal, PFM = poultry by-product and feather meal, SBM = soybean meal; NL = new litter, UL = used litter.

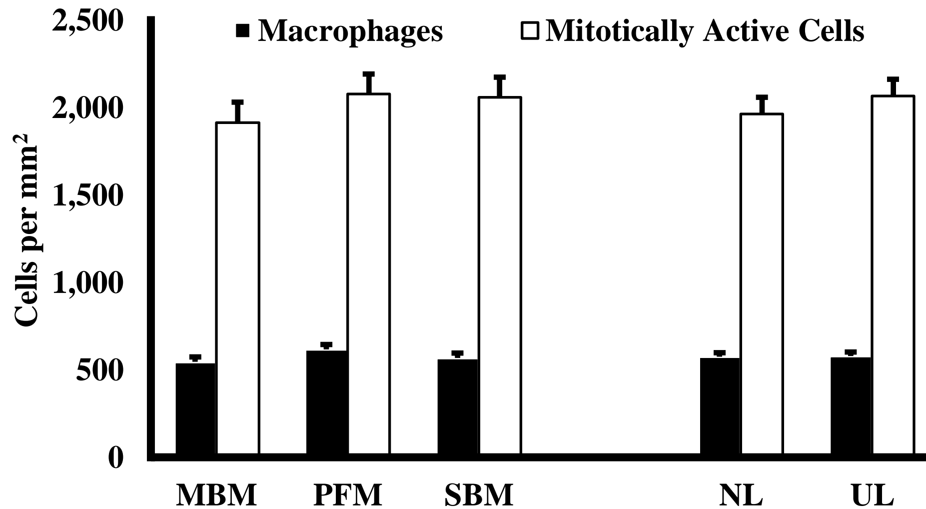

Figure 8. Effect of dietary protein source and litter condition on macrophage and mitotically active cell densities in the ileum of 11 d old broilers. (n = 6)
MBM = meat and bone meal, PFM = poultry by-product and feather meal, SBM = soybean meal; NL = new litter, UL = used litter.

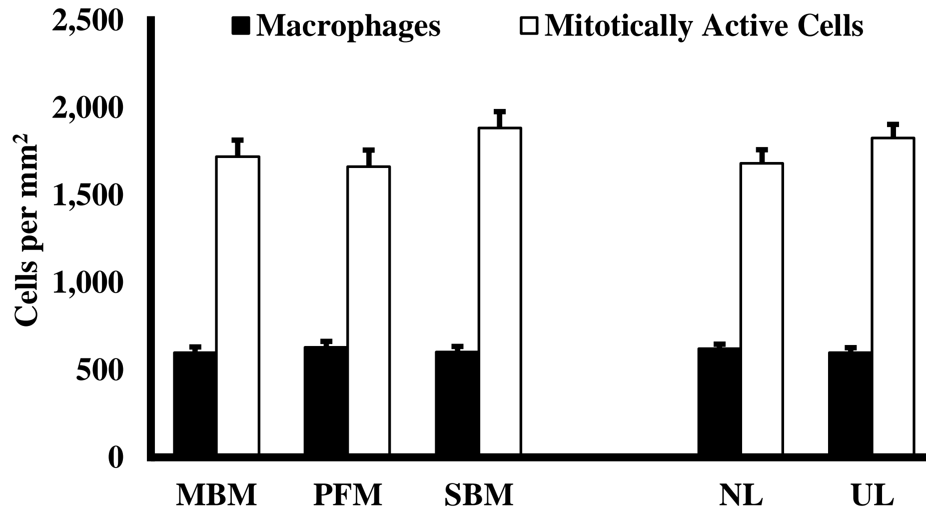

Figure 9. Effect of dietary protein source and litter condition on macrophage and mitotically active cell densities in the ileum of 15 d old broilers. (n = 6)
MBM = meat and bone meal, PFM = poultry by-product and feather meal, SBM = soybean meal; NL = new litter, UL = used litter.

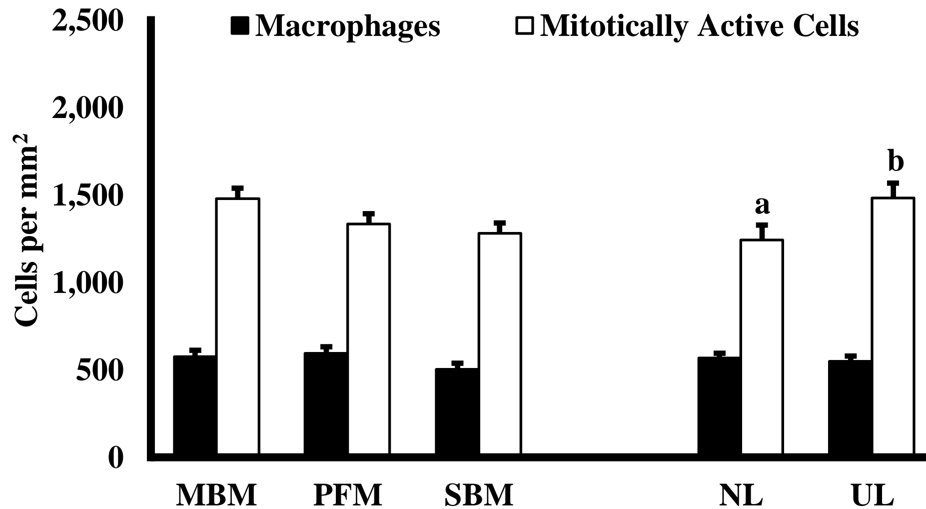

Figure 10. Effect of dietary protein source and litter condition on macrophage and mitotically active cell densities in the ileum of 21 d old broilers. (n = 6)
MBM = meat and bone meal, PFM = poultry by-product and feather meal, SBM = soybean meal; NL = new litter, UL = used litter. [a-b]Means within either main effect with different superscripts, differ $P < 0.05$.

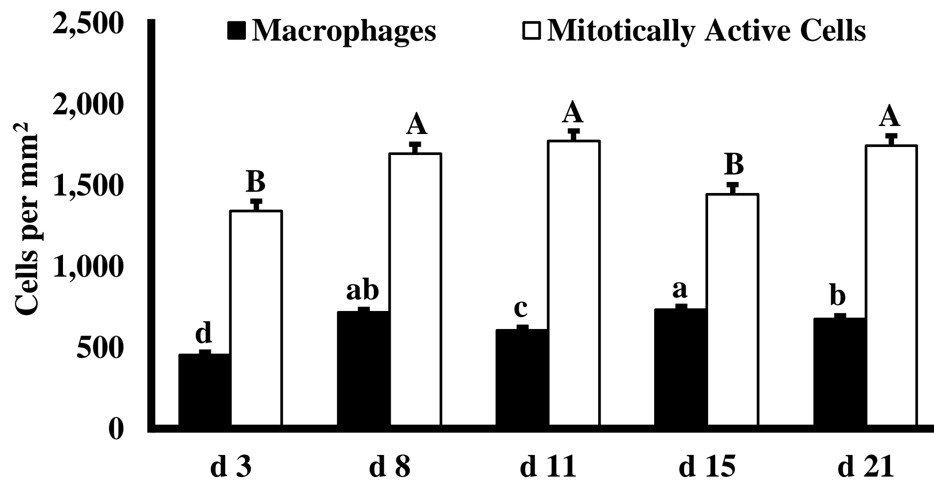

Figure 11. Effect of day of age on macrophage and mitotically active cell densities in the duodenum broilers. (n = 36)
a-dMean macrophage density with different superscripts differ P < 0.05. A-BMean mitotically active cell density with different superscripts, differ P < 0.05.

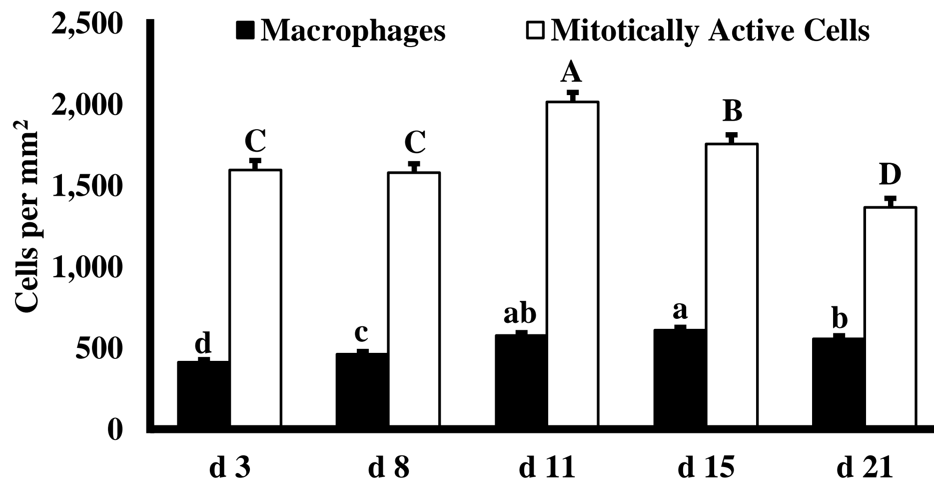

Figure 12. Effect of day of age on macrophage and mitotically active cell densities in the ileum broilers. (n = 36)
a-dMean macrophage density with different superscripts differ P < 0.05. A-CMean mitotically active cell density with different superscripts, differ P < 0.05.