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Intestinal Inflammation Model Inducing Gut Leakage by Different Methods in Broiler Chickens Using FITC-d as a Marker

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ABSTRACT

Traditionally, antibiotic growth promoters (AGP) have been used in food animals to reduce enteric inflammation and maintain intestinal homeostasis, thus improving performance. Due to increasing restrictions regarding the use of AGP, precise and high throughput enteric inflammation models and markers to search for effective alternatives are urgently needed. Oral administration of fluorescein isothiocyanate dextran (FITC-d) and its passage into blood can be used as a marker for tight junction permeability. FITC-d is a large molecule (3-5 kDa) which does not usually leak through the intact gastrointestinal tract barrier. However, when conditions disrupt the tight junctions between epithelial cells, the FITC-d molecule can enter circulation as demonstrated by an increase in trans-mucosal permeability associated with chemically induced disruption of tight junctions by elevated serum levels of FITC-d after oral administration. In chapter one, we evaluated the dose titration of FITC-d for optimal measurement of enteric inflammation in broiler chicks using the following models: a) 24h feed restriction (FR); b) dextran sodium sulfate (DSS); or c) rye-based diet (RBD). In these experiments, FR, DSS, and RBD significantly increased ($P < 0.05$) serum concentrations of FITC-d, suggesting that FITC-d may be a good indicator of permeability as has been shown in some mammalian models. In chapter two, a series of experiments were conducted to evaluate the effect of dexamethasone (DEX) treatment in feed on systemic (serum) FITC-d levels, total numbers of aerobic bacteria in liver as an index of bacterial translocation (BT), differential white blood cell counts, and immune organs in broiler chickens. DEX-treated chickens showed a significant increase in serum FITC-d and BT, again indicating that stress increased paracellular leakage across the gut epithelium associated with dissolution of tight junctions. Additionally, heterophil/lymphocyte ratio was significantly increased and relative spleen and bursa of

Fabricious weight ratios were significantly decreased in DEX-treated chicks. These results provide a robust measurement model for enteric inflammation model in broiler chickens to further evaluate candidate non-antibiotic anti-inflammatory treatments as candidate AGPs.

Key words: fluorescein isothiocyanate dextran, feed restriction, dextran sodium sulfate, rye diet, dexamethasone, permeability

TABLE OF CONTENTS

I. INTRODUCTION

Increasing grain prices, concerns over subtherapeutic antibiotic feed additives, and concerns for diminished animal well-being have driven a need for new sustainable disease management practices and improved feed efficiency for the poultry industry. In recent years, non-antibiotic feed additive and probiotic research has surged, but measuring the effect of these strategies has traditionally been limited to gross performance or pathological evaluation. The critical need for effective antibiotic growth promoter (AGP) alternatives was the focus of a recent OIE/USDA conference where issues related to increasing environmental footprint of monogastric animal agriculture, increased costs associated with production, and decreased animal wellbeing were clearly associated with the current and impending loss of these important drugs in many parts of the world (Seal et al., 2013).

Although the mechanism by which subtherapeutic AGP result in modulations of microbial presence, absence and abundance patterns within the gut are in doubt, the effects of inclusion of AGP are clear (Butaye et al., 2003). Modulations of the gut microflora by AGP include suppression of bacterial pathogens, reduction of nutrient use, increased production of vitamins and other nutrients, and reduced production of ammonia (Butaye et al., 2003) and AGP have a profound impact on growth rate and feed efficiency, possibly through effects on stabilization of the microbial populations (Gunal et al., 2006). Importantly, these AGP are known to reduce intestinal disease frequency and severity, reduce mortality, while reducing feed usage and improving rate of gain in monogastric animals, including poultry (Dibner and Richards, 2005). Recent poultry research has also shown that leakage of enteric bacteria into circulation results in non-gastrointestinal diseases (Tellez et al., 2009; Borst et al., 2012; Wideman, 2013).

An emerging and compelling hypothesis implicates reduced enteric inflammation as an underlying common modality for benefits associated with the addition of AGP (Niewold, 2007). Intestinal imbalances, or dysbiosis, are the result of changes, such as diet, infection, or even stresses that affect the intestinal microflora, and inflammation is obligatorily associated with intestinal disease, regardless of etiology (Lambert, 2009). When dysbiosis occurs, some populations of bacteria are reduced or even absent, while others may become overabundant, in addition to increased inflammation of the intestinal tract. Niewold (2007) compiled compelling evidence suggesting that the effects of AGP on gut microflora may be due to effects on gut inflammatory status, rather than direct effects on the microflora *per se*. Central to the hypothesis of Niewold (2007), is that AGP may not benefit animals directly through an antimicrobial effect because they are provided at sub-minimal inhibitory concentrations, levels known to not inhibit affected pathogen levels within the ingesta of poultry. Additionally, the ability of antibiotics to affect growth performance, regardless of the class of antibiotics used, and their target bacterial populations, suggests that the effects may not be directly due to antimicrobial activity. The microbial populations of the intestinal tract are immensely diverse, and the ability of an antibiotic targeted towards specific classes of bacteria does not explain how the activity is consistent throughout the entire life of animals experiencing periods of changing microbial populations (Lu et al., 2003). Niewold (2007) also pointed out that many popular AGP are classes that accumulate in phagocytes with known attenuation of the innate inflammatory response. This hypothesis is consistent with the observation that the intestinal walls of AGP-fed animals are thinner, which could be attributed to a reduced influx and accumulation of inflammatory cells (Jukes et al., 1956).

Some probiotics and prebiotics, as potential AGP alternatives, have been shown to increase performance and have anti-inflammatory effects, through a variety of mechanisms including increased production of volatile fatty acids (VFA), known to have profound anti-inflammatory activity (Liu et al., 2013). For example, work from our laboratories has indicated that increased VFA production was associated with lactose supplementation (Tellez et al., 1993), and improved production, especially in combination with specific lactic acid bacterial cultures (Vicente et al., 2007; Torres-Rodriguez et al., 2007a,b). These studies have shown that application of these cultures reduced *Salmonella* colonization and improved performance of poultry under laboratory and field trial conditions. Interestingly, inflammation of the gut mucosal epithelium has been shown as a key mechanism for mucosal colonization by several pathogens, and is supported by emerging data, primarily from rodent studies, indicating that inflammation in the gut directly results in dysbiosis where the overall diversity and abundance of bacteria are reduced (Craven et al, 2012). With increased inflammation, regardless of cause, more nutrients and colonization niches are made available to pathogens. For example, *Salmonella*, *Shigella flexneri*, *Vibrio cholerae*, and *Citrobacter rodentium* may exploit inflammation in order to reduce competition from native microflora and promote their own success in colonization and pathogenesis rodent models (Stecher et al., 2007; Winter et al., 2010). In contrast, reduced inflammation has been associated with stabilization of beneficial commensal bacterial populations known to reduce expression of inflammatory cytokines and reduce goblet cell size and mucus secretion, supporting the premise that inflammation is a precursor to dysbacteriosis and necrotic enteritis (Fraune and Bosch, 2010; Kau et al., 2011; Maslowski et al., 2011; Salzman, 2011). This may explain why multiple classes of antibiotics and effective probiotics enhance performance, and indicate that effective AGP alternatives,

through anti-inflammatory activity, could replace AGP for improved performance and animal well-being.

Thus, measuring intestinal inflammation in the search for AGP replacements may be crucial to the selection process. Availability of such assay(s), specific for intestinal inflammation, is also expected to be useful for identifying causes and prophylactics for inflammation under a variety of nutritional programs and environmental conditions. Development of AGP alternatives has been met with many failures due to a lack of complete understanding of mechanisms of action and because *in vivo* effectiveness is most commonly measured through performance trials or disease resistance studies which are imprecise and cumbersome for high throughput screening. This manuscript describes attempts at establishing non-infectious models of enteric inflammation in broiler chickens and evaluates the use of a large molecular weight fluorescent marker for high-throughput screening for induction or amelioration of enteric inflammation.

II. LITERATURE REVIEW

A. BARRIER FUNCTIONS OF THE GASTROINTESTINAL TRACT

The mucosal barrier of the gastrointestinal tract (GIT) represents the largest body surface in contact with the external environment, playing a crucial role in toleration of the microbiome as well as nutrients and water that also need to be allowed to enter the body (Galley and Bailey, 2014; Pijls et al., 2013). Barrier function is a critical aspect of gut heath. Oxidative stress, poorly digestible protein or energy sources and coccidiosis are some examples that can cause gut barrier failure (Pastorelli et al., 2013; Sharma et al., 2007; Williams, 2005; Latorre et al., 2014; Tellez et al., 2014). However, as a consequence of the removal of antimicrobial growth promoters, new multifactorial diseases causing enteritis and gut disorders of unknown origin have emerged in broilers, causing negative impacts in health and performance (Gholamiandehkordi et al., 2007; Yegani and Korver, 2008; Castanon, 2007; Dahiya et al., 2006). Among them, dysbacteriosis, defined as the presence of a qualitatively and/or quantitatively abnormal microbiota, that promote a cascade of reactions in the GIT including reduced nutrient digestibility and impaired intestinal barrier function, increasing the risk of bacterial translocation and inflammatory responses (Teirlynck et al., 2011). However, more recently, poor gut health has also been associated with bacterial chondronecrosis with osteomyelitis (BCO) lesions and lameness in broilers chickens and broiler breeders (Wideman et al., 2015; Wideman et al., 2012; Wideman et al., 2011). On a daily basis, the intestine is exposed to an unlimited number of antigens including dietary components, toxins, commensal and pathogenic microorganisms, therefore, the GIT serves as a selective barrier to take up nutrients and fluids into the body, while excluding undesirable molecules and pathogens (Sharma et al., 2010; Groschwitz and Hogan, 2009; Pastorelli et al., 2013). Hence, proper gut

barrier function is essential to maintain optimal health and balance throughout the body and represents the first line of defense against these foreign antigens from the environment (Jeon et al., 2013). The first layer of gut barrier is the extrinsic mucus layer comprised of outer layer associated with bacteria, loosely attached to epithelium, and an inner layer with high concentrations of secretory IgA and mucin which are adherent to the second layer of gut barrier, the intestinal epithelial cells (IECs). The IECs are represented by a single layer of epithelial cells that separate the intestinal lumen from underlying lamina propria (Sakamoto et al., 2000; Kim and Ho, 2010; Johansson et al., 2010). These epithelial cells must be able to rapidly regenerate in the event of tissue damage (Audy et al., 2012; Iizuka and Konno, 2011; Groschwitz and Hogan, 2009). The enterocytes are responsible for absorption of nutrients. Tight junctions (TJ) seal the paracellular space between adjacent epithelial cells near the apical surface, which regulates the permeability of the intestinal barrier by preventing paracellular diffusion of microorganisms and antigens across the epithelium (Ulluwishewa et al., 2011; Sander et al., 2005). Since IECs are the primary cell type coming into contact with the external environment, they act as the host's first line of the defense. In spite of their non-hematopoietic derivation, IECs represent a core element of innate immunity within the gut associated lymphoid tissue (GALT), displaying a wide array of immune functions. In fact, IECs are able to recognize pathogens through the expression of innate immune receptors, release anti-microbial molecules, secrete a wide number of hormones, neuro transmitters, enzymes, as well as cytokines and chemokines that link innate and adaptive immune responses (Ballard et al., 1995; Alverdy et al., 2005; Edelblum and Turner, 2009). Therefore, any direct or indirect damage on IECs may cause a breakdown in gut barrier and consequently, disruption of normal mucosal immune homeostasis that can potentially lead to uncontrolled chronic intestinal and systemic

inflammation (Ilan, 2012; Schulzke et al., 2009). Several investigators have described the pathways associated with the disruption of the protein networks that connect epithelial cells by inflammatory mediators (hormones, oxygen free radical species, enzymes as well as multiple pro inflammatory cytokines) caused by pathogens, diet ingredients or stress (Steed et al., 2010; Schulzke et al., 2009; Hu et al., 2013). Other factors can also induce gut barrier loss. Feeding oxidized/unpreserved fat has been shown to increase intestinal epithelial turnover rates and increase apoptosis at villus tips in poultry and swine (Dibner et al., 1996).

B. STRESS AND THE GUT-BRAIN AXIS

The impact of the central nervous system, sometimes called the "gut-brain axis," is not fully elucidated. Clearly, there are important relationships between ingested and microbe-manufactured chemicals, the microbes that reside in the gut, and potential effects on the brain. Conversely, the central nervous system can impact the GIT. In commercial poultry, genetic selection for growth parameters in meat type chickens gives rise to a parent stock (broiler breeders) that tends to lack the ability to self-regulate feed intake (Yang et al., 2011; de Jong et al., 2003). Nonetheless, the high body mass is associated with lameness, and high mortality rates (often due to skeletal and/or cardiovascular disease). In order to regulate weight gain, limit health risks, and also maintain high fertility, husbandry practices for the parent stock of broiler chickens include a high degree of feed restriction (FR) (Rajman et al., 2006; Zulkifli et al., 1993). However, chronic FR represents a permanent stress for any animal, particularly for birds with relatively high metabolic requirements, where increased plasma corticosterone concentrations are often associated with chronic stress observed in FR programs (Washburn et al., 1980; Mounier et al., 1998). Stress can induce a variety of changes in normal gastrointestinal function, including changes in gut motility and permeability, as well as

alterations in ion, fluid, and mucus secretion and absorption (Alverdy and Aoys, 1991; Collins and Bercik, 2009; Verbrugghe et al., 2011; Karavolos et al., 2013). Animal models of acute and chronic stress have shown that stress induces changes in intestinal barrier function including increased transcellular and paracellular intestinal permeability, caused by a temporary dissociation of TJ proteins (Maejima et al., 1984; Assimakopoulos et al., 2011; Koh et al., 1996; Matter and Balda, 2007). These changes have been linked to Mast cells, important effectors of the brain-gut axis that initiate a consequent release of a wide range of neurotransmitters and pro-inflammatory cytokines, causing marked effects on gastrointestinal physiology (Bailey et al., 2011; Groschwitz and Hogan, 2009).

C. IMPORTANCE OF DEVELOPING A MODEL FOR GUT INFLAMMATION IN BROILERS

Antibiotic as growth promoter in the feed of different animal species has been used for many years to improve feed efficiency and growth (Castanon, 2007). The mechanisms on how antibiotics can promote growth are now thought to include non-antimicrobial mechanisms such as the ability to reduce innate inflammatory responses (Niewold, 2007). Indeed, some antibiotics have been demonstrated to inhibit one or more mechanisms of inflammation involving both inflammatory cells and pro-inflammatory cytokine production (Dahiya et al., 2006). These observations led to the emerging hypothesis that commonly-used AGP might reduce the negative consequences of inflammation, allowing rediversion of energy toward growth and muscle accretion (Niewold, 2007). Although the use of AGP in animal production has advantages, extensive use has of these molecules has been claimed to contribute to the emergence of antimicrobial resistance in zoonotic pathogens, resulting in a ban by the European Union (Castanon, 2007). The removal of AGP from livestock production has increased cost,

reduced feed efficiency and increased the incidence of some enteric diseases, which has encouraged the search for alternatives such as effective probiotics incidence forcing animal husbandry to find alternatives including probiotics (Yegani and Korver, 2008). The major functional effect of nutraceuticals, probiotics and/or prebiotics is the balance of both GIT microflora and innate immune responses, and control of pro-inflammatory and anti-inflammatory cytokines responsible for the negative impact of inappropriate inflammation (Joerger, 2003). The purpose of the present thesis was to study and develop models for measuring the consequence of inflammation, leaky gut, within the GIT of broiler chickens.

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III. CHAPTER I

A. Dose titration of FITC-D for optimal measurement of enteric inflammation in broiler chicks

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B. ABSTRACT

Traditionally, antibiotic growth promoters (AGP) have been used in food animals to reduce enteric inflammation and maintain intestinal homeostasis, thus improving growth and performance. Due to increasing restrictions regarding the use of AGP, precise and high throughput enteric inflammation models and markers to search for effective alternatives are urgently needed. Oral administration of fluorescein isothiocyanate dextran (FITC-d, 3-5kDa) and its passage into blood can be used as a marker for tight junction permeability. In study 1, 10d broilers were assigned to control, 24h feed restriction (FR), or dextran sodium sulfate (DSS) (0.75% in water for 5d), and received an oral gavage of FITC-d 2.5h before sample collection. FITC-d in serum and intestinal samples (duodenum and ceca) were higher ($P<0.05$) after FR than in DSS and control groups. In study 2, FR was evaluated for effect on mucosal leakage and an oral dose of FITC-d of 0.5, 1.1, or 2.2 mg/chick was used to measure GIT permeability at 6d of age. The amount of FITC-d remaining in duodenal tissue of control birds increased with dose, only 1.1 mg FITC-d/chick dose resulted in differences ($P<0.05$) between control and FR. No differences were noted between control and FR, regardless of FITC-d dosage in cecal recovery of FITC-d. Additionally, FR increased FITC-d serum levels when compared to controls and in a dose-dependent manner. Experiment 3 compared serum levels after administration of 0.55 and 1.1 mg/chick doses of FITC-d in birds treated with FR, rye-based diet (RBD), and DSS. Intestinal sections were collected for FITC-d recovery in the 1.1 mg dosage group. All inflammation treatments significantly increased serum FITC-d levels at both dosages. Only FR resulted in increased ($P<0.05$) FITC-d recovery from duodenum, ileum, and ceca. In conclusion, FR, DSS, and RBD affect GIT tight junction integrity, suggesting their value for enteric inflammation models, and FITC-d may be a good indicator of permeability.

Key words fluorescein isothiocyanate dextran, feed restriction, dextran sodium sulfate, rye diet, enteric inflammation

C. INTRODUCTION

The gastrointestinal tract (GIT) has numerous physiological functions including effective absorption of dietary nutrients and plays a key role as a barrier against enteric pathogens, thus integrity and repair are vital for protection of animals against disease and optimal production performance. The mucous layer, tight junctions between epithelial cells, and gut-associated lymphoid tissue help to maintain a homeostasis between dietary antigens, in addition to enteric pathogens and beneficial microorganisms (Van Der Hulst et al., 1998). Disruption of gut health and its barrier results in nonselective permeability and, could lead to malabsorption of nutrients and translocation of a greater amount of enteric bacteria to various internal organs which may lead to diseases and reduced growth performance (Quinteiro-Filho et al., 2012). Furthermore, increasing grain prices, concerns over subtherapeutic antibiotic feed additives (AGP), and concerns for diminished animal well-being have driven a need for new sustainable disease management practices and improved feed efficiency for the poultry industry.

Although the mechanism by which subtherapeutic AGP result in modulations of microbial presence, beneficial performance effects of inclusion of AGP are clear (Butaye et al., 2003). Possible mechanisms by which AGP modulate gut microflora by AGP include suppression of bacterial pathogens, reduction of nutrient use, increased production of vitamins and other nutrients, and reduced production of ammonia (Butaye et al., 2003) and AGP have a profound impact on growth rate and feed efficiency, possibly through effects on stabilization of the microbial populations (Gunal et al., 2006). Importantly, these AGP are known to reduce intestinal disease frequency and severity, reduce mortality, while reducing feed usage and improving rate of gain in monogastric animals, including poultry (Dibner and Richards, 2005).

In the case of poultry, it has been proposed that one benefit of AGP is functional control of enteric inflammation (Niewold, 2007). Niewold (2007) also pointed out that many popular AGP are classes that accumulate in phagocytes with known attenuation of the innate inflammatory response. This hypothesis is consistent with the observation that the intestinal walls of AGP-fed animals are thinner (Jukes et al., 1956), which could be attributed to a reduced influx of inflammatory cells. However, models of enteric inflammation are not currently well-developed in poultry, and hinder the search for a complete understanding of the roll of AGP in production and alternative additives that can provide the same, or better, functions. Thus, there is an urgent need to develop enteric inflammation and permeability models in poultry that can both aid in elucidating the performance-enhancing effects of AGP and in the search for alternatives.

Recent publications describing rodent models of enteric inflammation and leaky gut have described fluorescein isothiocyanate–dextran (FITC-d) as a marker of enteric leakage (Shah et al., 2007; Brandl et al., 2009; Yan et al., 2009). Yan et al. (2009) demonstrated an increase in pericellular leakage associated with dextran sodium sulfate (DSS) treatment by measuring the serum levels of FITC-d 4h after FITC-d oral gavage. Similarly, starvation in mice resulted in reduced gut epithelial cell proliferation and increased apoptosis (Chappell et al., 2003). These findings suggest that induction of gut inflammation and measurement of gut leakage using FITC-d could be an effective method to study gut health.

The studies herein were designed to evaluate multiple inducers of enteric inflammation and dosages of FITC-d as a marker of leaky gut. Feed restriction (FR) is a common practice for breeder stock because genetic selection for growth parameters in meat type chickens gives rise to a parent stock (broiler breeders) that tends to possess high body mass associated with excessive

fat deposition, lameness, and high mortality rates often due to skeletal and/or cardiovascular disease. In order to regulate weight gain, limit health risks, and also maintain high fertility, husbandry practices for the parent stock of broiler chickens include a high degree of FR (Lee et al., 1971; Yu et al., 1992). Well-being and disease susceptibility during FR are a concern for producers, thus FR was evaluated for its impact on enteric permeability. Additionally, alternative feedstuffs such as wheat and rye are not uncommon, and often contain high levels of non-starch polysaccharides (NSP; Choct et al., 1995). Such diets often lead to higher susceptibility to necrotic enteritis and increased digesta viscosity (Annett et al., 2002; Tellez et al., 2014). The purpose of the present studies was to evaluate alterations in gut permeability under inflammation and stress induced by FR, high NSP diet, or DSS, previously studied inducers of leaky gut in broilers (Kuttappan et al., 2014a, b; Latorre et al., 2014; Menconi et al., 2014; Tellez et al., 2014). Additionally, multiple doses of FITC-d were evaluated for serum and intestinal recovery to help optimize optimal detection of gastrointestinal permeability as a marker of enteric inflammation.

D. MATERIAL AND METHODS

Experimental Animals

Day of hatch broiler chicks were obtained from a primary breeder and randomly assigned to treatment groups. Chicks were housed in brooder battery cages with wire floors. Throughout each experiment, chicks were provided a diet that met or exceeded NRC requirements (National Research Council, 1994), except FR groups, and water *ad libitum* throughout the research period. A previously determined dose for drinking water administration of DSS (MW 40,000; Alfa Aesar, Ward Hill, MA) was provided as a 0.75% solution *ad libitum* as described below in experimental design (Menconi et al., 2014). The rye-based diet (RBD) was formulated as

previously described as an inducer of leaky gut, and both control and rye-based diet are described in Table 1 (Tellez et al., 2014). Rye diet was provided as the sole source of nutrition during d5-d7 in Experiment 3, described below.

To detect enteric leakage, chickens were dosed with FITC-d (MW 3,000-5,000; Sigma Aldrich Co., St. Louis, MO) by oral gavage at concentrations described below. Previous unpublished data has suggested that 2.5h after oral gavage with FITC-d is the optimal blood collection time point for broilers. Birds were killed by CO_2 inhalation, and blood and intestinal tissue samples were collected post-mortem. All animal handling procedures were in compliance with Institutional Animal Care and Use Committee regulation at the University of Arkansas.

FITC-d Fluorescence in Blood

For detection of FITC-d in serum, blood was kept at room temperature for 3h to allow clotting, and centrifuged (1,000Xg for 15min) to separate serum. Fluorescence levels of diluted serum (1:1 in PBS) were measured at excitation wavelength of 485nm and emission wavelength of 528nm (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., Vermont, USA), and FITC-d concentration per mL of serum was calculated based on a standard curve.

Tissue Levels of FITC-d

Measurement of FITC-d released from intestinal tissue was accomplished by collection of 2.5 cm sections of GIT which were cleaned by flushing with Hanks buffered salt solution, weighed and placed in tubes with 10mL of Hanks buffer containing glutamine (0.3g/L) and antimicrobial agents (Penicillin 100µ/mL; Streptomycin 0.01mg/mL; Amphotericin B 0.25µg/mL). Duodenal samples were collected from the descending duodenum, ileal samples from immediately proximal to the Meckel's diverticulum, and a single entire cecum for tissue sampling. The tubes were incubated for 2h at 42°C and sampled by collecting 100µL of buffer

from each tube. FITC-d levels of buffer in the tubes were determined by fluorescence measurement as described above for serum, with the final concentration reported as µg/g of ileal tissue.

Experimental Design

Experiment 1. Evaluation of feed restriction and Dextran sodium sulfate on enteric leakage. Thirty chicks were comingled in a single wire-floor brooder battery cage through d10, at which point they were randomly separated into three separate cages according to treatment group (n=10/group) – control (CON), FR, or DSS for the remainder of the experiment. On d10, DSS group began receiving 0.75% DSS in drinking water, and continued treatment through the end of the experiment. On d13 FR began and continued for 24h through the end of the experiment. Fluorescein isothiocyanate–dextran (2.2 mg/bird) for serum detection was administered by oral gavage on d14.

Experiment 2. Evaluation of three doses of FITC-d in serum and GIT tissue after feed restriction. Forty-eight day of hatch chicks were randomly separated into wire-floor brooder battery cages (24 chicks/cage) and reared with feed and water *ad libitum* through d5. On d6 feed was removed from one group, FR, for the remainder of the experiment (24h). Concentrations of FITC-d at 0.55 mg/chick, 1.1 mg/chick, or 2.2 mg/chick were administered by oral gavage to 8 chicks each in CON and FR groups on d7. Blood, duodenum, and ceca were collected 2.5 h after administration of FITC-d.

Experiment 3. Evaluation of two doses of FITC-d in serum and GIT tissue after feed restriction, DSS, or rye-based diet. Eighty day of hatch chicks were randomly separated into wire-floor brooder battery cages (20 chicks/cage) and reared with feed and water *ad libitum* through d5. On d5, 0.75% DSS treatment in drinking water or RBD were initiated for two cages

and continued throughout the remainder of the experiment. On d6 feed was removed from one group, FR, through termination of the experiment (24 h). Concentrations of FITC-d at 0.55 mg/chick, or 1.1 mg/chick were administered by oral gavage to 10 chicks each in CON, FR, DSS, and RBD groups on d7. Blood was collected from all groups, plus duodenum, ileum, and ceca were collected from 1.1 mg/chick FITC-d group 2.5 h after administration of FITC-d.

Statistical Analysis

Data were analyzed using ANOVA (SAS 9.3, SAS Institute Inc., Cary, NC), and means were separated with Duncan's significant difference test with $P < 0.05$ considered as significant. Individual birds were considered as the experimental unit for the entire analysis. In all trials, data are expressed as mean ± standard error.

Data from serum FITC-d levels were analyzed in 2 X 2 factorial (Treatment X Dose) using JMP Pro 11.2.0 (SAS Institute Inc.) and main effects of treatment, dose, and interaction between treatment and dose were evaluated. Serum FITC-d data showed occasional, but random, high values which were not representative of the respective group means. Although, the reason/s for such high FITC-d values is/are still not clear, it does not seem to be related to treatment. Since these values were creating noise in analysis, outliers from treatment groups were identified as above or below two standard deviations from mean, based on empirical or 68-95-99.7 rule. Identified outliers were trimmed, or truncated, according to (Ghosh and Vogt, 2012). Analysis of Experiments 2 and 3 serum (0.55 and 1.1 mg/chick doses) from CON and FR were completed both with unedited and truncated data, and results are reported as mean ± standard error (SE) and P values for the respective main effects to describe this phenomenon. Main effects with P-value less than 0.05 were considered significant. All other reported data for serum and tissue samples represents truncated values, with number of outliers removed reported.

E. RESULTS

Serum FITC-d

Various inducers of leaky gut were compared for ability to increase FITC-d passage into serum, including FR, DSS, and RBD. In experiment 1, both FR and DSS, with recovery levels of 0.364±0.017 and 0.279±0.01 µg/mL respectively, resulted in higher ($P<0.05$) levels of FITC-d (2.2mg/bird) than control chicks at 0.182±0.015 µg/mL (Table 2). Feed restriction also caused higher ($P<0.05$) levels of serum FITC-d than controls, at 0.218±0.007 vs. 0.131±0.02 µg/mL, in experiment 2 when administered at 0.55mg/bird, but not at other doses. Additionally, all three tested enteric inflammation treatments – FR, RBD, and DSS, showed significantly increased FITC-d recovery compared to control serum, regardless of FITC-d dose in experiment 3.

Furthermore, multiple doses of FITC-d were tested to determine optimal concentration for measuring enteric leakage. In experiment 2, three doses, 0.55 mg/bird, 1.1 mg/bird, and 2.2 mg/bird were evaluated after 24h FR, with 0.55 mg FITC-d/bird being the only dose that resulted in significant difference from controls with recovery levels at 0.218±0.007 µg/mL in FR group and 0.131±0.02 µg/mL in the control group (Table 2). It was also noted that as FITC-d dosage increased, the level of recovery from control serum also rose in a dose-dependent manner of 0.131±0.014, 0.201±0.035, and 0.302±0.032 µg/mL of serum for 0.55, 1.1, and 2.2 mg FITC-d/bird, respectively. Also, the difference between control and FR groups became less apparent as mg of FITC-d increased (Figure 1B). While 0.55 mg/bird dose of FITC-d also resulted in significant differences from control in Experiment 3, 1.1 mg/bird dose of FITC-d resulted in the greatest difference from control for FR, RBD, and DSS in Experiment 3 (Table 2).

Tissue FITC-d

Experiments 2 and 3 evaluated tissue recovery of FITC-d by oral gavage to help determine which regions of GIT are affected by each method of induction. Three doses of FITC-d, 0.55, 1.1, and 2.2 mg/bird, were tested for recovery in duodenum and cecum in Experiment 2. Only the dosage of 1.1 mg/bird of FITC-d results in a significant ($P<0.05$) difference from control after 24h FR, with 1.245±0.071 µg/g and 1.709±0.176 µ/g of tissue in control and FR groups, respectively (Table 4). No differences were measured between control and FR in cecal levels of FITC-d. In contrast, FR increased recovery of FITC-d from tissue in all three GIT tissues in Experiment 3, in which only 1.1 mg/bird FITC-d was measured. Duodenum tissue recovery increased from 1.267±0.217 µg/g in control birds to 3.057±0.288 µg/g in FR birds, ileum recovery of FITC-d was 3.541±0.664 µg/g for control group vs. 11.40±3.099 µg/g in FR group, and cecal increased from 6.082±0.916 µg/g in control birds to 15.071±1.943 µg/g in FR birds. Both RBD and DSS failed to result in elevated FITC-d tissue recovery for all intestinal sections.

F. DISCUSSION

Increasing grain prices, concerns over subtherapeutic antibiotic feed additives, and concerns for diminished animal well-being have driven a need for new sustainable disease management practices and improved feed efficiency for the poultry industry. In recent years, non-antibiotic feed additive and probiotic research has surged, but measuring the effect of these strategies has traditionally been limited to gross performance or pathological evaluation. The critical need for effective AGP alternatives was the focus of a 2012 OIE/USDA conference where issues related to increasing environmental footprint of monogastric animal agriculture, increased costs associated with production, and decreased animal wellbeing were clearly

associated with the current and impending loss of these important drugs in many parts of the world (Seal et al., 2013). The ongoing diminution of AGP usage is estimated to reduce feed efficiency of monogastric animals by approximately 6-10% (reviewed by Thomke and Elwinger, 1998; Bedford, 2000).

Although the mechanism by which subtherapeutic AGP result in modulations of microbial presence, absence and abundance patterns within the gut are in doubt, the effects of inclusion of AGP are clear (Butaye et al., 2003). Modulations of the gut microflora by AGP include suppression of bacterial pathogens, reduction of nutrient use, increased production of vitamins and other nutrients, and reduced production of ammonia (Butaye et al., 2003) and AGP have a profound impact on growth rate and feed efficiency, possibly through effects on stabilization of the microbial populations (Gunal et al., 2006). Importantly, AGP are known to reduce intestinal disease frequency and severity, reduce mortality, while reducing feed usage and improving rate of gain in monogastric animals, including poultry (Dibner and Richards, 2005). An emerging and compelling hypothesis implicates reduced enteric inflammation as an underlying common modality for benefits associated with the addition of AGP (Niewold, 2007). Intestinal imbalances, or dysbiosis, are the result of changes, such as diet, infection, or even stressors that affect intestinal microflora. The resulting inflammation from these imbalances is thus obligatorily associated with intestinal disease, regardless of etiology (Lambert, 2009). Niewold (2007) also noted that many popular AGP are classes that accumulate in phagocytes and are known to diminish innate inflammatory responses. This hypothesis is consistent with the observation that the intestinal walls of AGP-fed animals are thinner (Jukes et al., 1956), which could be attributed to a reduced influx and accumulation of inflammatory cells. While it is well-known that intestinal microflora are important potentiators of the intestinal host defenses,

modulation of inflammatory responses is critical, as common production problems related to inflammation are commonly related to an excessive, rather than insufficient, innate immune response (McCracken and Gaskins, 1999; Tracey, 2002).

Thus, measuring intestinal inflammation in the search for AGP replacements may be crucial to the selection process. Availability of such assay(s), specific for intestinal inflammation, is also expected to be useful for identifying both causes and preventatives for inflammation under a variety of nutritional programs and environmental conditions. Animal models for human GIT diseases, such as irritable bowel disorder (IBD), have been previously developed, and have well-established measurement protocols (Yan et al., 2009). A common molecule for quantification of enteric inflammation is FITC-d, a molecule which does not usually leak through the intact gastrointestinal tract barrier (Rose et al., 2012). However, when conditions disrupt tight junctions between epithelial cells, FITC-d can enter circulation, as demonstrated by an increase in trans-mucosal permeability associated with enteric inflammation treatment in these studies. Progressive detection of FITC-d in serum (Table 2) and gut tissue retention (Table 4) is consistent with leakage from the lumen and suggests the presence of a change in paracellular permeability rather than transcellular transport. *Ex vivo* evaluation of GIT permeability has been used as a method to determine gut leakage in animal models. For many of such studies, either an everted or non-everted intestinal section and a marker dye, like FITC-d or phenol red in buffer, and the rate of passage of dye across intestinal walls was estimated to determine gut leakage (Nakamaru et al., 1997; Lambert, 2009; van der Meer et al., 2012). Specially designed apparati, such as Ussing chambers, are required to perform some permeability assays, which are slow and often lead to low number replications in experimental results (Dixit et al., 2012), and are obviously devoid of circulatory, endocrine, and cytokine feedback

mechanisms. The present studies suggest that decreased mucosal integrity can be measured in the simple FITC-d assay as described.

Occasional outliers were noted in both serum and tissue FITC-d detection assays, and were presently reported with their inclusion or after removal from reported values when outliers were ± 2 standard deviations from the mean, as described by Ghosh and Vogt (2012; Figure 1 and Table 3). These random extremely high values created noise in data that made interpretation difficult, and although the cause of these outliers is not yet known, they do not seem related to FITC-d dose or treatment, as shown in Table 3. Treatment X dose interaction was not significant for either truncated or unedited data in Experiment 2 and 3, but removal of outliers did occasionally change the P-value from $P>0.05$ to $P<0.05$, as shown for Experiment 2 treatment values. Furthermore, removal of two outliers from Experiment 2 changed a dose response curve that was uninterpretable (Figure 1A) to one that followed expected results and can be interpreted to discern appropriate dosage for FITC-d detection of enteric leakage (Figure 1B). In fact, the curve presented in Figure 1B suggests that tested doses of FITC-d may be at the top of the dose-response curve, and further testing of lower concentrations may be warranted, because 0.55 mg/kg was the only concentration on the linear portion of the curve, and without truncation, this observation would not have been noted.

While 1.1 mg/kg FITC-d was the only concentration tested that resulted in significant differences in tissue recovery (Table 4), a lower dose of 0.55 mg/kg FITC-d appeared to more consistently result in a greater difference from the mean for serum samples (Table 2). Feed restriction effects, as measured at 0.55, 1.1, and 2.2 mg/kg FITC-d, were 166%, 141%, and 128% greater than the respective control values for Experiment 2, suggesting that background leakage decreases with FITC-d dose, a primary objective of these studies. Although regional enteric

effects of the selected insults was not a primary goal of the present studies, tissue evaluation of regional enteric FITC-d provided some insight into relative inflammation of selected portions of the GIT.

These studies suggest that circulating levels of FITC-d can be used for rapid evaluation of inflammatory-associated enteric epithelial leakage, which may be useful in the search for AGP alternatives in future studies. Future evaluation and comparison of regional FITC-d recovery with specific enteric pathogen-induced enteric inflammatory insults may allow for determination of the value of FITC-d tissue retention following post-mortem sample collection, which may have value for diagnostic or investigations related to idiopathic infectious etiologies.

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Table 1 Ingredients (%) of corn and rye diets.

Ingredient	Corn diet	Rye diet
Ingredients		
Corn	**55.53**	0.0
Rye	0.00	**58.27**
Soybean meal	**35.69**	**31.16**
Vegetable oil	4.22	6.29
Dicalcium phosphate	1.82	1.79
Calcium carbonate	1.12	1.05
Salt	0.38	0.38
DL-Methionine	0.37	0.35
Vitamin premix[1]	0.20	0.20
L-Lysine HCl	0.28	0.22
Choline chloride 60%	0.20	0.10
Mineral premix[2]	0.10	0.10
Selenium 0.6%	0.02	0.02
Propionic acid	0.02	0.02
Antioxidant	0.05	0.05
Total	100.00	100.0

[1]Vitamin premix supplied the following per kg: vitamin A, 20,000,000 IU; vitamin D3, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K3, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g (Nutra Blend LLC, Neosho, MO 64850).

[2]Mineral premix supplied the following per kg: manganese, 120 g; zinc, 100 g; iron, 120 g; copper, 10–15 g; iodine, 0.7 g; selenium, 0.4 g; and cobalt, 0.2 g (Nutra Blend LLC, Neosho, MO 64850).

[3] Ethoxyquin.

Table 2 Serum FITC-d levels after enteric inflammation treatments of dextran sodium sulfate (DSS), feed restriction (FR), or rye diet (RBD). DSS was administered in drinking water for 5 days, FR was conducted for 24 h, and RBD provided for 3 days before oral gavage with FITC-d on d14, blood was collected 2.5 h post-gavage. Values reported as mean ± standard error.

FITC-d Dose	Treatment	Serum FITC-d (µg/mL) Mean±SE	# Outliers Removed[1]
Experiment 1			
2.2mg/bird	Control	0.182±0.015[c]	
	FR	0.364±0.017[a]	
	DSS	0.279±0.010[b]	1
Experiment 2			
0.55mg/bird	Control	0.131±0.020[b]	
	FR	0.218±0.007[a]	
1.1mg/bird	Control	0.201±0.035[a]	1
	FR	0.283±0.020[a]	
2.2mg/bird	Control	0.234±0.026[a]	
	FR	0.302±0.032[a]	1
Experiment 3			
0.55mg/bird	Control	0.193±0.014[b]	1
	FR	0.281±0.017[a]	1
	RBD	0.288±0.047[a]	1
	DSS	0.281±0.016[a]	1
1.1mg/bird	Control	0.163±0.023[c]	1
	FR	0.307±0.022[a]	1
	RBD	0.224±0.009[b]	1
	DSS	0.251±0.013[b]	

[1] Data was truncated to remove outliers ±2SD from the group mean

[a-c]values with different superscripts within each experiment and treatment dose are significantly different ($P<0.05$)

Table 3 Effect of treatment (control or feed restriction, FR), dose of FITC-d (0.55 or 1.1 mg/chick), and interaction between treatment and dose on serum FITC-d (µg/mL) levels in Experiments 2 and 3. FR was conducted 24h immediately prior to oral gavage with FITC-d, blood samples were collected 2.5h after dosage for detection. Serum FITC-d levels reported as mean ± SE (µg/mL).

FITC-d Dose		**Experiment 2**		**Experiment 3**	
		With Outliers	**Truncated**	**With Outliers**	**Truncated**
0.55 mg/bird	Control	0.131±0.022	0.131±0.020	0.227±0.036	0.193±0.014
	FR	0.218±0.008	0.218±0.007	0.299±0.024	0.281±0.017
1.1 mg/bird	Control	0.278±0.089	0.201±0.035	0.216±0.057	0.163±0.023
	FR	0.283±0.022	0.283±0.020	0.334±0.033	0.307±0.022
Pooled SE		0.04	0.02	0.04	0.02
P-value					
Treatment		0.3016	0.0007*	0.0211*	<0.0001*
Dose		0.0234*	0.0049*	0.7616	0.9222
Treatment X Dose		0.3609	0.9178	0.5737	0.149

*significant (P<0.05) effect of the main effect/s

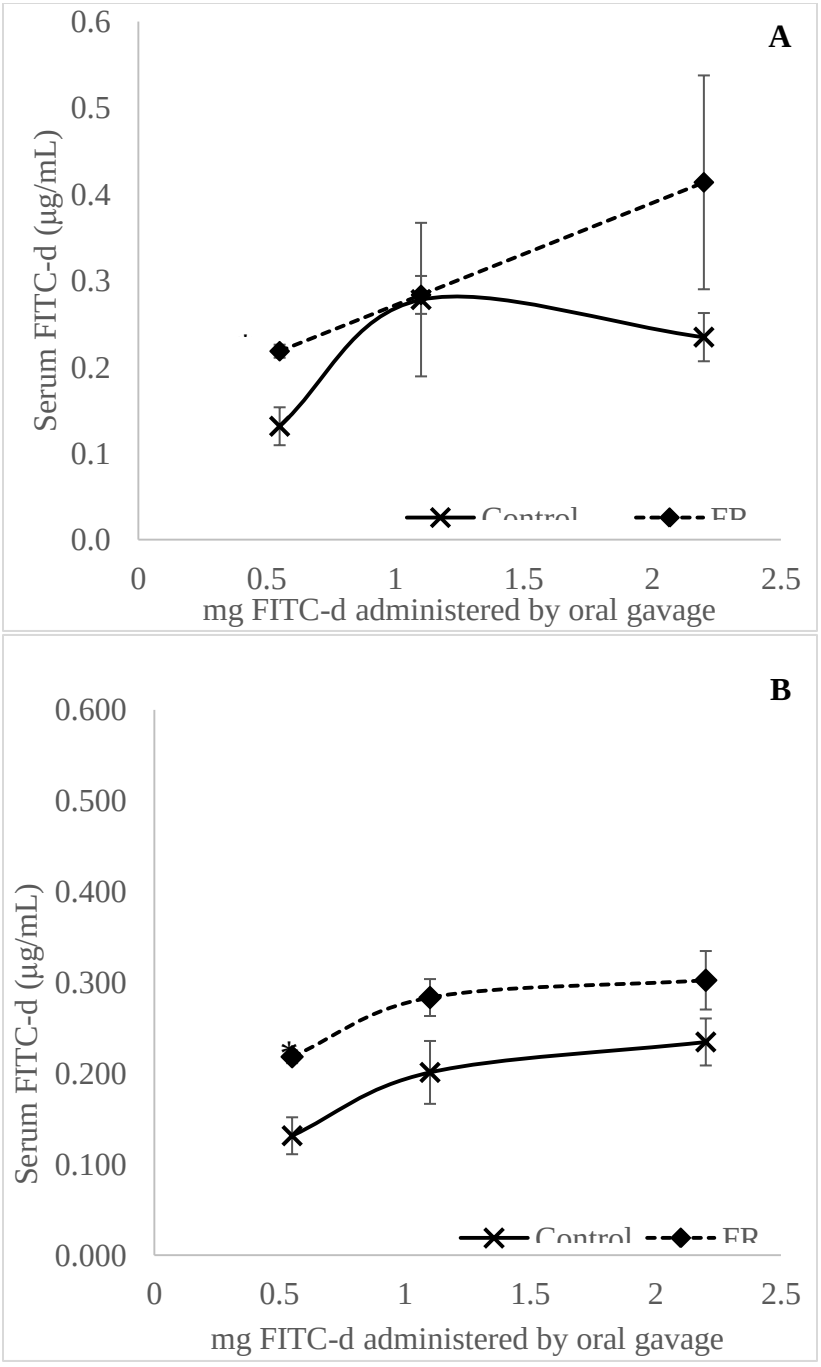
Table 4 Tissue recovery of FITC-d (µg/g tissue) from duodenum, ileum, and cecum from broilers after enteric inflammation treatment. 2.5h after FITC-d administration by oral gavage, chicks were killed and tissue sections incubated in Hank's Buffer for 2.5h. Levels reported are µg of FITC-d recovered from incubation buffer per g of tissue, truncated mean ± SE (±2SD outliers removed). FR = 24h feed restriction, RBD = rye based diet, DSS = 0.75% DSS in drinking water.

FITC-d Dose		Duodenum	Ileum	Cecum
Experiment 2				
0.55mg/bird	Control	1.042±0.111[a]	-ND-	3.220±0.583[a]
	FR	1.694±0.375[a]	-ND-	6.307±2.201[a]
1.1 mg/bird	Control	1.245±0.071[b]	-ND-	6.310±1.177[a]
	FR	1.709±0.176(1)[a]	-ND-	6.104±0.405(1)[a]
2.2mg/bird	Control	1.832±0.357[a]	-ND-	6.390±0.713[a]
	FR	1.837±0.197(1)[a]	-ND-	6.813±2.450[a]
Experiment 3				
1.1 mg/bird	Control	1.267±0.217[b]	3.541±0.664[b]	6.082±0.916[b]
	FR	3.057±0.288[a]	11.40±3.099[a]	15.071±1.943[a]
	RBD	2.620±0.148(1)[a]	4.915±0.881(1)[b]	5.918±1.783[b]
	DSS	2.575±0.247[a]	5.221±0.866[b]	5.829±0.554[b]

ND = no data collected

[a-b] values with different superscripts within each experiment, tissue, and treatment dose are significantly different ($P<0.05$)

Figure 1 Detection of FITC-d in serum after 24h feed restriction (FR) without (A) and with (B) truncation of ± 2 SD from mean. Chickens were subjected to 24h FR immediately prior to oral gavage with 0.55, 1.1, or 2.2 mg FITC-d and blood collected 2.5 h post-gavage (Experiment 2).



IV. CHAPTER 2

A. Effect of dexamethasone in feed on intestinal permeability, differential white blood cell counts, and immune organs in broiler chicks

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B. ABSTRACT

We have previously shown that intestinal barrier function can be adversely affected by poorly digested diets or feed restriction, resulting in increased intestinal inflammation-associated permeability. Three experiments were conducted to evaluate the effect of dexamethasone (DEX) treatment on systemic Fluorescein isothiocyanate-dextran (FITC-D; 3-5kDa) levels, indicative of increased gut epithelial leakage. Exp 1 compared DEX injections of 1 mg/kg, once per day, on d3, 5, and 9 with feed administration at 0.57, 1.7, or 5.1 ppm d4-10, on FITC-D serum concentrations 2.5 h after gavage with 4.16 mg/kg FITC-D. All DEX treatments resulted in marked (2-6X; $P<0.05$) increased serum FITC-D levels. Feed DEX administration resulted in greater ($P<0.05$) gut permeability than injection at any dose, with numerically optimal effects at the lowest dose tested. In Exp 2-3, chicks were randomly assigned to starter ration containing either control (CON) or DEX treated feed (0.57 ppm/kg; d3-10 Exp 2, d4-10 Exp 3). At d10, all chicks were treated by oral gavage with FITC-D and serum samples were obtained as described above. Samples of the liver were aseptically collected, homogenized, diluted 1:4 wt/vol in sterile saline, and serial dilutions were plated on tryptic soy agar to evaluate total numbers of aerobic bacteria in liver as an index of bacterial translocation (BT). In both experiments, FITC-D absorption was significantly enhanced ($P<0.05$) in DEX-treated chicks, again indicating increased paracellular leakage across the gut epithelium associated with dissolution of tight junctions. Exp 2 differential cell counts showed an increased heterophil/lymphocyte ratio, and immune organ (spleen and bursa of Fabricius) weights for Exp 2 and 3 were decreased ($P<0.05$) from controls. In Exp 2 and 3, dietary DEX administration resulted in numerically (Exp 2) or significantly ($P<0.05$) increased enteric BT to the liver, supporting the observation that dietary DEX causes a stress-like inflammatory GI response, which may contribute to subclinical or

clinical disease, and may be a useful model for ongoing disease mitigation research related to stress-related diseases of GIT origin.

Key words Chickens, dexamethasone, stress, bacterial translocation, permeability

C. INTRODUCTION

The intestinal mucosa consists of a single layer of epithelial cells that serves multiple functions including secretion of mucus and enzymes, absorption of nutrients, and barrier function between external and internal environments. Thus, maintenance of epithelial integrity, from both nutrition and disease perspectives, is a major component of rearing healthy feed efficient poultry. Barrier function is influenced by a variety of factors such as enzymes, infectious agents, toxins, and hormones (Söderholm and Perdue, 2001; Lamprecht and Frauwallner, 2012). Continuity of the epithelium is important for controlling both paracellular and transcellular permeability, and is largely controlled by tight junction (TJ) distribution and integrity. Animal models of acute and chronic stress have demonstrated induced changes in intestinal permeability associated with a temporary redistribution of TJ (Maejima et al., 1984; Matter and Balda, 2007). Other enteric changes associated with stress include gut motility, permeability, and alterations to ion, fluid, and mucus secretion (Alverdy and Aoys, 1991; Karavolos et al., 2008).

These changes have been linked to Mast cells which are important effectors of the brain-gut axis and translate stress signals into the release of a wide range of neurotransmitters and pro-inflammatory cytokines, with dramatic effects on gastrointestinal physiology (Groschwitz and Hogan, 2009; Bailey et al., 2011; Lamprecht and Frauwallner, 2012). Studies have shown that dexamethasone can modulate enteric tight junction integrity (Boivin et al., 2007; Tenenbaum et al., 2008), but it is also regularly used as an inducer of opportunistic diseases with translocation of bacteria from the gastrointestinal tract to blood circulation (McGruder et al., 1995; Huff et al., 1999; Wideman and Pevzner, 2012). Studies in poultry directly testing effects dexamethasone on enteric permeability have yet to be reported, though studies using dexamethasone for induction of skeletal diseases with etiologies partially involving enteric bacteria have been published (Huff

et al., 1998; Wideman and Pevzner, 2012). We have previously shown that intestinal barrier function can be adversely affected by poorly digested diets (Tellez et al., 2014) and feed restriction (Kuttappan et al., 2014; Vicuña et al., 2014), resulting in increased intestinal inflammation-associated permeability. The purpose of the present study was to evaluate the alterations in gut permeability under stress induced by administration of dexamethasone in feed in broiler chicks.

D. MATERIAL AND METHODS

Experimental Animals and Diets

Three experiments were conducted to evaluate the effect of stress on intestinal permeability induce by administration of dexamethasone in feed. In all experiments, broiler chickens were obtained from Cobb-Vantress (Siloam Springs, AR, USA). For each experiment, chickens were randomly assigned to control or dexamethasone groups. Chickens were placed in a controlled age-appropriate environment with unrestricted access to feed and water. In each experiment, chickens received an antibiotic free corn/soybean based diet meeting the nutritional requirements of poultry recommended by National Research Council (1994). All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas.

Serum determination of FITC-D leakage

Fluorescein isothiocyanate dextran (FITC-D; MW 3-5 KDa; Sigma Aldrich Co., St. Louis, MO) levels were detected in serum. After collection from chickens, blood was kept at room temperature for 3h, centrifuged (1,000 X *g* for 15 min) to separate serum from red blood cells, and diluted 1:1 in PBS. Levels of FITC-D in serum were measured at excitation wavelength of 485 nm and emission wavelength of 528 nm (Synergy HT, Multi-mode microplate

reader, BioTek Instruments, Inc., Vermont, USA). Fluorescence measured was then compared to a standard curve with known FITC-D concentrations. Gut leakage for each bird was reported as μg of FITC-D/mL of serum.

Bacterial translocation

To measure bacterial translocation (BT) from the intestinal tract to blood circulation, the right half of the liver was aseptically removed from each chicken, collected in sterile bags, homogenized, weighed and 1:4 wt/vol dilutions were made with sterile 0.9% saline. Serial dilutions of each sample were made and plated on tryptic soy agar for determination of total aerobic bacteria translocation levels (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD).

Experiment 1

Day of hatch broiler chicks were randomly assigned to 5 groups (n = 25). The aim of this experiment was to compare dexamethasone injection (DEXINJ) at a known stress-response-inducing dose of 1 mg/kg of BW (positive control) on d3, d5, and d9 to feed administration of dexamethasone at 0.57 ppm (DEXF1X), 1.71 ppm (DEXF3X), or 5.13 ppm (DEXF9X) for d4-10 of the experiment. This dosage was based on calculations that would provide approximately 1 mg/kg of BW daily by oral consumption for DEX1X treatment. A control group without dexamethasone was included as a negative control. Effects on enteric permeability were measured by FITC-D serum concentrations. At d10, chickens in all treatment groups were given an oral gavage dose of FITC-D (4.16 mg/kg), and killed by CO_2 inhalation 2.5h later for blood collection from the femoral vein.

Experiment 2

Day of hatch broilers were weighed and randomly assigned (n = 25) to wire floor brooders with a starter ration. On d3, the DEXF treatment group was changed to feed containing

DEXF1X (0.57 ppm) for the remainder of the experiment. At d10, all chickens were weighed and blood samples were collected after CO_2 asphyxiation from the femoral vein of 12 chickens from each group. Differential cell counts were conducted with a Cell-Dyne 3500 System (Abbott Laboratories, Chicago, IL) that has been standardized for differential counts of poultry blood. Hematologic measurements of heparin anti-coagulated blood included total counts of white blood cells (WBC), heterophils, lymphocytes, monocytes, eosinophils, and basophils. Heterophil/lymphocyte ratios (H/L), an indicator of stress in birds (Gross and Siegel, 1983), were calculated by dividing the number of heterophils in 1 mL of peripheral blood by the number of lymphocytes. The remaining chickens in both groups were orally gavaged with FITC-D and serum samples were obtained 2.5h post-gavage and liver samples collected for BT measurement, as described above. Spleen and bursa of Fabricius were removed and cleaned of adherent tissues. The weight of both organs was measured and expressed as relative to final body weight ((organs weight/final BW) × 100).

Experiment 3

Day of hatch broilers were weighed and randomly assigned (n = 25) to wire floor brooders with a starter ration. On d4, the DEXF treatment group was changed to feed containing DEX1X for the remainder of the experiment. On d10 all chickens were weighed and orally gavaged with FITC-D. Blood samples were obtained 2.5h post-gavage and liver samples collected for BT measurement, as described above. Spleen and bursa of Fabricius were removed and cleaned of adherent tissues. The weight of organs were measured separately and expressed as relative to final body weight ((organs weight/final BW) × 100).

Statistical analysis

Serum FITC-d data showed occasional, but random high values which were not representative of respective group means. Although the reason/s for such high FITC-D values are still not clear, it does not seem to be related to our treatments. Since these values were creating noise in analysis, outliers from treatment groups were identified as above or below two standard deviations from the mean, based on empirical or 68-95-99.7 rule. Identified outliers were truncated according to Ghosh and Vogt (2012) and the number of removed samples reported below. Samples removed due to high FITC-D levels were not included for all other data analysis. All data were subjected to Analysis of Variance (Experiment 1) or One Way Analysis of Variance (Experiments 2 and 3) as a completely randomized design using the General Linear Models procedure of SAS (SAS Institute, 2002). In all trials, data are expressed as mean ± standard error. Significant differences among the means were determined by using Duncan's multiple-range test at $P \leq 0.05$.

E. RESULTS

Three levels of DEXF were tested and compared to DEXINJ or negative control for ability of dexamethasone administered in feed for seven days to affect intestinal mucosal leakage as measured by FITC-D. Each feed inclusion treatment, DEXF1X (0.57 ppm), DEXF3X (1.71 ppm), and DEXF9X (5.13 ppm) resulted in 0.300±0.026, 0.276±0.016, and 0.256±0.010 µg/mL FITC-D in serum, respectively, compared to 0.059±0.011 µg/mL in negative control and 0.126±0.012 µg/mL in DEXINJ (Figure 1). All groups except negative control were truncated by removal of one sample <2SD from the group mean. Each DEXF group was statistically higher than control and DEXINJ, suggesting that indicating increased paracellular leakage across the gut epithelium associated with dissolution of tight junctions, however treatment-associated

clinical signs, such as lethargy and pallor, and mortality were noted in DEXF3X and DEXF9X (data not shown). Similarly, serum FITC-D significantly increased from 0.22±0.01 in control group to 0.29±0.03 in DEXF group for experiment 2 (Table 1) and from 0.33±0.01 in controls to 0.63±0.04 in DEXF for experiment 3 (Table 3). One sample from each group was removed for truncation (<2SD) in experiment 2 and no samples were truncated for experiment 3.

Detectable total liver aerobic bacteria was significantly ($P<0.05$) higher at 0.00±0.00 vs. 1.26±0.45 Log_{10} in experiment 2 (Table 1), and 0.00±0.00 vs. 2.58±0.35 Log_{10} (Table 3) supporting the observation that dietary DEX causes a stress-like inflammatory GI response. Final BW and BWG were significantly decreased in DEXF groups in experiments 2 and 3 (Table 1, Table 3). Similarly, relative spleen and bursa of Fabricius weight ratios in DEXF treated chickens were significantly decreased in experiments 2 and 3 when compared to non-treated controls (Tables 1 and 3).

Results of the evaluation of DEXF (0.57 ppm) on total leukocyte counts (WBC), and percentages of heterophils, lymphocytes, monocytes, eosinophils, and basophils of peripheral blood of 10-day-old broilers from experiment 2 are summarized in Table 4. Significant increases were observed in the percentages of heterophils, eosinophils and H/L ratio, as well as a significant decrease difference in the percentage of lymphocytes in the DEX treated chickens when compared with control chickens (Table 4). Increased H/L from 0.55±0.21 in non-treated chickens to 1.34±0.39 in DEXF chickens was consistent with previous reports describing effects of dexamethasone on leukocyte counts. However, an increase in eosinophils from 0.08±0.04 in control birds to 0.31±0.1 was unexpected, but may be due to lack of differentiation of granulocytes by the cell sorter (personal communication, Sonia Tsai).

F. DISCUSSION

Blood corticosteroid concentration is a known indicator of stress in chickens, and the glucocorticoid dexamethasone is well-studied as an inducer of cell-mediated immunosuppression, especially related to decreased disease resistance (Rose, 1970; Gross and Siegel, 1983; Huff et al., 1998). Furthermore, dexamethasone has been used as a mediator of opportunistic diseases in poultry including colibacillosis, turkey osteomyelitis, and bacterial chondronecrosis with osteomyelitis (Bayyari et al., 1997; Huff et al., 1998; Wideman and Pevzner, 2012). Manifestation of these conditions may be related to decreased macrophage bactericidal activity (Schaffner, 1985; Schaffner and Schaffner, 1988), and all require translocation of bacteria from the gastrointestinal tract into blood circulation, where they eventually manifest. Stress is also a well-known immunosuppressing event that can lead to opportunistic infections in both humans and animal models, which may be related to depression of pituitary adrenocortical function (Westerhof et al., 1996). The pathophysiology of stress-induced intestinal disturbances is known to be mediated by corticotrophin releasing factor, which increases intestinal paracellular permeability via mast cell dependent release of TNF-α and proteases (Taché and Perdue, 2004; Teitelbaum et al., 2008). In these permeability experiments, the progressive detection of FITC-D in serum (Figure 1, Tables 1 and 3) is consistent with leakage from the lumen and suggests the presence of a change in paracellular permeability.

Alterations in enteric mucosal permeability are connected with bacterial translocation in the portal and/or systemic circulation in several types of intestinal permeability syndromes leading to systemic bacterial infections (Seki and Schnabl, 2012; Ilan, 2012). Fluoroscein isothiocyanate dextran is a molecule (3-5 kDa) which is not normally absorbed by an intact gastrointestinal tract barrier. However, when conditions disrupt tight junctions between epithelial

cells, FITC-D molecules can enter circulation, as demonstrated by an increase in trans-mucosal permeability associated with DEX treated chickens and elevated serum levels of FITC-D after oral administration (Yan et al., 2009; Tellez et al., 2014). The fact that elevated permeability was significantly higher in DEX treated chickens in all experiments suggest that this stress practice has a strong impact on the epithelial barrier, altering gut permeability in broiler chickens (Figure 1, Tables 1 and 3).

In the present study, a significant increase in total detectable aerobic bacteria in the liver was observed in DEXF treated chickens when compared with control chickens, which could be related to either decreased bactericidal activity of macrophages, or increased epithelial permeability (Tables 1 and 3). Furthermore, stress-induced bursal atrophy has been suggested to be caused by an increased corticosteroid production (Huff et al., 1999). In the present study, BW, BWG and the relative lymphoid organ (bursa of Fabricius and spleen combined) were significantly reduced in DEXF treated chickens (Tables 1 and 3), and these results are in agreement with previous investigations (Gross and Siegel, 1983; Huff et al., 1999; Shini et al., 2009; Wideman and Pevzner, 2012).

Increased H/L is considered an indicator of chronic stress in birds (Gross and Siegel, 1983), which was consistent with decreased levels of lymphocytes in chicken blood samples and increased heterophils in response to DEXF treatment. This suggests that administration of dexamethasone in feed may be an acceptable manner for delivery of glucocorticoids to chickens to mimic stress and provide a means by which to study the effects of stress conditions on enteric inflammation parameters such as mucosal integrity. Furthermore, this model has potential to serve as a method of induction of diseases for which bacterial translocation is a component of

pathogenesis, such as colibacillosis, spondylolisthesis, or bacterial chondronecrosis with osteomyelitis.

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Table 1 Evaluation of administration of dexamethasone in feed (DEXF) on serum FITC-D, bacterial translocation, body weight and lymphoid organ weight in birds from Experiment 2. Treated chickens received control feed supplemented with 0.57 ppm of dexamethasone d4-10. On d10 chickens in both groups were gavaged with FITC-d (4.16 mg/kg) and blood samples were collected 2.5h later. Lymphoid organ ratio includes bursa and spleen and is expressed as a percentage of body weight. All samples were collected on d10. Data are expressed as mean ± standard error. [a–b] Means within a column with different superscripts differ (P< 0.05).

	Serum FITC-D (µg/mL)	**Total Liver Aerobic Bacteria (Log_{10} cfu/g)**	**Final BW (g)**	**BWG (g)**	**Lymphoid Organ Ratio (%)**
Control	0.22 ± 0.01^{b}	0.00 ± 0.00^{b}	171.44 ± 2.87^{a}	103.28 ± 2.82^{a}	0.24 ± 0.01^{a}
DEXF	0.29 ± 0.03^{a}	1.26 ± 0.45^{a}	152.07 ± 2.09^{b}	84.37 ± 1.57^{b}	0.16 ± 0.01^{b}

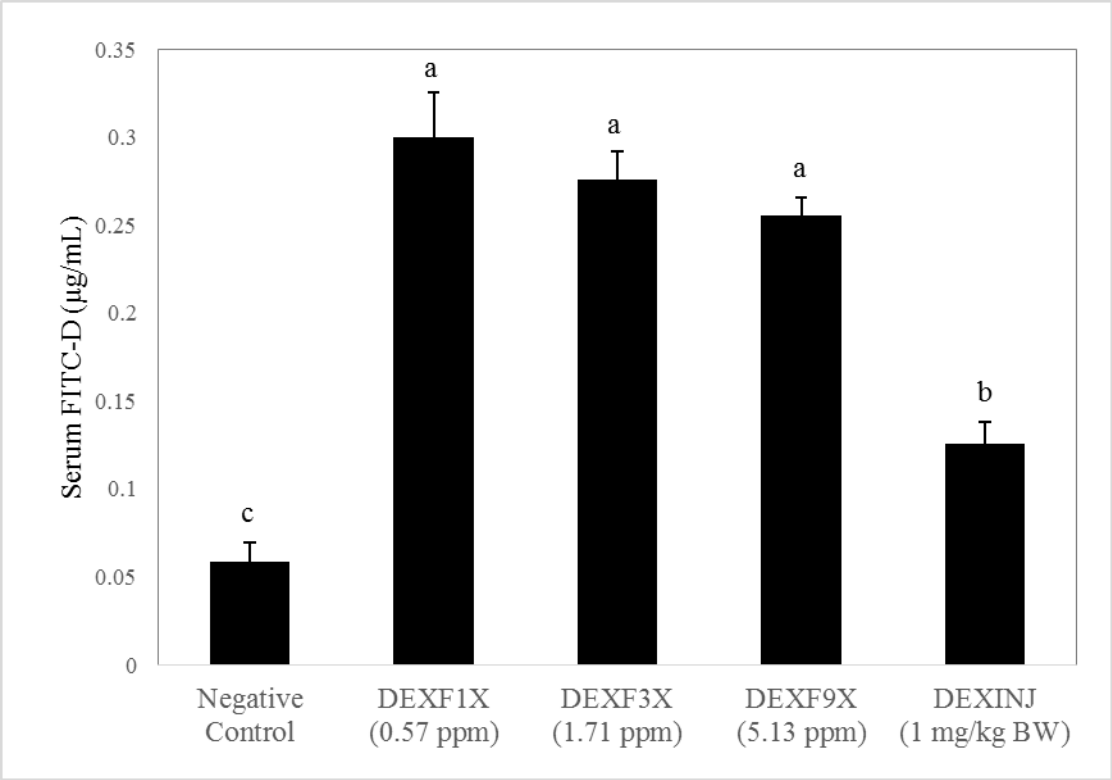
Table 2 Evaluation of administration of dexamethasone in feed on total leukocyte counts (WBC), and percentages of heterophils, lymphocytes, monocytes, eosinophils, and basophils of peripheral blood, Experiment 2. Treated chickens received control feed supplemented with 0.57ppm d4-10. Data are expressed as mean ± standard error. [a–b] Means within a row with different superscripts differ ($P<0.05$).

	Control	**Dexamethasone**
WBC ($\times 10^3/\mu L$)	17.20 ± 4.85^a	21.62 ± 2.51^a
Heterophils (%)	24.02 ± 7.08^b	41.6 ± 6.23^a
Lymphocytes (%)	59.58 ± 8.26^a	34.21 ± 8.06^b
Monocytes (%)	12.51 ± 2.17^a	9.82 ± 1.63^a
Eosinophils (%)	0.08 ± 0.04^b	0.31 ± 0.1^a
Basophils (%)	2.84 ± 0.35^a	3.02 ± 0.20^a
Heterophil:Lymphocyte	0.55 ± 0.21^b	1.34 ± 0.39^a

Table 3 Evaluation of administration of dexamethasone in feed (DEXF) on serum FITC-D, bacterial translocation, body weight and lymphoid organ weight in birds from Experiment 2. Treated chickens received control feed supplemented with 0.57 ppm of dexamethasone d4-10. On d10 chickens in both groups were gavaged with FITC-d (4.16 mg/kg) and blood samples were collected 2.5h later. Bursa and spleen weight rations are expressed as a percentage of body weight. All samples were collected on d10. Data are expressed as mean ± standard error. [a–b] Means within a column with different superscripts differ (P< 0.05).

	Serum FITC-D (µg/mL)	**Total Liver Aerobic Bacteria (Log_{10} cfu/g)**	**Final BW (g)**	**Bursa Weight Ratio (%)**	**Spleen Weight Ratio (%)**
Control	0.33 ± 0.01^{b}	0.00 ± 0.0^{b}	225.40 ± 4.75^{a}	0.15 ± 0.006^{a}	0.11 ± 0.007^{a}
DEXF	0.63 ± 0.04^{a}	2.58 ± 0.35^{a}	146.71 ± 2.42^{b}	0.07 ± 0.005^{b}	0.07 ± 0.006^{b}

Figure 1 Levels of serum FITC-D after treatment with Dexamethasone in feed (DEXF) at three different levels or injection (DEXINJ). Treated feed was provided d4-d10 and injections were administered every other day from d4-d10. FITC-D (4.17 mg/kg) was administered by oral gavage 2.5h before blood sample collection. Truncation of data included removal of one sample (<2SD from mean) from each DEXF and DEXINJ group. Treatments with different superscripts indicate statistical difference (P<0.05).



V. CONCLUSIONS

Barrier function is a critical aspect of gut heath. On a daily basis, the intestine is exposed to a nearly unlimited number of antigens and potential insults, including dietary components, toxins, and commensal and pathogenic microorganisms. The GIT serves as a selective barrier to take up nutrients and fluids into the body, while excluding undesirable molecules and pathogens. Hence, proper gut barrier function is essential to maintain optimal health and balance throughout the body and represents the first line of defense against these foreign antigens from the environment. Oxidative stress, poorly digestible protein or energy sources and coccidiosis are some examples that can cause gut barrier failure. However, as a consequence of the removal of antimicrobial growth promoters, new multifactorial diseases causing enteritis and gut disorders of unknown origin have emerged in broilers, causing negative impacts in health and performance. Additionally, commercial poultry are commonly exposed to a plethora of stressors that can induce a variety of changes in normal gastrointestinal function, including changes in gut motility and permeability, as well as alterations in ion, fluid, and mucus secretion and absorption. These changes have been linked to Mast cells which are important effectors of the brain-gut axis and which translate the stress signals into the release of a wide range of neurotransmitters and pro-inflammatory cytokines, with dramatic effects on gastrointestinal physiology. In this thesis, we evaluated several intestinal inflammation models to induce gut leakage by different methods in broiler chickens using FITC-d as a marker. In chapter one, we evaluate the dose titration of FITC-d for optimal measurement of enteric inflammation in broiler chicks using the following models: a) 24h FR; b) DSS; or c) RBD. All three models evaluated, FR, DSS, and RBD increased significantly ($P < 0.05$) serum concentrations of FITC-d, suggesting their value for enteric inflammation models, and that FITC-d absorption from the GIT

into the circulation may be a good indicator of permeability. In chapter two, a series of experiments were conducted to evaluate the effect of dexamethasone (DEX) treatment in feed on systemic (serum) FITC-d levels, total numbers of aerobic bacteria in liver as an index of bacterial translocation (BT), differential white blood cell counts, and immune organs in broiler chickens. DEX-treated chickens showed a significant increase in FITC-d serum levels and BT, again indicating that stress increased paracellular leakage across the gut epithelium associated with dissolution of tight junctions. Additionally, heterophil/lymphocyte ratio was significantly increase and relative spleen and bursa of Fabricius weight ratios were significantly decreased in DEX-treated chicks. The results of these studies provide the baseline of an enteric inflammation model in broiler chickens to further evaluate anti-inflammatory properties of nutraceuticals as alternatives to the use of AGP.

APPENDIX

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Dr. Lisa Bielke

FROM: Craig N. Coon, Chairman
Institutional Animal Care and Use Committee (IACUC)

DATE: September 8, 2014

SUBJECT: IACUC APPROVAL
Expiration date: September 14, 2017

The Institutional Animal Care and Use Committee (IACUC) has APPROVED protocol 15006: *Development of enteric inflammation models for investigation of antibiotic alternatives in poultry*

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond September 14, 2017 you must submit a new protocol prior to that date. By policy the IACUC cannot approve a study for more than 3 years at a time.

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

CNC/aem

cc: Animal Welfare Veterinarian

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