The Effects of Feeding Reduced-oil DDGS to Broilers when Challenged with *C. perfringens* and *Eimeria* spp. on Necrotic Enteritis and Intestinal Microbiome.

by

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Abstract

Clostridium perfringens-associated necrotic enteritis (NE) costs the international poultry industry an estimated $6 billion annually. Traditionally, the incidence of NE has been controlled by the use of antibiotic growth promoters (AGPs). However, increased prevalence of NE has been observed alongside the removal of AGPs due to consumer demands. Modification of diets is an alternative method for control, as dietary influences can disrupt the intestinal microbiota composition. Recent implementation of oil-extraction technology by ethanol plants has resulted in reduced-oil DDGS (R-DDGS) sources with varying nutrient content entering poultry diets. Appropriate use of these R-DDGS sources requires information of nutrient utilization and their influence on broiler intestinal microbiome. For these experiments, the objective was to determine the effects of three different R-DDGS sources on broiler performance, intestinal microbiome, and severity and incidence of intestinal lesions after a NE challenge. The four treatments consisted of control (corn-soy) and R-DDGS diets fed across broilers either challenged or unchallenged with *Eimeria* spp. (d 18) and *C. perfringens* (d 21 to 23), in a 2 x 2 factorial arrangement of treatments. Treatments were applied to 9 replicate cages of 10 chicks each. Body weight and feed intake were measured over the 28-day feeding period, and body weight gain (BWG) and mortality corrected feed conversion ratio (AFCR) were calculated. Birds were raised in Petersime batteries cages up to 28 d, then euthanized, lesion scored, and jejunum samples collected for analysis by 16S rRNA gene sequencing. A two-way ANOVA was used to determine diet and challenge main effects and interactions ($P \leq 0.05$). A Man-Whitney U Test was used to determine pairwise comparisons of treatment microbiome alpha-diversity indices ($P \leq 0.05$). Birds consuming the low-oil DDGS (L-DDGS; 4.29% crude fat) diet experienced reduced performance at 14 to 27 d of age (BWG, AFCR) and 0 to 27 d of age (AFCR) ($P \leq 0.05$), but challenge had no effect and there was no interaction. When unchallenged, the L-DDGS diet led to a lower Species Richness and Shannon Index value than the control diet ($P \leq 0.05$). The medium-oil DDGS (M-DDGS; 6.6% crude fat) diet resulted in greater NE incidence and average lesion score, in which challenge also had an effect ($P \leq 0.05$). An interaction effect

of diet and challenge was observed on NE severity and NE incidence ($P \leq 0.05$). In the third experiment, the M-DDGS diet (7.07% crude fat) led to reduced BWG at 0 to 28 d of age ($P \leq 0.05$), but challenge had no effect and there was no interaction. Greater NE incidence and average lesion score was observed in those birds consuming the M-DDGS diet ($P \leq 0.05$), with no interaction. When feeding R-DDGS products, amino acid content/quality must be accounted for, as it can result in increased NE incidence and reduced live performance. Future research on the effects of lactate- and butyrate-producing bacteria on NE development could be beneficial, as the industry continues to reduce the use of AGPs.

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List of Abbreviations

NE	Necrotic enteritis
AGPs	Antibiotic growth promoters
DDGS	Distillers dried grains with solubles
R-DDGS	Reduced-oil distillers dried grains with solubles
L-DDGS	Low-oil distillers dried grains with solubles
M-DDGS	Medium-oil distillers dried grains with solubles
FI	Feed intake
BW	Body weight
BWG	Body weight gain
AFCR	Mortality corrected feed conversion ratio
GIT	Gastrointestinal tract
EE	Ether extract
TSA	Tryptic soy agar
AF	Aflatoxin
DON	Deoxynivalenol
FUM	Fumonisin
OTA	Ochtratoxin A
ZEA	Zearalenone
CP	Crude protein
ADF	Acid detergent fiber
NDF	Neutral detergent fiber
CF	Crude fiber
TDN	Total Digestible Nutrients
NE	Net energy
ME	Metabolizable energy
DE	Digestible energy

PCR	Polymerase chain reaction
OTU	Operational taxonomic unit
BIOM	Biological observational matrix
H’	Shannon Index
GLM	General linearized model
ANOVA	Analysis of variance
d	Day
spp.	Species
CFU	Colony forming units
bp	Base pairs
TAS	Targeted amplicon sequencing
ME	Metabolizable energy
U.S.	United States
E.U.	European Union
FDA	Food and Drug Administration
et al	*et alib*

Chapter 1.0 Introduction

The development of *Clostridium perfringens*-associated necrotic enteritis (NE) occurs almost exclusively in broilers between two to six weeks of age (Cooper and Songer, 2010). Under current control measures, NE has been estimated to cost the international poultry industry up to $6 billion (US) annually (Wade and Keyburn, 2015). However, increased prevalence of NE has been observed alongside the removal of antibiotic growth promoters (AGPs; McDevitt *et al.*, 2006) due to consumer demands. Consequently, this has elicited interest in understanding the pathogenicity of this enteric disease, in order to seek alternative methods for control.

Although *C. perfringens* has been established as the main causative agent, a simple infection is not sufficient to onset the disease. Additional predisposing factors are necessary to facilitate the proliferation of *C. perfringens* (Williams, 2005). It has been proven that dietary factors, among others, strongly influences NE in broilers (Annett *et al.*, 2002). Therefore, a refined nutritional formula that makes the broiler gut environment less susceptible is critical for maintaining broiler optimal health and live performance (Croom *et al.*, 2000; Dahiya, 2006). However, feed continues to account for over 70% of the total cost of poultry production (Yegani and Korver, 2008). With feed prices continuing to rise, the incorporation of co-products has become of interest to the broiler industry (Yegani and Korver, 2008). One of the major co-products is distillers dried grain with solubles (DDGS) (Saunders *et al*., 2009).

The inclusion of DDGS in poultry diets has become common over the past 5 to 10 years, due to its competitive price. However, over 80% of dry-grind ethanol plants have recently implemented oil-extraction technologies to generate increased revenue. This has resulted in reduced-oil DDGS (R-DDGS) sources becoming more available to the poultry industry. Traditional DDGS contains approximately 12.55% oil content (Liu, 2010). However, R-DDGS sources can vary in oil content from 4 to 12% (CEPA, 2012). This variation in oil content, results in additional changes to protein and fiber content. Therefore, appropriate use of R-DDGS sources in broiler diets requires reassessment of nutrient utilization and their influence on broiler intestinal microbiome. Previously, our lab had demonstrated that increasing concentrations of traditional DDGS in broiler diets (7.5% vs. 15%) could potentially increase NE incidence and

severity (Macklin *et al.*, 2011). Therefore, the objective of these experiments was to determine the effects of feeding R-DDGS sources on broiler performance, intestinal microbiome, and incidence and severity of lesions after a NE challenge.

Chapter 2.0 Review of Literature

2.1 Poultry Gut Health

The poultry industry has undergone remarkable changes and growth over the last 30 years. As growth rate and FCR have improved, the bird's nutrition and health care have become more demanding (Choct *et al.*, 1999). The nutritional and health status of poultry are interlinked with what is referred to as gut health (Kelly and Conway, 2001; Yegani and Krover, 2008). Gut health is highly complex, in which it encompasses: the macro- and micro- integrity of the gut, the balance of the gut microflora and the status of the immune system (Choct, 2009). The gut harbors more than 640 different species of bacteria, contains over 20 different hormones, digests and absorbs the vast majority of nutrients, and accounts for 20% of body energy expenditure (Kraehenbuhl and Neutra, 1992). Thus, anything that effects the gut will undoubtedly influence the animal as a whole. Interest from the scientific community has been growing towards the identification of new alternatives to in-feed antibiotics, as maintenance or enhancement of gut health is essential for the welfare and productivity of food animals (Choct, 2009).

2.1.1 The Gastrointestinal Tract

As the largest immunological organ in the body, the gastrointestinal tract (GIT) is continuously exposed to a wide variety of potentially harmful substances (Mathew, 2001). Not only is the gut the major organ for nutrient digestion and absorption, it also works as the first protective mechanism to exogenous pathogens (Mathew, 2001). McCracken and Lorenz (2001) describe the GIT ecosystem as "a precarious alliance that embraces a sound epithelium, immunological preparedness, and a favorable balance of transient microbes". The GIT system includes the digestive system itself, the organs that provide digestive secretions (liver, pancreas and gallbladder) and associated digesta, microflora, and the immune system (Dibner and Richards, 2004).

2.1.1.1 The Digestive System

The anatomy of the chicken GIT is unique. The feed is ingested whole, where it enters the esophagus and travels to the crop. The crop serves as temporary storage, along with some bacterial fermentation, resulting in the formation of lactic acid (Turk, 1982). The mixture then progresses through the proventriculus-gizzard to macerate and improve aqueous compatibility (Moran, 2016). The proventriculus, commonly referred to as the "glandular stomach", secretes gastric juices including pepsin and hydrochloric acid. In the gizzard, larger food particles are ground to small particles capable of being taken into the intestinal tract for further digestion and absorption (North and Bell, 1990). The small intestine in poultry is relatively simple and short, but highly efficient. It focuses on digestion-nutrient absorption, and is separated into three parts: duodenum, proximal small intestine (jejunum), and distal small intestine (ileum) (Dibner and Richards, 2004). The duodenum is the principle site of nutrient absorption, where chemical action by pancreatic enzymes assists digestion. Additional assistance comes from bile, intestinal secretions and various microbes harboring the gut lumen (Duke, 1986). The jejunum and ileum are differentiated by their location in reference to Meckel's diverticulum (Andrew and Hickman, 1974). The ileocecal junction, found at the base of the ileum and the top of the large intestine, is where twin cecal pouches join the linear portion of the intestine. It is location of the largest element of the gut immune tissue—the cecal tonsils (Dibner and Richards, 2004). The ceca are thin-walled pouches that contain the anaerobic microflora responsible for fermentation within the bird (Dibner and Richards, 2004). The large intestine is relatively short, approximately 4 inches in length, and manages waste and water re-absorption (Moran, 2016; North and Bell, 1990). At the end of the digestive tract, there is a cloaca, which serves as a common pathway for removal of excretory, reproductive, and digestive wastes (Denbow, 2000).

2.1.1.2 Accessory Organs

Certain organs, including the pancreas, liver and gall bladder are directly associated with digestion, as their secretions empty into the intestinal tract (North and Bell, 1990). The pancreas is a long, narrow gland that lies between the duodenal loop, providing endocrine and exocrine secretions. The endocrine secretions include both insulin and glucagon; furthermore, regulating cellular uptake of glucose and amino acids after ingestion. The exocrine secretions consist of digestive enzymes and electrolytes (Dibner and Richards, 2004). The liver provides exocrine

secretions to the digestive tract, particularly bile, which functions to emulsify fats and raise the pH of the duodenal digesta. Bile in the gall bladder undergoes concentration, by the reabsorption of water and inorganic salts (Dibner and Richards, 2004).

2.1.1.3 Microflora

The intestinal track of poultry consists of a complex and dynamic microbiome, consisting primarily of bacteria (Zhu *et al.*, 2002). The diversity and composition of the poultry intestinal microbiome were first investigated using cultivation-based methodologies (Barnes *et al.*, 1972; Salanitro *et al.*,1974), as 16S rRNA gene-target analyses were not applied until the 2000s (Zhu *et al.*, 2002). The diversity of bacterial species in the gut is one of the most important factors for the establishment of a stable ecosystem in the intestinal tract. This is suggested by the observation that, until the bacterial populations are fully established, young animals have fewer bacterial species in the intestinal tract than adult birds, making them more susceptible to disturbances than of adult animals (Mead, 1989). The gut flora is also believed to both protect against the intestinal colonization of pathogens and to stimulate the immune response (Mead, 1989). The microflora of the intestinal tract of broiler chickens influences digestion, gut morphology, immune responses and health; therefore, a range of factors, such as diet, feed additives, host, and litter management, can affect it (Pan and Yu, 2014). There is significant diversity in bacterial populations among different parts of the GIT, where each region develops its own unique microbial profile. The population densities tend to increase from the proximal to distal GIT (Pan and Yu, 2014).

Bacterial species differ due to their substrate preferences and growth requirements; therefore, the chemical composition and structure of the digesta largely determines the microbiome species distribution (Hume *et al.,* 2013). The crop has characteristic microflora that are largely dominated by *Lactobacillus*, establishing only after a few days of hatching (Rubio *et al.*, 1998). The proventriculus and the gizzard bacterial populations are lower than in other parts of the GIT, where *Lactobaccilli* also dominate (Oakley *et al.*, 2014). This is most likely due to the low pH, requiring any surviving microorganisms to show a high degree of acid tolerance (Gabriel *et al.*, 2006). The small intestine harbors large bacterial populations dominated by *Lactobacillus*, *Enterococcus*, and various *Clostridiaceae* (Van der Wielen *et al.*, 2002; Rehman

et al., 2007). The majority of the organisms isolated from the duodenum and ileum are Gram-positive (Salanitro *et al.,* 1974). Anaerobes compromise 39% of the total number of isolates obtained with the greatest range occurring in the duodenum, where obligate anaerobes included species of cocci and *Clostridium* (Salanitro *et al.*, 1974). The ceca are evacuated only 1-2 times per day, providing a relatively stable condition for microbial proliferation (Gabriel *et al.,* 2006). At least 38 different types of anaerobic bacteria of different strains have been isolated from chicken ceca (Barnes *et al.*, 1972). The composition of cecal microflora can vary between flocks, between birds within a single flock, and even within a single bird examined at different times (Salanitro *et al.*, 1974).

2.1.1.4 Immune System

The bird is protected against invasion by opportunistic pathogens located within the gut microflora tight junctions, a layer of mucin, and gut-associated immune tissue, of the gut epithelium (Dibners and Richards, 2004). Intestinal mucus serves as the frontline of defense, in which mucins, the main structural component of the intestinal mucus layer, are secreted by goblet cells. Mucins are large glycoproteins, which assemble into a protective gel-like layer of the epithelial surface (Kim and Khan, 2013). It is well established that a beneficial microbial community has an important role in maintaining normal physiological homeostasis, the host immune system, and influencing organ development and host metabolism (Sommer and Backhed, 2013). The bursa of fabricius, found on the dorsal surface of the large intestine, is the primary immune organ for B-lymphocyte proliferation (Leslie, 1975). The gut itself is also heavily populated with B- and T-lymphocytes, found in the cecal tonsils, Meckel's diverticulum, and the epithelial lining of the intestinal lumen (Jeurissen *et al.*, 1989). The gut microbiota also controls B-cell response and IgA production. IgA secreted into the lumen plays an important role in pathogen binding and removal (Macpherson and Harris, 2004). Gut immune homeostasis is maintained by a complex network of cells and their secreted soluble products (Kamada *et al.,* 2013).

2.2 *Clostridium perfringens*

Clostridium perfringens, previous referred to as *Clostridium welchii*, is the etiological agent of necrotic enteritis in poultry (Timbermount *et al*., 2009). As the causative agent of a prominent enteric disease in broiler flocks, it leads to a significant financial loss for poultry producers (Buzby and Roberts, 1997). *C. perfringens* strains have been shown to be susceptible to various anti-microbial drugs both in *in vitro* and *in vivo* studies (Devriese *et al*., 1993; Geier *et al*., 2010). However, recent omission of their commercial use in poultry production, due to either legal requirement or production of market-favorable products, has led to a resurgence of this bacterium (McDevitt *et al*., 2006; Van Immerseel *et al*., 2009). Consequently, replacements for anti-microbial drugs that can reduce the prevalence of *C. perfringens* in poultry are warranted.

2.2.1 Epidemiology in Poultry Operations

C. perfringens is a ubiquitous bacterium found in poultry houses and its surroundings, including water, poultry feces, feed, soil and air (Craven, 2001). It is suggested that colonization of poultry occurs early in life, as *C. perfringens* contamination found on processed broiler carcasses can originate in the breeder operation, and be transmitted through the hatchery and grow-out operations (Craven *et al*., 2003). In environmental samples from poultry farms, the highest incidences have been detected in wall swabs (53%), fan swabs (46%), fly strips (43%), dirt outside the entrance (43%), and swabs of boots (29%) (Craven *et al*., 2001). *C. perfringens* lack the ability to produce 13 of the 20 essential amino acids. Therefore, it is associated with protein-rich foods, where 75% of the foodborne outbreaks can be traced to raw and processed foods, particularly meat and poultry (Johnson and Gerding, 1997). When the intestinal contents of broiler chickens were analyzed for the presence of *C. perfringens*, approximately 75% to 95% of the animals were found positive (Miwa *et al*., 1998; Craven *et al*., 2001). The *C. perfringens* population is found to normally be less than 10^2 to 10^4 colony-forming units (CFU) per g of the intestinal contents in the small intestine of healthy chickens compared to 10^7 to 10^9 CFU/g in diseased birds (Kondo F, 1988). It is believed that a small number of *C. perfringens* enterotoxin (CPE) positive *C. perfringens* bacteria co-exist with a large number of non-enterotoxigenic *C. perfringens* bacteria in chicken (Miwa *et al*., 1996). When poultry meat was analyzed, up to 84% of positive meat samples were reported, of which 12% tested positive for the CPE strains (Bean *et al*., 1996; Miwa *et al*., 1998; Craven *et al*., 2001).

2.2.2 Growth Characteristics

C. perfringens is a Gram-positive, spore forming, rod-shaped anaerobic bacterium that is encapsulated and non-motile (Canard *et al*., 1992). On the surface of nutrient agar plates, colonies appear large, round, slightly opaque, and shiny (Brynestad and Granum, 2002). Colonies usually demonstrate double-zone hemolysis on nutrient blood agar plates, with a clear inner theta-toxin zone and a hazy outer zone caused by alpha-toxin production. The maximum and minimum growth temperatures for *C. perfringens* are dependent on strain, pH, growth medium, and presence or absence of other microorganisms. *C. perfringens* can grow in and on a wide variety of culture media including Shahidi Ferguson perfringens agar (SFPA), tryptose sulphite cycloserine (TSC) agar, nutrient blood agar, nutrient agar, blood heart infusion broth (BHI) and cooked-meat medium (CMM). Typical growth occurs between 15 and 50°C, with an optimum of 45°C for most strains. *C. perfringens* grow at pH values ranging from 5 to 9, with an optimum between 6 and 7 (Labbe and Juneja, 2006). Under proper conditions, they display the shortest generation time (G_t) of any bacterial pathogen. The G_t for most strains at temperatures between 33 and 49°C is below 20 minutes; however, G_t of 8 minutes has been reported (Labbe, 2000). The adverse implications for food safety of an organism with a G_t of 8 minutes are noteworthy when one considers that a >100,000-fold increase in population can occur within 2 hours (Labbe and Juneja, 2006). Although this organism is an anaerobe, it will usually grow at an oxidation-reduction potential (E_h) below +350 mV, while the final levels can reach below -400 mV (Labbe, 2000). *C. perfringens* is especially intolerant to low water activity (a_w), where different strains will stop growing between 0.95 and 0.97 (Labbe, 2000).

The ability to sporulate is an important property of *C. perfringens*. It factors in its classification, allowing for the survival of conditions that would destroy non-spore forming bacteria and the resumption of vegetative cell growth, during which high levels of enterotoxin can be formed and released (Labbe and Juneja, 2006). The mature spore (usually located sub-terminally) demonstrates characteristic structures including the core cortex, spore coat layers and sub-coat region (Gombas and Labbe, 1985). The bacterial sporulation process is divided into stages I-VII, with stage VII corresponding to the release of the mature spore. The sporulation process is highly strain dependent, as well as media dependent under laboratory conditions. Low

levels of spores are usually produced in CMM; however, the levels of sporulation are insufficient for high CPE yields (Labbe and Juneja, 2006). The medium of Duncan and Strong (1968), or minor modifications of it, has been most widely utilized. Optimal conditions are often determined, as a specific protocol and medium may not apply for all strains. In general, it is recommended to use 37°C, as certain strains possess decreased amylase activity above 40°C (Garcia-Alvarado *et al*., 1992).

2.2.3 Virulence Factors

C. perfringens can produce over 13 different toxins although each bacterium only produces a subset of these (Petit *et al*., 1999). *C. perfringens* strains are classified into five main toxinotypes (A, B, C, D and E), based on the production of four major toxins (alpha, beta, epsilon and iota) (Songer, 1996; Petit *et al*., 1999). In principle, all toxinotypes produce alpha-toxin, a phospholipase C sphingomyelinase that hydrolyzes phospholipids and promotes membrane disorganization (Titball *et al*., 2000). Necrotic enteritis is caused by *C. perfringens* Type A, and to a lesser extent, Type C. *C. perfringens* Type A strains produce the chromosomal-encoded alpha-toxin, while *C. perfringens* Type C produce alpha-toxin together with beta-toxin (Petit *et al*., 1999). Toxinotypes B, D and E do not contribute to poultry disease (Petit *et al*., 1999).

2.2.4 Pathogenesis in Necrotic Enteritis

In healthy birds, several different types of *C. perfringens* exist, whereas not all isolates of *C. perfringens* from diseased birds are pathogenic (Van Immerseel *et al*., 2009). This indicates that different strains of *C. perfringens* may vary in their virulence, where only specific strains have the ability to produce NE, with extensive proliferation of these specific types (Cooper and Songer, 2009). The A toxinotype of *Clostridium perfringens* appears to be the most likely culprit for broilers, along with its alpha-toxin, NetB toxin (or both) (Keyburn *et al*. 2008). NetB appears to be continuously released during the cell's vegetative phase, followed intermittently by the alpha-toxin after sporulation (Moran, 2014).

2.2.4.1 Alpha-toxin

For more than 20 years, alpha-toxin has been proposed to be the main virulence factor for NE in poultry (Van Immerseel *et al*., 2008). However, it's significance in the pathogenesis of NE

remains questionable (M'Sadeq, 2015). The alpha-toxin is the first known bacterial protein that has both enzymatic activity and toxic properties (Titball, 1999). This bi-functional phospholipase C and sphingomyelinase enzyme consists of two separate domains: the *alpha*-helical N-terminal domain and the *beta*-sandwich C-terminal domain. The N-terminal domain contains the active site, with catalytic activity. The C-terminal domain involves the binding of the enzyme to the cell membrane, through which it interacts with the phospholipid content of the cell membrane, resulting in toxicity (Titball, 1999). Alpha-toxin's hemolytic, cytotoxic, and necrotic properties directly influence the host metabolism. However, the toxin does not have hemolytic activity in the absence of the binding domain (Titball, 1999). Alpha-toxin is believed to play a vital role in the severity of NE primarily due to its ability to impair the intestinal mucosal barrier (Truscott and Alsheikhly, 1977). Alpha-toxin promotes membrane disorganization, damage, and inflammation through the hydrolysis of lecithin. This consequently results in the formation of diacylglycerol—stimulating the activation of protein kinase C and the subsequent arachidonic acid cascade (Titball, 1999).

The positive findings that indicate alpha-toxin's role in pathogenesis of NE, are that the crude supernatant from *C. perfringens* type A cultures induced NE lesions in broilers and that antibodies to *C. perfringens* alpha-toxin prevented the development of lesions. In one *in vitro* study, a significantly higher amount of alpha-toxin was noted in isolates from birds with NE that isolates from healthy birds (Hofshagen and Stenwig, 1992). Recently, more convincing evidence for the possible involvement of alpha-toxin has come from immunization with a purified alpha-toxin that induced protection against experimental induced NE. Against these findings, other recent studies have indicated that alpha-toxin plays no direct part in the pathogenesis of NE (M'Sadeq *et al*., 2015). Histological analysis of tissue damage occurring in the early stages of lesion development is not consistent with the phospholipase C or sphingomyelinase activities of alpha-toxin. However, the most convincing evidence comes from studies using an alpha-toxin negative mutant of *C. perfringens*, associated with an NE outbreak. In virulence trials, both the alpha-toxin negative mutant and the wild-type strain had an impaired ability to cause NE lesions. However, these mutants were not complemented with a functional alpha-toxin gene, so it was not clear that the reduced virulence was due to the impairment of alpha-toxin production (Keyburn *et*

al., 2006). With this contradicting evidence, there was a need to re-evaluate previous work and the opportunity to search for other factors important in pathogenesis.

2.2.4.2 NetB Toxin

Necrotic enteritis toxin B-like (NetB) was discovered by Keyburn *et al*. (2008) as a virulence factor for the development of NE. The toxin was identified from *C. perfringens* type A strain (EHE-NE18) isolated from an infected chicken. It was screened for proteins from the supernatant of *C. perfringens* cultures that were cytotoxic for chicken hepatocellular carcinoma cells (LMH) *in vitro*. NetB is a pore-forming toxin that belongs to the B-pore-forming toxin family (Savva *et al*. 2013). This toxin showed limited amino acid similarity with pore-forming *beta*-toxin (38%) and the *alpha*-toxin of *Staphylococcus aureus* (31%) (Keyburn *et al*., 2010). The *netB* structural gene was identified by genomic sequencing of an NE isolate (Keyburn *et al*., 2008). Against the LHM cell line, the mechanism of action appears to include the formation of a hydrophilic pore in the cell membrane with a functional diameter of 1.6-1.8 nm. Additional evidence to verify the contribution of NetB to the pathogenesis comes from the observation that chickens suffering from NE carry the *netB* gene and produce highly conserved NetB toxin (Keyburn *et al*., 2010). Surveys conducted in North America demonstrated that *C. perfringens* isolates from birds infected with NE carried the *netB* gene, while only a small percentage of the isolates from healthy birds carry the gene (Chalmers *et al*., 2008; Martin and Smyth, 2009). This suggests that the presence of NetB is a critical factor in the development of NE; however, it is possible that other virulence factors also contribute to the disease that may have yet to be identified.

2.3 Necrotic Enteritis in Poultry

Necrotic enteritis (NE) is a globally significant welfare and economic issue, as it currently the most common clostridial enteric disease in poultry (Cooper *et al*., 2013). The disease was first recorded in Australia in 1930, and was fully characterized in the United Kingdom by 1961. NE is characterized by necrosis and inflammation of the gastrointestinal tract with a significant decline in growth performance, and in clinical cases, a significant increase in flock mortality (Van der Sluis, 2000). The disease occurs almost exclusively in broilers,

between 2-6 weeks of age, but is also seen in layers mainly during 2 -24 weeks of age (Long, 1973). The causative agent is *Clostridium perfringens*, a Gram-positive, spore forming anaerobic bacterium that is a normal inhabitant of the gastrointestinal tract. However, additional predisposing factors contribute to pathogenesis of the disease, as these elements can directly influence the physical properties of the gut, disrupt the gut microbiota, and alter the immune system (Baba *et al*., 1977). The standard for diagnosing NE in poultry includes necropsy and bacteriologic culture, with genotyping of isolates (Moore, 2015). The overall effect of this disease has been estimated to cost the global poultry industry US $5—6 million per annum in production losses and current control measures (Wade and Keyburn, 2015).

2.3.1 History

The discovery of NE dates back to 1930 in Australia, when Bennetts (1930) isolated what he considered to be *Bacillus welchii*, later renamed *Clostridium welchii* and then *C. perfringens*, from intestinal lesions found in a Black Orpington pullet. He considered this an entertoxemia that had contributed to the bird's death. Mann (1945) described what he considered the "six-day disease", in which he had observed *C. perfringens* invading the intestinal walls of chicks. He also found that roughage and protein stimulated proliferation of the anaerobic intestinal bacteria, particularly those resembling *C. perfringens* (Mann, 1947). However, it was a year before this that McGaughey (1944) was able to reproduce the disease by feeding cultures of *C. welchii* to day-old chicks; furthermore, suggesting the cause of death was *C. welchii* intoxication. Finally, Parish (1961) described an intestinal disorder in a flock of six to seven-week-old cockerels in England, naming the disease NE. He considered it a disease of entertoxemia possibly caused by *C. welchii*, dividing the disease into chronic and acute forms. He was able to isolate *C. perfringens* from the intestinal tract of the affected birds, finding the extracts to be lethal to chickens and mice (Parish, 1961). These isolates are now believed to have been Type C (Long 1973). It was not until after 1977 that NE began to emerge as a worldwide problem. As the number of outbreaks of the disease increased around the world, researchers began to investigate methods for treatment and prevention (Williams, 2005).

2.3.2 The Types and Clinical Signs

Under field conditions the disease exists in two forms, the acute clinical form and the mild sub-clinical form. The clinical form of NE is associated with signs such as ruffled feathers, relative immobility, depression, anorexia, diarrhea and decreased appetite (Long 1973; Al-Sheikhly and Al-Saieg, 1980). Birds displaying clinical signs will typically die within a few hours, with mortality rates up to 1% per day (Helmboldt and Bryant, 1971). In the sub-clinical form of NE there is no peak in mortality and no clinical signs present (Nairn and Bamford, 1967). A reduction in performance is commonly associated; however, this allows the subclinical form of NE to remain unnoticed, as it is only detected due to the rejection of carcasses at the processing plant (Kalshusdal and Hofshagen, 1992). During this sub-clinical infection, *C. perfringens* can reach the portal blood stream and bile ducts; consequently, leading to cholangiohepatitis, or diseased livers (Timbermont *et al.,* 2011). During meat inspection at the slaughter house, these infected diseased livers can be found without any prior clinical signs from the flock (Timbermont *et al.,* 2011). Although the clinical forms of NE may cause higher levels of mortality, the subclinical forms are more economically detrimental due to the investment placed in the flock throughout grow-out, until the point of rejected carcasses at the processing plant.

There is controversy as to whether wet litter can be used as an early indicator of the disease. Kaldusdal and Hofshagen (1992) noted "dark and moist" wood-shaving litter when birds had subclinical NE. Elwinger and Teglof (1991) found a direct correlation between sticky droppings and poor litter conditions. Diarrhea may be associated with the acute clinical form of NE; however, it is not always of *C. perfringens* origin as it may just be that the water to food intake ratio is being consequently increased. Adverse effects such as leaky drinkers, feed quality, mycotoxins, house temperature, ventilation and stocking density may contribute (Babu, 1992).

2.3.3 Gross Lesions

Gross lesions as a result of birds infected with NE, are usually restricted to the small intestine. They are found primarily on the jejunum, sometimes extending into the duodenum or the ileum (Parish, 1961; Kaldusdal and Hofshagen, 1992; Helmboldt and Bryant, 1971). Rarely, lesions may also occur in the colorectum, caecal tonsils or necks (Long *et al*., 1974). Normally, the mucosal surface of the intestinal tract should appear smooth and shiny, reflecting an intact

mucosal epithelial barrier. With erosion and ulceration occurring, a complex of subtle mucosal dullness, ulceration, acute hemorrhage, subacute hemorrhage, and the formation of individual mucosal fibrin plaques that cannot be removed with a finger may be observed (Shojadoost *et al.,* 2012). As the disease progresses a large-scale accumulation of fibrin, necrotic tissue debris, and inflammatory cells coalesce to form a layer of fibro-necrotic material covering the mucosa, often two thirds of the small intestine. This is commonly found under field conditions (Nairn and Bamford, 1967; Long *et al.,* 1974). Under experimental conditions, where *C. perfringens* is being directly administered to the bird, the first appearance of intestinal damage has been reported as early as 6 hours post-challenge (Truscott and Al-sheikhly, 1977). There is an overall marked distension of the intestine, with the lumen usually filled with gas and dark brown fluid material. The intestinal wall will be friable, and easily torn (Nairn and Bamford, 1967; Long *et al.,* 1974). Microscopic examination shows a strong inflammatory response to *C. perfringens* in the early stages of NE.

Microscopic examination of lesions of NE indicated that in clinical cases, cellular degeneration may reach the submucosa, or even the muscularis mucosa (Nairn and Bamford, 1967). In the sub-clincal form of the disease, microscopic changes are mostly seen in the small intestine, sometimes in the liver, but rarely ever in the ceacum. Small affected areas of degeneration will appear merged with areas of normal, unaffected intestine. The degenerative process progresses down the villi, until many of the villi will appear equally affected. Degenerative changes begin with congestion of blood vessels, followed by the enlargement of the epithelial cells of the villi. In some extreme cases, most commonly associated with field conditions where NE is apparent in the clinical form, the necrotic tissue is clearly differentiated from the normal by line of segregation (Kaldhusdal *et al.,* 1995). The villi may even sloth off, accompanied by coagulation necrosis (Olkowski *et al.,* 2006). In the final stages of the degeneration process, the nuclear material can flow out of the individual cells in the form of irregular deposits that disappear once all layers of the intestine are effected (Al-sheikhly and Al-Saieg, 1980; Long, 1973).

Regenerative changes in the intestinal tract in field cases, are characterized by proliferation of epithelial cells, production of connective tissue network at the inflammatory

zone, decreased numbers of goblet and columnar epithelial cells, and increased number of cuboidal cells (Parish, 1961; Long *et al*., 1974). Large numbers of gram-positive rod-shaped bacteria can be seen in the sloughed epithelium. The intestinal villi are left shortened and flattened with reduced absorptive surface (Long *et al*., 1974).

2.3.4 Treatment and Prevention Through the Use of AGPs

Poultry producers for decades have primarily controlled and treated NE by adding antimicrobials to feed. The use of antibiotics in animal feed was approved for use by the U.S. FDA in 1951 (Jones and Ricke, 2003). The disease has been treated primarily in water with linomycin, bacitracin, oxytetracycline, penicillin, and tylosin. In feed, it has been treated with bacitracin, lincomycin, virginiamycin, penicillin avoparcin, and nitrovin (Lanckriet *et al.*, 2010). AGPs are hypothesized to contribute to animal health by suppressing microbial growth and inflammation which may induce subclinical disease, reduce microbial destruction of essential nutrients, and increase the availability of vitamins and growth factors due to decreased competition (Heikinheimo and Korkeala, 2005). Since 1951, such use has grown dramatically worldwide. A 1999 survey by the Animal Health Institute found that 20.42 million pounds of antibiotics were used on animals annually in the U.S. (Jones and Ricke, 2003). The dramatic increase in the use of AGPs has consequently resulted in rising concerns from consumers in regard to the overall effect of these products on human health. Therefore, in 2010 the FDA called for a strategy to phase out production use of medically important antimicrobial products and to bring the remaining therapeutic uses under oversight of a veterinarian.

The largest concerns are of the development of antimicrobial resistance by bacterial pathogens of animals; however, there are also concerns of drug residues in foods for human consumption, as well as the environment (Takeda *et al.*, 1995). This has resulted in retailers and food production companies to proclaim to go antibiotic free in the near future in the US. However, beginning in 1970s, countries of what is now the EU began eliminating the use of AGPs in animal feedstuff. The negative effect of the withdrawal of AGPs from poultry feed has resulted in increased number of outbreaks of NE and other clostridial diseases, particularly in western Europe (Van Immerseel *et al.*, 2004). In replacement of AGPs, Europe has controlled NE primarily by the use of ionophores, enhanced managerial practices and modified diets

(Engberg *et al.*, 2000). In recent years there has been an explosive interest in understanding the pathogenesis of NE and investigating how it can be preventing with antibiotic alternatives. Although *C. perfringens* is well documented as the causative agent of NE, other contributory factors are essential required that predispose birds to this complex, multi-factorial disease.

2.4 Predisposing Factors: Controlling Necrotic Enteritis Post-Antibiotic Era

It is well accepted that NE is a multi-factorial disease process in which a number of cofactors are usually required to outbreak the disease; however, these predisposing factors that lead to *C. perfringens* proliferation and progression to the disease are numerous and ill-defined. Overall, reported factors that predispose birds to NE can be divided into three major categories: infectious, non-infectious, and management.

2.4.1 Infectious Predisposing Factors

2.4.1.1 Coccidiosis

Coccidiosis has been studied for over a century and is still considered as the most economical important parasitic condition affecting poultry worldwide (Williams, 2005). Coccidiosis is a disease that is caused by protozoan parasites of the genus *Eimeria*, developing within the intestine of most domestic and wild animals and birds. Eight species of *Eimeria* (*E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, *E. tenella, and E. mivati*) are recognized as infecting chickens (De Gussem, 2007). Generally, it is agreed upon that from the species recognized in broiler chickens, the most pathogenic are *E. acervulina*, *E. maxima*, and *E. tenella* (De Gussem, 2007). Each species causes a separate disease, each exhibiting a characteristic degree of pathogenicity. Most infestations under field conditions are mixed, but one species will be dominant (Hafez, 2008). Unlike bacteria and viruses, coccidia have a genetically fixed, self-limiting life cycle. Therefore, the severity of each coccidiosis is positively correlated with the number of infective oocysts ingested (Johnson and Gerding, 1997). Overall, the protozoan parasites of the genus *Eimeria* multiple in the intestinal tract causing tissue damage, which results in mortality, interruption of digestive processes or nutrient absorption, reduced weight gain, and increased susceptibility to other disease agents. Coccidiosis can be an important contributing factor in the development of necrotic enteritis in broiler chickens, as

consequential mucosal damage facilitates the establishment and multiplication of *C. perfringens* (Al-Sheikhly and Truscott, 1977).

Coccidia are spread by the faecal-oral route, in which the highly resistant oocysts can remain viable in the litter for many months (McDougald, 2003). Oocysts in the environment are practically ubiquitous where poultry occur. Unsporulated oocyts are expelled from the intestinal mucosa and excreted in the feces. Excreted oocyts, must sporulate to become infective, for which oxygen, moisture and warmth are necessary (Kheysin, 1972). The *Eimeria* cycle includes two distinct phases; (a) internal phase in which the parasite multiples in different parts of the intestinal tract and the oocyts are excreted in the feces; (b) the external phase during which the oocyst must undergo a final process called sporulation before they are again infective. The part of the intestinal tract and the total duration of the internal phase of the cycle is dependent on the species (Chapman and Jeffers, 2015).

Each disease may exhibit three increasing levels of severity: (1) coccidiasis, a mild infection, causing no adverse effects; (2) subclincal coccidiosis, resulting in a slight, but economically important reductions of growth rate and FCR; and (3) clinical coccidiosis. The less severe, clinical coccidiosis are caused by *E. praecox*, *E. mitis*, *E. acervulina* or *E. mivati*. The more severe coccidiosis is caused by *E. brunetti*, *E. maxima*, *E. necatrix*, and *E. tenella* (Williams, 2005). The most common effects of poultry coccidiosis are reduction of weight gain and an adverse effect on feed conversion ratio, where *E. mitis* and *E. praecox* may adversely affect performance, similarly to *E. acervulina* (Williams, 2005). *E. mivati* does not result in severe damage, where damage to the villus tip of those infected consists of cell sloughing and exposure of connective tissue. Coccidiosis usually increases passage time of digesta during the acute phase, where it is considered that reduced intestinal motility is caused by pH values ≤ 5.6 (Schildt and Herrick, 1955). Decreased digesta viscosity caused by *E. acervulina*, *E. tenella* and *E. praecox*, is commonly associated with poor performance (Morgan and Catchpole, 1996). Infections of the duodenum, jejunum or ileum can result in reduced absorption of nutrients by the intestinal epithelium (Turk, 1978). Reduced activities of digestive enzymes, as well as a general impairment of protein digestion has also been reported in birds infected with coccidiosis (Turk, 1978). During *E. acervulina* and *E. maxima* infections, villous degeneration may occur, further

contributing to malabsorption. Intestinal leakage of plasma proteins may additionally increase mucosal permeability (Rose and Long, 1969).

2.4.1.1.1 Diagnosis and Lesion Scoring

Coccidiosis is often extremely difficult to diagnose and can only be done in the laboratory (Conway and Mckenzie, 2007) by counting coccidia per gram of feces and/or examining the intestinal tract to determine the lesions scores as describes by Johnson and Reid (1970). Oocysts per gram (OPG) counts in feces or litter have poor relation with the impact of the parasite on the performance of a flock. Identification of different species based on the morphology of oocyst is very challenging and requires expertise (De Gussem, 2007). Lesion scoring is an interpretation based on macroscopic visible lesions caused by *Eimeria*, usually following a scoring system from zero to four (Johnson and Reid, 1970). The individual scores for all the species are usually complied for a certain number of birds per flock resulting in a Total Mean Lesion Score (TMLS) (De Gussem, 2007). An important debate is still ongoing on what levels are to be considered clinical and what levels are subclinical. Some consider lesions higher than 1.5 per species requiring treatment (De Gussem, 2007).

During necropsy, the entire intestine is usually pulled out unbroken from the bird, where the gizzard and rectum are left attached for orientation as to the location of lesions observed in various parts of the intestine. Lesions produced by *E. acervulina* and *E. mitvati* occur primarily in the duodenal loop and the upper part of the jejunum. *E. maxima* and *E. necatrix* produce their most severe lesions in the mid-intestinal area. *E. brunetti* invades the mucosa of the lower intestine and the rectum. Lesions of *E. tenella* are found mostly in the ceca, but occasionally some strain of *E. tenella* will cause lesions in the rectal area (Conway and McKenzie, 2007). When scoring mixed infections, four areas of the mucosal surface of the intestine are individually examined in addition to the serosal surface. A score of 0 to +4 is recorded for each of the four regions of the mucosal surface; the upper intestine, lower intestine, and the ceca (Conway and McKenzie, 2007).

2.4.1.1.2 Mechanism of Infection in the Pathogenesis of NE

NE and some *Eimeria* species may independently cause acute fatal disease in poultry; however, NE seems unlikely to predispose birds to coccidiosis, as destruction of enterocytes

would remove potential development sites for coccidian (Williams, 2005). However, various laboratory trials and cases suggest that coccidiosis predisposes birds to NE (Bennetts, 1930; Baba *et al.*, 1997). The precise mechanism of coccidial infection in the pathogenesis of NE is not clear; however, mucosal damage resulting from coccidiosis is a probable cause (Williams, 2005).

The sexual stage in the life cycle of the coccidian parasite results in extensive damage to the intestinal mucosa (Van Immerseel *et al.,* 2004). Intestinal damage during *Eimeria* infection will result in leakage of plasma proteins into the lumen of the intestinal tract. Subsequent degradation of proteins into amino acids by proteolytic enzymes in the GIT may cause an increase in the acidity of the digesta. An acidic pH would affect the permeability of the cell membrane, suppressing the intestinal motility, all of which favors *C. perfringens* proliferation and toxin production (Van Immerseel *et al.*, 2004). Collier *et al.* (2008) suggested that coccidial infection additionally induces muconeogenesis as a result of a host mucogenic response. *C. perfringens* has the ability to utilize mucus as its substrate; therefore, increasing the amount of mucus provides an additional growth advantage for *C. perfringens.* Overall, *Eimeria* infection disrupts the microbiota and results in physical changes to the GIT induced by coccidiosis, potentially playing an important role in NE predisposition (Moore, 2016).

2.4.1.2 Immunosuppression

Changes to the immune status of birds can increase the incidence of NE. The peak risk period for broiler birds is about 3 weeks of age, as this is the time when the maternal antibodies are disappearing from the circulation, potentially causing some susceptibility to infection or proliferation of *C. perfringens* (Moore, 2016). Infections with viruses such as Marek's disease virus, infectious bursal disease virus and chicken anemia virus can also have immunosuppressive effects (Hoerr, 2010).

2.4.2 Non-Infectious Predisposing Factors

2.4.2.1 Nutritional Factors

Diet is widely recognized as having a strong impact on the incidence of NE in broiler chickens, due to its potential to alter the GIT environment (Annett *et al.*, 2002). Evidence from several studies has shown that there is a relationship between cereal type used in the diet, dietary protein levels, and anti-nutritional factors on the incidence of NE (Drew *et al.*, 2004).

The level of crude protein, the protein source, and the amino acid content of the diet all have a significant effect on the intestinal population of *C. perfringens*. Therefore, poultry diets with high protein content or those rich in animal protein such as meat and bone meal or fish meal have been shown to predispose birds to NE (Williams, 2005). *C. perfringens* lacks many enzymes required for amino acid biosynthesis, making it necessary to obtain these from its host. Thus, a diet high in protein provides an exceptional amino acid source for *C. perfringens* proliferation (Drew *et al.*, 2004). Methionine and glycine, in particular, have long been known to stimulate growth and establishment of *C. perfringens*. The amounts of methionine and glycine, which have additionally been shown to increase alpha toxin *in vitro*, are higher than other amino acids in fish meal protein-based diets (Dahiya *et al.*, 2006). Another possible explanation of the association between fish or meat meal and NE could be related to their higher zinc concentrations. In an *in vitro* study Baba and others (1992) proposed that dietary zinc increased the production of alpha toxin. With increased dietary protein level, there will also be an increased activity of the enzyme trypsin in the small intestine. This in turn, will lead to faster release of coccidian from their oocyts. There are some protein sources such as raw soybean, cottonseed meal, and flax cakes, which contain varying amounts of anti-nutritional factors such as trypsin inhibitors. When ingested by the bird, they reduce the digestibility of protein and thus increase nitrogen concentration in the lower GIT, providing appropriate growth for *C. perfringens* (McDevitt *et al.*, 2006). The level of *C. perfringens* has been found highest with the greater amount of animal protein (40% crude protein/feed) and lowest in plant-source protein diets fed to chicks (Drew *et al.*, 2004).

The dietary fat source is also known to have an effect on the incidence of NE, since animal fat (a mixture of lard and tallow) has been shown to lead to high *C. perfringens* counts when compared to vegetable/soya oil (Knarreborg *et al.*, 2002). The type of fat source is suspected to indirectly influence gut microflora through its impact on viscosity of digesta, internal transit time, and digestion in the small intestine (Pan and Yu, 2014).

There is renewed interest in dietary fiber of poultry, in terms of both gut health and the effect on microflora. Most feed ingredients of plant origin contain considerable amounts of fiber (non-starch polysaccharides, NSP, plus lignin), with the majority being insoluble (Bach

Knudsen, 1997). In addition, a number of recent reports show that chickens consume a considerable amount of their bedding material, composed primarily of insoluble NSP plus lignocellulose compounds (Hetland *et al.*, 2003). Various fiber components can result in positive and negative attributes as a result of their beneficial effects on gut health and potential to modify the gut microflora. The difference in emphasis, relates to the inclusion level. At low inclusion levels (perhaps less than 1%) there may be advantages to using NSP as a means of beneficially modifying the gut microflora, especially is situations where AGPs are not used (De Lange, 2000). It has been hypothesized that monogastric animals have a fiber requirement, because their gut development requires physical stimulation by hard, solid particles of feed (Hetland *et al.*, 2004). On the other hand, it is widely believed that high levels of NSPs strongly influence the incidence of NE in broilers by increasing digesta viscosity, prolonging transit time, and decreasing nutrient digestibility (Choct *et al.*, 1999). Poor quality wheat at levels above 50% corn-replacement can increase susceptibility to NE. Birds fed diets that are enriched in cereals such as barley, wheat, rye or oats suffer more severe NE than birds fed maize-based diets (Hofshagen and Kaldhusdal, 1992). The NSPs and certain types of starch present in the cereals are not digestible by the enzymes in the birds GIT; therefore, they act as substrates for the gut microflora and provide an opportunity for pathogenic bacteria to proliferate (McDevitt *et al.*, 2006).

2.4.2.2 Mycotoxins

Mycotoxins may alter animal's susceptibility to infectious diseases by affecting intestinal health and the innate and adaptive immune systems. Mycotoxins are secondary fungal metabolites, where the ingestion of these fungal compounds can produce a variety of diseases, collectively called "mycotoxicosis" (Richard, 2007). Avian mycotoxicosis leads to severe losses not only in terms of performance, but also as an immunosuppressive agent, increasing the birds' susceptibility to mortality and diseases. The genera of most concern globally are *Aspergillus*, *Fusarium*, and *Penicillium*. The major toxins produced by these three genera that significantly affect the health of poultry species include: alfatoxins (AF), zearalenone (ZEN), ochratoxin A (OTA), fumonisins (FUM), deoxynivalenol (DON) and T-2 toxin (Murugesan *et al.*, 2015). The recognition that mycotoxins affect health and productivity of poultry has led to research on

counteracting methods, including detection and elimination or detoxification of mycotoxins over the last few decades (Murugesan *et al.*, 2015).

2.4.2.2.1 Aflatoxins

AF was isolated following the outbreak of Turkey X disease in the United Kingdom in 1960 (Blount, 1961). AF are mycotoxins that are produced by the *Aspergillus* species, including *A. flavus*, *A parasiticus* and *A. nominus* (Chen *et al.*, 2013). AF can be found in a wide range of feed commodities pre-harvest, depending on regional differences and climatic conditions. Significant quantities of AF can be found in maize, peanuts and tree nuts (Reddy *et al.*, 2009). In poultry, adverse effects include reduction in growth rate and feed efficiency, decreased egg production and hatchability, and increased susceptibility to disease. In addition, residues of AF from animals can appear in edible animal products for human consumption, which raises public health concerns (Chen *et al.*, 2013). The FDA has established guidelines for the maximum toxin level that can be safely fed to poultry, dependent on the class of animal and feed type. If feed is contaminated by multiple mycotoxins at the same time, AF has the capability of interactions with other mycotoxins to produce more severe effects on broiler performance. Birds fed diets that contain AF as low as 0.3 mg/kg start to show reductions in growth rate, feed intake and worsened feed efficiency. AF acts as an inhibitor or protein synthesis; consequently, suppressing the immune response in poultry. Limited data suggests that the absorptive surface of the small intestine deteriorates during chronic exposure to low levels of AFB_1 (Chen *et al.*, 2013).

2.4.2.2.2 Ochratoxins

Ochratoxin was isolated from species *Aspergillus* and *Penicillium* in 1965, by Van der Mere *et al.* Ochratoxins are a family of toxic compounds consisting of three members, A, B and C, which are structurally related and are produced as secondary metabolites of several species of fungus. The disease caused by Ocratoxin A (OTA) exposure is known as ochratoxicosis, where the primary target is the kidney. Ochratoxicosis occurs less frequently in poultry than alfatoxicosis, but is more lethal because of its acute toxicity (Ghimpeteanu *et al.* 2010). In general, OTA formation occurs mainly after harvesting on insufficiently dried cereal and cereal products (Denli and Perez, 2010). OTA has been found in cereal grains (maize, barley, wheat, oats, rye), hay and mixed feed (Battaglia *et al.*, 1996; EFSA, 2006). Young poultry are more

sensitive to ochratoxin ingestion than adults (Pattison *et al.*, 2008). Typical signs of poultry ochratoxicosis are reduction in weight gain, poor feed conversion ratio, reduced egg production, poor egg shell quality and nephrotoxicity (Denli and Perez, 2010). The effects are dependent on the level of the toxin and time exposure; however, numerous studies have shown that even exposure to low levels of OTA (0.5mg/kg feed) altered performance (Prior *et al.*, 1980; Wang *et al.*, 2009). One of the profound effects of OTA is its ability to alter the immune system, as well as inhibit protein synthesis. The enlargement of the liver and kidney in OTA intoxication is caused by the involvement of these organs in detoxification and elimination (Denli and Perez, 2010). The Commission of the European Communities has recommended guidelines for the maximum tolerable limits for OTA in complementary and complete feedstuffs for poultry to be 100 ug/kg (Off. J. Eur. Union, 2006).

2.4.2.2.3 Fusarium

Fumonisms (FUM) are a group of mycotoxins that were first isolated from cultures of *Fusarium moniliforme* and chemically characterized by Gelderblom *et al.* (1988). Six different FUM have been identified (A1, A2, B1, B2, B3, B4) (Bezuidenhout *et al.*, 1988). However, fumonisin B1 (FB1) has been reported to be the predominant form produced by *Fusarium verticillioides* (Norred, 1993). In most animals FUM impairs immune function, causes liver and kidney damage, decreases weight gains, and increases mortality rates (Jones *et al.*, 1994). The mechanism by which the FUM cause toxicity in animals appears to be due to the disruption of sphingolipid metabolism (Wang *et al.*, 1991). The specific conditions needed for the production of FUM are unknown, but it is suggested that drought stress followed by warm, wet weather during flowering seems to be important. Corn is the major grain commodity affected by this fungus (Richard, 2000).

2.4.2.2.4 Zearalenone

Zearalenone (ZEN) is a mycotoxin produced mainly by the fungi belonging to the genus *Fusarium* in foods and feeds (Bennett and Klich, 2003). ZEN mimics the effect of the female hormone estrogen and at low doses, increases the size or early maturity of mammary glands and reproductive organs. At higher doses, ZEN interferes with conception, ovulation, implantation, fetal development and the viability of newborn animals (Jones *et al.*, 1994). It is the most

prevalent mycotoxin in feed and raw materials available in the South Asian Regions, and primarily effects breeders and layers (Kannan, 2010). Chickens tolerate ZEN better than swine for example, but it has the potential adverse effects in bird performance and egg yield. ZEN at more than 0.5 ppm is detrimental to broiler breeders and layers that experienced a reduction in egg performance. In poultry layers and breeders it impacts: eggshell thickness, interior egg quality, oviduct enlargement, and lowered serum progesterone (Kannan, 2010).

2.4.2.2.5 Trichothecenes

Trichothecene mycotoxins are a group of fungal metabolites with the same basic backbone structure, including T-2 toxin and DON (vomitoxin). Toxic effects of *Trichothecenes* include oral lesions, growth retardation, abnormal feathering, decreased egg production and egg shell quality, regression of the bursa of Fabricius, changes in the liver, abnormal blood coagulation and immunosuppression (Lesson *et al.*, 1995; Danicke, 2002).

The T-2 toxin, produced mainly by *Fusarium tricinctum*, was the first *Trichothecene* to be found as a naturally occurring grain contaminant in the United States (Hsu *et al.*, 1972). Inn poultry, T-2 has been implicated to cause mouth and intestinal lesions, as well as impair the birds' immune response. This results in consequential egg production declines, decreased feed consumption, weight loss and altered feather patterns (Jones *et al.*, 1994). Concentrations of T-2 that result in oral lesions are lower (0.4 mg/kg) than concentrations reported to decrease chick performance (3-4 mg/kg) (Lession *et al.*, 1995). Danicke (2002) concluded broiler performance is affected at dietary concentrations of 3-4 mg/kg of T-2 toxin.

Vomitoxin, also called deoxynivalenol (DON), is stable, survives processing and milling and occurs in feed prepared from contaminated corn and wheat. The most common producer of DON is *Fusarium graminearum* (Marasas *et al.*, 1984). The level of DON that affects chick performance is still debated, with some researchers (Huff *et al.*, 1986) reporting toxic effects at 16 mg/kg diet, whereas others (Moran *et al.*, 1982) report no toxic effect until dietary concentrations exceed 116 mg/kg of DON. This mycotoxin acts as an inhibitor of the protein synthesis at the ribosomal level whereby rapidly proliferating cells in tissues with high protein turnover rates, such as the immune system and small intestine, are most affected (Neirinckx *et al.*, 2011). As a consequence, feeding DON contaminated diets can lead to greater susceptibility

to enteric infections (Grenier *et al.*, 2013). Specifically, the intake of DON contaminated feed at contamination levels below the EU guidance level, is a predisposing factor for the development of NE in broiler chickens due to the negative influence on the epithelial barrier, and to increased intestinal nutrient availability for clostridial proliferation (Antonissen *et al.*, 2015).

2.4.2.2.6 Testing and Control

Since the 1960's, many analytical methods have been developed for the testing of mycotoxins in human food and animal feeds due to the concern of toxicity for human health (Truckseess, 2000). Mycotoxins present a major analytical challenge due to the range of chemical compounds that they represent and the vast array of feed matrices in which they are found (Cole, 1986). However, valid determination of mycotoxins and their metabolites is a crucial step in any intervention, mitigation, or remediation strategy to cope with the deleterious effects of mycotoxins to poultry. In general, methods for mycotoxin determination can be divided into chromatographic methods, immunochemical methods, and "other" methods which include direct spectroscopic methods. Among them, the methods for thin-layer-chromatography (TLC), enzyme-linked immunosorbent assay (ELISA) and immunosensor-based methods have been widely used for rapid screening. Meanwhile, high-performance liquid chromatography (HPLC) with fluorescent detection (FD) and mass spectrometry detection (MS) have been used as confirmatory and reference methods (Krska *et al.*, 2008). When considering testing DDGS for mycotoxin contamination, it is essential to use approved analytical procedures to get accurate results. HPLC is the preferred method to determine the presence of mycotoxins in animal feeds (Krska *et al.*, 2008).

2.4.2.3 Stress

Any stressful condition has the potential to predispose birds to NE, as it can potentially change the intestinal environment in such a way that the risk of induction is raised (McDevitt *et al.*, 2006). For example, overcrowding, environmental ammonia, high stocking density, and alternation in feeding regime all have the potential to place additional stress on the bird (Hoerr, 2010; McDevitt *et al.*, 2006).

2.5 Distillers Dried Grains with Solubles (DDGS)

In the U.S., yellow dent corn is the predominant source of starch used to produce ethanol. Manufacturers use two major processes to produce alcohol or other starch based products, dry-milling and wet-milling. In dry milling, the grain is cleaned and ground dry to reduce the particle size, used in fermentation (Chen *et al.*, 1999). The wet milling procedure removes the maximum amount of starch from the kernel by first adding water to the grain allowing it to seep so the starch can be removed (May, 1987). Co-products from the dry milling and/or wet milling of corn have important nutrients for diet formulation. The primary co-product from dry milling are distillers dried grains with solubles (DDGS; U.S. Grains Council, 2008).

Distillers dried grains with solubles (DDGS) consists of the non-fermentable residues (i.e., protein, fiber, fat, and ash) from ethanol manufacture. DDGS is defined as the product obtained after removal of ethyl alcohol by distillation from the yeast fermentation of a grain or a grain mixture by condensing and drying at least 75% of the resultant whole stillage (AAFCO, 2000). DDGS has been widely used for several years as a valuable source of energy, amino acids, and phosphorous in poultry diets (U.S. Grains Council, 2008). Today, the production of this co-product has increased dramatically due to the considerable demand for ethanol (Batal and Dale, 2006; Lumpkins *et al.*, 2005). Although DDGS has been available as a feed ingredient for decades, it varies in nutrient content. In addition, this co-product can concentrate up to three times preexisting mycotoxins present in the source maize (Zhang *et al.*, 2009). Various methods have been used in the preparation of DDGS, causing the end product to vary in physical appearance and chemical composition. Therefore, this has led a need for reassessment in regard to their inclusion in poultry diets.

2.5.1 History of DDGS in Poultry

The use of DDGS in poultry feed dates back to the 1930's when Cooley (1938) and Boruff and Miller (1938) invented the first methods of producing DDGS; however, it has been used much more extensively in the past two decades. The inclusion of DDGS in diets for laying hens was first conducted by Matterson *et al.* (1996). Additional findings from Harms in 1969, found that DDGS could be added to laying hen diets at a concentration of 10%. When analyzing the protein quality of DDGS, Parsons and Baker (1983) concluded that at least 20% soybean meal (SBM) could be replaced by DDGS without any detrimental effects. In 2004, Lumpkins *et*

al. determined that it could be successful included at 6, 12, and 15%, respectively, in starter, grower, and finisher periods. Waldroup *et al.* (1981) reported that there were no significant differences in body weights and FCR at 42 days of age, when DDGS was included in the diets up to 25%. Lumpkins *et al.* (2005) went on to concluded that DDGS could be used up to 12% in commercial laying diets. However, Masa'deh (2011) observed DDGS concentrations up to 25% in laying hen diets from 24 to 76 weeks without any detrimental effects. Few studies have been conducted analyzing the effects of DDGS on carcass composition. Wang *et al.* (2007) reported that DDGS did not affect carcass composition of broilers fed up to 15% DDGS. Corzo *et al.* (2009) found there was no significant effect of 8% DDGS inclusion on color, pH, cooking loss, and shear values compared to the negative control. Studies have shown that broilers fed 8% DDGS have had greater linoleic and total polyunsaturated fatty acids, making it more susceptible to oxidation. There is currently limited research on the effects of feeding DDGS on poultry gut health (Perez *et al.*, 2011; Macklin *et al.*, 2011); however, some research has been conducted in regard to enteric diseases in swine (Perez *et al.*, 2009, 2010).

2.5.2 Amino Acid Variability

Distillers dried grains with solubles can be a good source of protein, but has been demonstrated to be highly variable (Kim *et al.*, 2008). When considering the process for production, yeasts will utilize some amino acids from the soluble protein fraction (Garnsworthy and Wiseman, 2009). In addition, high temperatures during the drying process can damage proteins and reduce available amino acids (Cromwell *et al.*, 1993). Furthermore, the Mallard reaction can result in low and variable levels of lysine, arginine and cysteine in DDGS, specifically. The amount of solubles being in the DDGS can also influence the amino acid profile (Garnsworthy and Wiseman, 2009). Subsequently, one of the major causes of amino acid variability is that the solid and liquid streams of DDGS production are not highly regulated (Belyea *et al.*, 1988). Overall, variability in amino acid profiles can provide a challenge when determining DDGS sources economic and feeding value for poultry. Furthermore, amino acid variation can result in undigested amino acids traveling down the gastrointestinal tract of broilers. Intestinal bacteria, including *C. perfringens*, can utilize these undigested amino acids as a substrate for proliferation and subsequent disease induction (Drew *et al.*, 2004).

2.5.3 Reduced-oil DDGS

Corn DDGS have typically contained 10 to 11% ether extract (EE) with a ME content similar to corn (Stein and Shurson, 2009). However, the majority of the US ethanol plants have recently implemented oil extraction technology due to the rise in value of corn oil. The current value of corn oil is approximately 88 cents/kg; whereas, a typical DDGS costs around 22 cents/kg (Musser, 2012). The greater portion of corn oil that is extracted from DDGS can either be utilized in biodiesel production or as a commodity in different industries (Saunders and Rosentrater, 2009). Consequently, this technology has led to a remaining corn DDGS with a greater range of EE (4 to 12%) (CEPA, 2011). As a result, NRC (2012) nutrient values for DDGS are based on oil content and are characterized as low (>4% oil), medium (between 6 and 9% oil), or high oil (>10%; NRC, 2012).

The process through which the corn oil is being extracted is referred to as "back-end oil extraction". This technology was discovered after 2007, and removes the corn oil after the entire corn kernel has been fermented to produce ethanol (Jung and Batal, 2009). The concentrated silage is heated, followed by the use of centrifuge technology to extract crude corn oil. The residual DDGS, or reduced-oil DDGS is the consequential result. Removing oil from DDGS will alter the chemical nature of these coproduct feed materials and may affect the physical properties as well. Because the feeding value of DDGS may be largely based on its energy content, changing the oil content of DDGS may more specifically affect growth performance (Graham *et al.*, 2014). Oil contains 2.25 times more energy than carbohydrates; therefore, removal of oil likely reduces the ME content in the DDGS source, effecting its economic value and dietary inclusion rates (Kerr *et al.*, 2013). Dale (2013) indicated a decrease of 17.5 kcal/kg for each 1% decrease in DDGS oil content. Limited research has been conducting involving low-oil DDGS sources in poultry. Recent research by Purdum *et al.* (2014) concluded that low oil DDGS sources can be used in laying hen diets, but they will have less value as more oil is taken out. Therefore, low oil DDGS products are more likely to fit into laying hen rations, which are lower in ME, compared with meat bird diets such as those for broilers or turkeys. Producers of low oil DDGS should not expect as high an economic value associated with low oil products compared with higher oil products in the poultry feed industry (Purdum *et al.*, 2014).

2.5.4 DDGS Endogenous Fiber and Poultry Gut Health

The fiber composition of DDGS is primarily insoluble (42.2%) vs. soluble (0.7%; Shurson *et al.*, 2000). Feeding diets low in soluble non-starch polysaccharides can reduce proliferation of pathogenic organisms in the intestine (Hampson *et al.*, 1999). Fiber influences the secretory function of the epithelium, impairing bacterial adhesion (Smith and Halls, 1968), and also has a cleansing effect in the gut as a result of reducing the viscosity of the digesta (Lawrence, 1972). Recent observations show that DDGS or cellulose can speed recovery in young pigs from enteric disease after an experimental challenge with pathogenic *Escherichia coli* (Perez *et al.*, 2009, 2010). Perez *et al.* (2011) went on to determine if the inclusion of increasing concentrations of dietary DDGS could ameliorate the effects of *Eimeria acervulina* infection in young chicks. It was concluded that concentrations up to 20% of the diet did not prevent *Eimeria acervulina* infection or promote a faster recovery. Whitney *et al.*, (2006) conducted a disease challenge study with pigs fed diets containing DDGS, and determined that a DDGS inclusion of 10% in the diet would reduce the incidence and severity of *Lawsonia intercellularis* challenge compared with an antibiotic regimen. Pigs fed diets without DDGS and antibiotics had a higher proportion of ileal cells infected by *L. intercellularis*. On the other hand, it has also been implied that having lower levels of DDGS in diets of chickens is actually better at reducing the incidence and severity of the enteric disease, NE. In a study conducted by Macklin *et al.* (2011) it was postulated that undissociated VFFA could be diffusing across the cell membrane and then dissociating—resulting in a reduction of the cellular pH leading to cell damage. The damage could be resulting in an immune response, causing *C. perfringens* to become more virulent thus leading to an increase in NE.

Chapter 3.0 Effects of feeding low-oil distillers dried grains with solubles (L-DDGS) to broilers from 0 to 27 days post-hatch when challenged with *C. perfringens* and *Eimeria* spp. on necrotic enteritis and intestinal microbiome.

Introduction:

Necrotic enteritis (NE) is the most common clostridial enteric disease in poultry (Cooper *et al.*, 2013), leading to an estimated $6 billion in losses worldwide each year (Wade and Keyburn, 2015). It is characterized by necrosis and inflammation of the gastrointestinal tract (GIT) with a significant decline in growth performance and, in clinical cases, a severe increase in flock mortality (M'Sadeq *et al.*, 2015). Traditionally, NE has been controlled by antibiotic growth promoters (AGPs). However, legal requirements or production of market favorable products has resulted in omission of their commercial use, which has led to an increased NE incidence (McDevitt *et al.*, 2006; Van Immerseel *et al.*, 2009; Moran, 2014). Therefore, knowledge on the pathogenicity of this disease must be enhanced to seek alternative methods for control.

In order to develop NE, predisposing factors that facilitate the proliferation of *Clostridium perfringens* are required (Williams, 2005). Dietary factors, among others, have been widely recognized as having a strong impact on NE incidence. Furthermore, unabsorbed dietary components can serve as substrates for growth of intestinal bacteria (Annett *et al.*, 2002; Drew *et al.*, 2004; Pan and Yu, 2014). Distillers dried grains with solubles (DDGS) is a co-product of ethanol production and has become a common ingredient in poultry diets over the past decade (Lumpkin *et al.*, 2004; Shim *et al.* 2011). It serves as a partial replacement for energy, phosphorous and amino acids at a competitive price (Shurson, 2006; Purdum *et al.*, 2014). However, ethanol plants have implemented oil extraction technologies for increased revenue, resulting in reduced-oil DDGS (R-DDGS) sources varying in oil content from 4% to 12% (CEPA, 2012). Therefore, the value of these R-DDGS sources as an energy and nutrient source in poultry diets must be reassessed. In addition, the onset of NE has been associated with a shift in microbiota present within the gastrointestinal tract (GIT) (Antonissen *et al.*, 2015). Further

studies on poultry intestinal microbiome and its interaction with host and diet can be important for future nutritional strategies for disease control.

Whitney *et al.* (2006) determined that a DDGS inclusion of 10% of the diet would have similar effects to that of an antimicrobial regimen against *Lawsonia intercellularis* enteric disease development in swine. Macklin *et al.* (2011) implied contradicting results, where feeding increasing concentrations of DDGS (7.5% vs. 15%) in broiler diets, increased the incidence of NE. However, neither of these studies reported the use of R-DDGS sources. Therefore, the objective of this study was to determine the effects of feeding low-oil DDGS (4.29% crude fat) on broiler performance, intestinal microbiome, and severity and incidence of intestinal lesions after a NE challenge.

Materials and Methods:

Chicks and Management

Day-old broiler chicks were obtained from a commercial hatchery and transported to the Auburn University Poultry Research Farm. The chicks were unsexed and unvaccinated against coccidiosis. Upon arrival, the chicks were weighed and randomly distributed throughout four Petersime wire batteries (36 pens total, 10 birds per cage) in a temperature controlled room maintained at 35 ± 2°C during the first week and reduced 2-3°C weekly, for four weeks. The birds were fed a two-phase feeding program, consisting of a crumble starter diet (d 0 to 14) and a pelleted grower diet (d 14 to 27). Diets did not contain antibiotics. Birds were allowed *ad libitum* access to both feed and water throughout the duration of the experiment (27 d total). The DDGS source was provided by a commercial ethanol manufacturing plant located in the US. The manufacturers provided nutrient profiles were used in dietary formulation.

Experimental Design

The experiment consisted of a block randomized design with a 2 (diet: control or L-DDGS) × 2 (challenge: challenged or unchallenged) factorial arrangement of treatments. The control diet consisted of a corn-soy diet without L-DDGS. The L-DDGS diet consisted of a corn-soy diet, containing the L-DDGS source at 5% of the starter diet and 7.5% of the grower diet.

(Table 3.2). Challenged birds were housed separate from unchallenged birds. Each of the 4 treatments were represented by 9 replicate pens, containing 10 birds each.

Overall Challenge Scheme

At d 18 of age, broilers were given a 1 mL oral gavage of an *Eimeria* spp. solution. From d 21 to 23, a 1 mL oral gavage of *C. perfringens* was administered. Intestinal lesion scoring and jejunum tissue sample collection were performed during necropsy, 9 d post-*Eimeria* challenge as described below.

Eimeria Challenge

ADVENT® (Huvepharma) coccidiosis vaccine was diluted with sterile water and administered at a 10 x recommended dose per bird. The ADVENT® coccidiosis vaccine contains live oocysts of *E. acervulina*, *E. maxima*, *E. tenella*. It contains gentamicin and amphotericin B as preservatives. One mL of this *Eimeria* spp. solution was given to the challenged birds on d 18, via an oral gavage using a 1 mL Tuberculin syringe (Thermo Fisher Scientific).

Clostridium perfringens Challenge.

A *C. perfringens* strain, with NetB toxin producing ability, was isolated from a bird that had been diagnosed with NE (Bailey *et al.* 2013). The strain was maintained as a frozen stock in glycerol at -80°C until further application. The frozen bacterial isolate was revived by streaking a loop-full of frozen culture on reduced tryptic soy agar (rTSA) containing 5% sheep blood, incubated under anaerobic conditions (5%CO_2, 5%H and 90%N_2) at 37°C for 24 hours. After 24 hours, each plate was checked for purity. A single colony with typical *C. perfringens* morphology was picked, inoculated in brain heart infusion broth (100 mL) and incubated anaerobically for 24 hours at 37°C. After incubation, the culture was diluted (1:100) to produce an approximately 10^7 CFU/mL inoculum. The inoculum was orally administered to broilers of the challenged treatments groups on d 21, 22, and 23. Fresh inoculum was prepared for each challenge day. Inoculum levels were verified by serially diluting and spread plating the inoculum on duplicate onto rTSA containing 5% sheep blood plates, incubated anaerobically for 24 hours at 37°C and viable colonies showing typical colony morphology were counted.

Measurements and Sample Collection

Body weight (BW) and feed intake (FI) were measured on d 0, 14, and 27 on a per pen basis. Morbid birds were removed, euthanized, and BW recorded daily; mortalities were also removed daily and recorded. Mortality corrected feed conversion ratio (AFCR), body weight gain (BWG) per bird and FI per bird were calculated based off of the removed body weights where appropriate. On d 27, five birds were randomly selected from each pen and euthanized by CO_2 gas inhalation, followed by cervical dislocation. A jejunum sample (2 cm in length) was collected 15 cm proximal from the Meckel's diverticulum per bird, prior to lesion scoring for sterility. Samples were then pooled by pen and stored at -80°C until DNA extraction, as described below. NE lesions were scored in the duodenum, jejunum, and ileum using a modified scale of Prescott *et al.* (1978). Lesions were scored on a scale of 0 to 4, where 0 = no apparent lesions, 1 = thin friable intestines; 2 = focal necrosis, ulceration or both; 3= patchy necrosis; and 4= severe extensive mucosal necrosis. Coccidiosis lesions were scored according to the methods described by Johnson and Reid (2007). The duodenum, jejunum, and ileum were scored for *E. acervulina* and *E. maxima* on a scale of 0 to 4.

Diet and Mycotoxin Analyses

Prior to formulation, corn was analyzed for mycotoxin concentrations (Trilogy Laboratory Inc.; Table 3.3). Diet samples were analyzed for moisture, dry matter, crude protein (CP), crude fat, acid detergent fiber (ADF), neutral detergent fiber (NDF) and crude fiber (CF) (Midwest Laboratories Inc.; Table 3.2). Following termination of the experiment, a sample of the L-DDGS source was analyzed for CP, CF, ADF and NDF (Midwest Laboratories Inc.; Table 3.1). Geometric particle size was determined by a Tyler Ro-Tap shaker (Auburn, AL; ASAE Standard S319.4, 2009; Table 3.1).

DNA Extraction, Amplification, and 16S rRNA Gene Sequencing Data Analysis

Jejunum tissue samples were removed from the -80C freezer, thawed, and bacterial DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI) according to manufacturer instructions. Extracted DNA was tested for concentration and purity with a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific) according to manufacturer instructions. The standard for purity was a 260nm/280nm ratio between 1.8 - 2.0. Extracted DNA was stored in -20°C until further analysis.

Stage 1 of PCR amplification was performed at Auburn University using an iQ5 thermocycler (Bio-Rad, Hercules, CA), with reaction conditions described in Table 3.4. Eurofins Genomics® 16S rRNA gene, universal target primers (CS1/515F and CS2/926R) were utilized for amplification (Eurofins Genomics LLC). A touchdown protocol was performed, as described in Table 3.5. The DNA denaturation step was chosen based on the half-life of Taq (Innis and Gelfand, 2012). Annealing conditions were dependent on the base composition and length of the primers, where the primer extension parameters were based on the optimal temperature of Taq and length of the target sequence. Amplification of Stage 1 PCR yields were verified by gel electrophoresis in a 2% agarose gel. Running buffer and gels were made using 1x concentration of AccuGENE TBE buffer (Lonza Group, Basel, Switzerland). Electrophoresis was performed at 75v for ~1 hour or until sufficient separation between products was obtained. Lonza® 100 bp Extended Range DNA Ladder was used as a DNA size standard. Stage 1 PCR yields were stored at 4°C until further analysis.

Stage 1 PCR yields were sent to University of Illinois Chicago DNA Services Facility (UIC DNAS) facility for targeted amplicon sequencing (TAS). An 8 cycle Stage 2 PCR reaction (Table 3.5) was performed. The variable regions V3-V4 of the 16S rRNA gene were amplified using Fluidigm's Access Array™ Barcoded primers (0.4 μM concentration), in preparation for Illumina MiSeq sequencing (Illumina, Inc.) (Table 3.4). After sample amplification, purification and normalization were performed using SequalPrep plates (Thermo Fisher Scientific). The final pooled libraries were quantified using a formula based on Qubit 3.0 Fluorometer quantification (ThermoFisher Cat#Q33217), and diluted to 2 nM. The libraries were then denatured with 0.2 NaOH, according to Illumina MiSeq protocol. The volume of the final sample loading mixture is 600 μl, including the appropriate library concentration and spike in phiX control (generally 20%).

Forward and reverse reads were merged using the software package PEAR (Zhang *et al.*, 2014). Ambiguous nucleotides were trimmed from the ends and reads with internal ambiguous nucleotides were discarded. Primer sequences were identified using Smith-Watermann alignment, where reads that lacked either primer sequence were discarded. Sequences were then trimmed based on quality scores using a modified Mott algorithm with PHRED quality threshold

of P = 0.01. After trimming, any sequences < 400 bases were discarded. Chimeric sequences were identified using the USEARCH algorithm with GreenGenes 13_8 reference sequences (Edgar, 2010; McDonald *et al.*, 2012).

The software package QIIME was then used to generate operational taxonomic unit (OTU) clusters in a *de novo* manner using UCLUST algorithm with a 97% similarity threshold (Edgar, 2010; Caporaso *et al.*, 2010). Taxonomic annotations for each OTU were generated using the UCLUST algorithm and the GreenGenes 13_8 reference database with a minimum similarity threshold of 90% (Edgar, 2010; McDonald *et al.*, 2012). Taxonomic and OTU abundance data were merged into a single OTU table, as a biological observation matrix (BIOM) (McDonald, 2012). Species alpha-diversity indices (Shannon index (H') and Margalef Species Richness) were calculated from the OTU table, in BIOM format. 100% stacked column histograms of experimental treatment averages based on phylogenic classification were used to assess bacterial community composition (Figure 3.1). In order to further reduce noise within the data, less abundant OTUs which represented < 1% in all treatment averages, were categorized as "Other".

Data Analysis

All statistical analyses, outside of 16S rRNA gene sequencing, were facilitated using SPSS version 22 software. Significant differences were reported at $P \leq 0.05$. A two-way ANOVA was used to determine main effect and interactions, with diet and challenge as main effects when analyzing the performance and NE results. A Mann-Whitney U Test was used to determine pairwise comparisons of treatment microbiome alpha-diversity indices.

Results and Discussion:

Table 3.1 demonstrates the provided nutrient profile of the L-DDGS source, as well as those values that were analyzed (Midwest Laboratories Inc.) following termination of the experiment. There were differences between the company provided values and those that were analyzed: crude protein (CP; 28.9 vs. 26.40%, crude fiber (CF; 4.85 vs. 5.86%), acid detergent fiber (ADF; 11.62 vs. 8.60), and neutral detergent fiber (NDF; 25.31 vs. 24.30%). The crude fat value of the L-DDGS source utilized in this study was 4.29%. Limited research including L-

DDGS in poultry diets has been conducted; however, this crude fat value was even lower than those values used in other studies (Purdum *et al.*, 2014; Guney *et al.*, 2013).

The ingredient, calculated, and proximate analysis nutrient composition (Midwest Laboratories, Inc.) of experimental diets are demonstrated in Table 3.2. Animal diets (particularly swine and poultry) are not formulated on CP and crude fat basis, but rather on a metabolizable energy (ME) and digestible amino acid basis. In the current experiment, provided L-DDGS nutrient values (Table 3.1) were used in dietary formulation, using a least cost formula, on a total amino acid basis (Creative Formulation Concepts, LLC). The ME content of traditional corn-DDGS, are similar to that of corn (Pedersen *et al.*, 2007). However, the process of oil extraction drastically changes the oil content of DDGS, which alters the concentration of protein and fiber (Saunders and Rosentrater, 2009; Liu and Rosentrater, 2012), thus altering energy content (Ren *et al.*, 2011; Graham *et al.*, 2014). The L-DDGS source was added to a corn-soy diet, requiring additional supplementation of fat (poultry oil) to meet target ME.

The feeding value of DDGS may be largely based on its energy content, where changing the oil content of DDGS may affect growth performance (Graham *et al.*, 2014). Cortes *et al.* (2015) determined that the inclusion of L-DDGS (6.54% or 5.39% EE) at 0%, 6%, or 12% had no detrimental effect on layer growth performance. Similar results were reported by Guney *et al.* (2013) when broilers were fed L-DDGS (7.52 or 6.74% EE) at 10 or 20% inclusion for 18 d. However, the inclusion levels in the current experiment, 5.0% L-DDGS (4.29% crude fat) inclusion in the starter and 7.5% in the grower, resulted in reduced broiler live performance (Table 3.6). Birds consuming the L-DDGS diet experienced reduced BWG at 14 to 27 d of age ($P = 0.013$) in comparison to birds consuming the control diet, but challenge had no effect and there was no interaction ($P > 0.05$). In addition, birds consuming the L-DDGS diet experienced increased AFCR at 14 to 27 d of age ($P = 0.010$) and 0 to 27 d of age ($P = 0.003$) in comparison to birds consuming the control diet. Again, challenge had no effect and no interaction was observed ($P > 0.05$). Mycotoxins were determined to be present in low concentrations in the L-DDGS source and therefore did not influence performance results (Table 3.3; Trilogy Laboratory Inc.). Table 3.6 shows that neither of these factors seemed to relate to palatability or voluntary consumption, as diet had no significant effect on FI during any period ($P > 0.05$). Indication of

an increased need for energy was noticed, as FI was slightly greater in the L-DDGS diet treatment birds than the control diet treatment birds, at 14 to 27 d (1.481 vs. 1.467) and 0 to 27 d (2.038 vs. 1.996) of age. Similar results were noted by Purdum *et al.* (2014) in laying hens, where lower fat DDGS resulted in a decreased weight gain, and a slightly increased feed intake was noticed. However, proximate nutrient analysis values of experimental diets implied otherwise, as ME was found to be greater in the L-DDGS diets than the control diets (Table 3.2). Performance results could be related to fiber content and amino acid variation of the source, as a result of oil-extraction. As previously mentioned, analyzed values of the L-DDGS source by Midwest Labs (Midwest Laboratories Inc.) differed from provided values included in dietary formulation. Attempts were made to formulate diets to balance all known nutrients and expected performances to be equal. Had analyzed values of the L-DDGS product been included in diet formulation and diets been calculated on a digestible amino acid basis versus a total amino acid basis, performance results may have differed.

The L-DDGS diet did not influence NE incidence, severity or average lesion scores, as diet had no effect and no interaction was observed ($P > 0.05$; Table 3.7). Similar results were observed by Perez *et al.* (2009), as dietary DDGS did not prevent infection in pigs challenged with pathogenic *Escherichia coli*. As expected, challenged birds experienced a greater NE severity in comparison to unchallenged birds ($P = 0.045$). Dietary L-DDGS provided no evidence of promoting a recovery from coccidiosis. These results were supported by the findings of Perez *et al.* (2011) who concluded that concentrations of DDGS up to 20% of the diet did not prevent *E. acervulina* infection or promote faster recovery in young chicks.

Targeted amplicon sequencing (TAS) was conducted to uncover the bacterial communities in the jejunum tissue of broilers at 27 d of age. The jejunum was the intestinal segment collected, as it is involved in both absorption of nutrients and minerals, harbors immune cells that are important for monitoring luminal content (Bar-Shira *et al.*, 2003; Shokker *et al.*, 2009), and is the segment in which NE lesions have been found to be most common (Truscott & Al-Sheikhly, 1977; Fukata *et al.*, 1991) and severe (Long *et al.*, 1973). 16S rRNA gene sequences were rarified to 400 sequences per sample due to high host-DNA presence in the stage 1 PCR product. Those samples that contained < 400 sequences were excluded from data analysis.

Consequentially, this could lead to a more conservative view of the abundances of less common taxa across samples (Goodrich *et al.*, 2014). Hence, a direct comparison of OTUs and taxonomic composition between reported and the current study may not be accurate due to differences in experimental approaches (Cisek and Binek, 2014; Lumpkins *et al.*, 2010).

Alpha-diversity indices were analyzed, according to Species Richness (Margalef) and Shannon Index (H'). Alpha-diversity indices provide information about the rarity and commonness of species in a community, in order to better understand its structure. Numerous relationships have been utilized as basis for species richness indices, in which Margalef suggests a logarithmic relationship: $R_1 = (S\text{-}1)/Log\ N$, where S is the number of taxa and N is the number of individuals. Therefore, the Margelf index implies no particular regularity in distribution, in which the number of individuals in all the species are taken into account (Margalef, 1969). Distinctive differences in Species Richness of the jejunum tissue microbiota was observed of unchallenged control treatment versus the unchallenged L-DDGS treatment (P = 0.027; Table 3.9). The Shannon Index accounts for both abundance and evenness of the species present (Beals *et al.*, 2000). It is calculated using the following formula: $H' = -\sum p_i \ln p_i$, where p_i is the proportion of individuals found in species i. Typical values are generally between 1.5 and 3.5, where the index is rarely greater than 4. The Shannon Index increases as both the richness and evenness of the community increase (Magurran, 2004), where it represents the uncertainty about the identity of an unknown individual. Distinctive differences in Shannon Index values of the jejunum tissue microbiota of the unchallenged control treatment versus the unchallenged L-DDGS treatment were observed (P = 0.015; Table 3.9). In a highly diverse and evenly distributed system like the unchallenged L-DDGS treatment, an unknown individual could belong to any species, leading to a high uncertainty in predictions of its identity (Table 3.8). Whereas in a less diverse system, like the unchallenged control treatment, it is easier to predict the identity of unknown individuals, as it is dominated by one or a few species (Shannon, 1948; Table 3.8). No significant differences were noticed on alpha-diversity indices of challenged treatments (P > 0.05), as a result of experimental *Eimeria* spp. and *C. perfringens* challenge (Table 3.9).

The OTU table, in BIOM format, revealed 9 phyla, 17 classes, 30 orders, and 54 families of known bacteria present in rarefied samples (n = 30). In order to further reduce noise within the data, less abundant OTUs which represented < 1% in all treatment averages, were classified as "Other". The 5 most common phyla were *Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia* (Figure 3.1). Other studies have found *Firmicutes, Bacteroidetes* and *Proteobacteria* to also account for the majority of the intestinal bacteria of chickens (Pan and Yu, 2013; Wei *et al.*, 2013; Xiao *et al.*, 2016). *Firmicutes* was the predominant phylum found in all treatment birds at 27 d of age, accounting on average, for more than 82% of all major OTUs. This supports the findings of other studies that have found *Firmicutes* to account for 60 – 70% of all bacterial sequences of chicken intestinal microbiome (Pan and Yu, 2013; Xiao *et al.*, 2016). *Verrucomicrobia*, on the other hand, has been more commonly found as a minor phylum than a major phylum, representing no more than several sequences, suggesting low abundance and prevalence in the gut of chickens (Wei and Yu, 2013). Therefore, this may have been a result of rarefication of the data set.

At the family level, *Rikenellaceae, Enterococcaceae, Lactobacillaceae, Clostridiaceae, Lachnospiraceae, Ruminococcaceae, Enterobacteriaceae*, and *Verrucomicrobiaceae* were most common in different proportions (Figure 3.1). Other studies have also found *Lactobacillaceae, Clostridiaceae,* and *Enterococcaceae* to be the most abundant in the intestinal tracts of chickens (Samsudin and Al-Hassani, 2013). At the genus level, *Lactobacillaceae Lactobacillus* has been found to be more common in small intestine, indicating its contribution towards intestinal functions related to nutrient absorption. It is considered one of the most important bacterial groups that maintains the equilibrium of the microbial ecosystem (Pryde *et al.*, 1999), where disturbances to microbial equilibrium can result in the onset of disease. Through interaction with the epithelial cells, *Lactobacilli* can offer protection at the intestinal barrier by antagonistic activities against pathogens, where some *Lactobacilli* can inhibit the growth of *C. perfringens* (Sengupta *et al.*, 2013; Dec *et al.*, 2014*). Lactobacillaceae* was found in increasing proportions, across all treatments, with that of *Clostridiaceae*, the family in which *C. perfringens* exists. Results imply *Lactobacillaceae* can have beneficial effects on the small intestine, against *C. perfringens*. However, their indirect effects on other bacteria may be of even greater importance.

Butyrate-producing members of the *Ruminococcaceae* and *Lachnospiraceae* family have been isolated from the ceca contents of chickens (Eeckhaut *et al.*, 2011), in which some strains may contribute to cytokine production that support small intestine integrity by affecting tight junction expression and epithelial cell proliferation (Furness *et al.*, 2013). An indirect effect of lactate-producing bacteria to cross-feed lactate-utilizing and butyrate-producing bacteria has been suspected to be of importance (De Maesschalck *et al.*, 2015). *Ruminococcaceae* (9.22 to 12.07%) and *Lactobacillaceae* (20.29 to 21.14%) proportions were similar among treatments. The unchallenged control treatment was an exception, where butyrate-producing families were reduced (9.34%, 5.47%), and increased proportions of *Lactobacillaceae* (23.41%) and *Clostridiaceae* (51.25%) were observed (Figure 3.1). Overall, results imply lactate-producing strains of bacteria and butyrate producing bacteria can have beneficial effects against *C. perfringens*. However, their indirect effects on each other, and whether this is a consequence or predisposing factor to NE development remains unclear.

Conclusion:

When including L-DDGS (4.29% crude fat) sources in broiler diets, nutrient values should be analyzed prior to diets being formulated on a digestible versus total amino acid basis. When left unconsidered, a 5% inclusion level in the starter diet and 7.5% inclusion level in the grower diet will have detrimental effects on performance (BWG, AFCR). In this experiment, results imply L-DDGS did not shift the intestinal microbiota, as it did not influence NE development. The relationship between lactate- and butyrate-producing bacteria should be further studied, as they appear to have beneficial effects on NE formation.

Table 3.1 Proximate nutrient composition of low-oil distillers dried grains with solubles (L-DDGS)[1]

Item	Value	Method of Analysis[2,3]
Protein, fat, fiber (%)		
Moisture	10.68	
Dry matter	89.33	
Crude protein	28.39	
Analyzed	26.40	AOAC official method 990.03
Crude fat	4.29	
Crude fiber	4.85	
Analyzed	5.86	ANKOM Tech./AOAC official method 2001.11
ADF	11.62	
Analyzed	8.60	AOCS Ba 6a-05
NDF	25.31	
Analyzed	24.30	ANKOM Tech. Method
Ash	4.74	
Minerals (%, unless otherwise indicated)		
Calcium	0.05	
Phosphorous	0.80	
Sodium	0.28	
Potassium	1.15	
Magnesium	0.34	
Sulfur	0.81	
Copper (ppm)	5.80	
Iron (ppm)	62.50	
Manganese (ppm)	12.00	
Zinc (ppm)	44.50	
Amino Acids (%)		
Alanine	1.97	
Arginine	1.08	
Aspartic acid	1.81	
Cystine	0.48	
Glutamic acid	4.71	
Glycine	1.10	
Histidine	0.74	
Isoleucine	1.02	
Leucine	3.07	
Lysine	0.92	
Methionine	0.61	
Phenylalanine	1.31	
Proline	2.26	
Serine	1.40	
Threonine	1.08	
Tryptophan	0.20	
Tyrosine	0.73	
Valine	1.34	
Particle size (microns)	499	ASAE Standard S319.4

[1]Low-oil DDGS (4.29% crude fat) on an as-fed basis.
[2]All method of analysis were determined by Midwest Laboratories, Inc. (Omaha, NE).
[3]AOAC = AOAC International (Gaithersburg, MD); ANKOM = ANKOM Technology (Macedon, NY); AOCS = The American Oil Chemists' Society (Urbana, IL); ASAE = American Society of Agricultural Engineers (St. Joseph, MI).

Table 3.2 Ingredient and nutrient composition of diets fed to broilers from 0 to 27 d of age

	Starter Diets		Grower Diets		
Ingredient (%)	Control	L-DDGS	Control	L-DDGS	Method of Analysis[4,5]
Corn	62.621	59.511	66.540	61.875	
Soybean Meal 48	28.494	26.044	24.830	21.156	
L-DDGS (4.29% ether extract)[1]	0.000	5.000	0.000	7.500	
Poultry Meal 65	4.000	4.000	4.000	4.000	
Poultry Oil	0.948	1.546	1.112	2.008	
Salt	0.404	0.372	0.405	0.357	
Dicalcium Phosphate	1.409	1.279	1.111	0.915	
Limestone	1.127	1.211	1.079	1.205	
DL-Methionine	0.362	0.355	0.314	0.304	
L-Lysine 98	0.193	0.235	0.246	0.310	
L-Threonine	0.192	0.196	0.112	0.120	
Vitamin Premix[2]	0.050	0.050	0.050	0.050	
Choline Chloride Dry	0.100	0.100	0.100	0.100	
Trace Minerals Premix[3]	0.100	0.100	0.100	0.100	
Calculated Nutrient Content (% unless otherwise indicated)					
ME (kcal/kg)	3,053	3,053	3,109	3,109	
Crude Protein	21.500	21.500	20.000	20.000	
Calcium	0.930	0.930	0.840	0.840	
Phosphorous, Available	0.450	0.450	0.390	0.390	
Sodium	0.200	0.200	0.200	0.200	
Proximate Analysis Nutrient Content (% unless otherwise indicated)					
Dry Matter	85.780	86.260	87.130	87.430	Calculation
Crude Protein	21.500	20.700	20.600	19.400	AOAC official method 990.03
Crude Fat	3.580	4.040	3.850	4.950	AOAC official method 945.16
Acid Detergent Fiber	3.600	3.800	3.600	3.300	ANKOM Tech. Method
Ash	4.720	4.990	5.140	4.590	AOAC official method 942.05
Crude Fiber	1.980	2.040	1.690	2.240	AOCS Ba 6a-05
Neutral Detergent Fiber	6.700	8.800	6.800	8.200	Ankom Tech./AOAC official method 2001.11

[1]L-DDGS = Low-oil DDGS (4.29% crude fat) on an as fed-basis.
[2]Vitamin premix includes per kg of diet: Vitamin A (Vitamin A acetate), 18,739 IU; Vitamin D (cholecalciferol), 6,614 IU; Vitamin E (DL-alpha tocopherol acetate), 66 IU; menadione (menadione sodium bisulfate complex), 4 mg; Vitamin B12 (cyanocobalamin), 0.03 mg; folacin (folic acid), 2.7 mg: D-pantothenic acid (calcium pantothenate), 31 mg; riboflavin (riboflavin), 22.1 mg; niacin (niacinamide), 88.2 mg; thiamin (thiamin mononitrate), 5.5 mg; D-biotin (biotin), 0.18 mg; and pyridoxine (pyridoxine hydrochloride), 7.7 mg.
[3]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, cypress excel Se yeast), 0.3 mg
[4]All methods of analysis were determined by the Midwest Laboratories Inc. (Omaha, NE).

[5]AOAC = AOAC International (Gaithersburg, MD); ANKOM = ANKOM Technology (Macedon, NY); AOCS = The American Oil Chemists' Society (Urbana, IL); ASAE = American Society of Agricultural Engineers (St. Joseph, MI).

Table 3.3 Mycotoxin concentrations of low-oil distillers dried grains with solubles (L-DDGS) and ground corn included in diets fed to broiler 0 to 27 d of age

Analysis[1,2]	Results	Method[3]
Experimental Corn (ppb, unless otherwise indicated)		
AF B1	ND[4]	Internal SOP-14-168
AF B2	ND	Internal SOP-14-168
AF G1	ND	Internal SOP-14-168
AF G2	ND	Internal SOP-14-168
DON, ppm	0.1	Internal SOP-14-168
FUM B1, ppm	3.1	AOAC 999.15 with modifications
FUM B2, ppm	1.0	AOAC 999.15 with modifications
FUM B3, ppm	0.5	AOAC 999.15 with modifications
OTA	ND	AOAC 2000.03 with modifications
T-2 Toxin	ND	Internal SOP-14-168
ZEN	ND	Internal SOP-14-168
L-DDGS[5] (ppb, unless otherwise indicated)		
Total AF	ND	Internal SOP-14-168
Total FUM, ppm	ND	AOAC 999.15 with modifications
OTA	ND	AOAC 2000.03 with modifications
DON, ppm	1.30	Internal SOP-14-168
T-2 Toxin, ppm	ND	Internal SOP-14-168
ZEN	ND	Internal SOP-14-168

[1]All methods of analysis were determined by Trilogy Analytical Laboratory, Inc. (Washington, MO).
[2]AF = Alfatoxin; DON = Deoxynivalenol; FUM = Fumonisin; OTA = Ochratoxin A; ZEN = Zearalenone; ppb = Parts per billion; ppm = Parts per million.
[3]SOP = Standard operating procedure; AOAC = AOAC International (Gaithersburg, MD).
[4]ND = None detected.
[5]L-DDGS = Low-oil DDGS (4.29% crude fat).

Table 3.4 Reaction conditions and primers for amplification of bacterial populations of jejunum tissue collected from broilers at 27 d of age by polymerase chain reaction (PCR)

Reaction conditions	1 x Rxn volume (µL)
Stage 1[1]	
Master Mix[2]	12.5
DdH_2O[3]	10.75
DNA Template[4]	1.0
Forward Primer[5]	0.25
Reverse Primer[6]	0.25
BSA[7]	0.25
Stage 2[8]	
Primers[9]	0.4
Stage 1 PCR Yield	1.0

[1]Performed at Auburn University Laboratory (Auburn, AL).
[2]Master Mix = Lucigen EconoTaq Plus 2x Master Mix (Middleton, WI): 0.1 units/µL of EconoTaq DNA Polymerase, Reaction Buffer (pH 9.0), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dATP, 3 mM MgCl2, and a proprietary mix of PCR Enhancer/Stabilizer and blue and yellow tracking dyes.
[3]DdH_2O = double distilled H_2O.
[4]DNA Template = pooled jejunum tissue sample (5 birds/pen, each 2 cm in length, 15 cm proximal from Meckel's diverticulum) bacterial DNA extracted using Wizard Genomic Purification Kit (Madison, WI).
[5]Forward Primer = Eurofins Genomics CS1/515F Universal Target 16S rRNA Gene (Louisville, KY): 5'-GTGYCAGCMGCCGCGGTAA-3', expected amplicon size with CS1 456, annealing temperature 50°C.
[6]Reverse primer = Eurofins Genomics® CS2/926R Universal Target 16S rRNA Gene (Louisville, KY): 5'-CCGYCAATTYMTTTRAGTTT-3', expected amplicon size with CS2 456, annealing temperature 50°C.
[7]BSA (Bovine Serum Albumin) = Purified BSA 100x (Lawrenceville, GA).
[8]Performed at University of Illinois Chicago DNA Services Facility (Chicago, IL).
[9]Primers = Fluidigm's AccessArray Barcoded Primers (San Francisco, CA): Read 1: Standard CS1: A+CA+CTG+ACGACATGGTTCTACA; Index Read: CS2rc: A+GAC+CA+AGTCTCTGCTACCGTA; Read 2: Standard CS2: T+AC+GGT+AGCAGAGACTTGGTCT (A "+" preceding a base indicates a Locked Nucleic Acids(LNA)-base).

Table 3.5 Polymerase chain reaction (PCR) conditions for amplification of bacterial populations of jejunum tissue collected from broilers at 27 d of age

Cycle Step	Temperature	Time	Cycles
Stage 1[1]			
Initial Denaturation	94°C	5 min	1x
Variable Temperature Annealing			15x, reduce 1°C/cycle
Denaturation	94°C	30 sec	
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Constant Temperature Annealing	94°C		30x
Denaturation	94°C	30 sec	
Annealing	55°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	7 min	1x
Hold	4°C	∞	
Stage 2[2]			
Initial Denaturation	95°C	5 min	1x
Variable Temperature Annealing			28x
Denaturation	95°C	30 sec	
Annealing	60°C	30 sec	
Extension	72°C	30 sec	

[1]Performed at Auburn University Laboratory (Auburn, AL) using a Bio-Rad IQ5 thermocycler (Hercules, CA).
[2]Performed at University of Illinois Chicago DNA Services Facility (Chicago, IL) in preparation for Illumina MiSeq (San Diego, CA).

Table 3.6 Growth performance[1] of broilers challenged[2] or unchallenged with *C. perfringens* and *Eimeria* spp. fed a control diet (corn-soy) or low-oil distillers dried grains with solubles (L-DDGS)[3] diet from 0 to 27 d of age

	BW Gain (kg/bird)			Feed Intake (kg/bird)			AFCR[4] (kg/kg)			Mortality[5] (%)		
Day	0 to 14	14 to 27	0 to 27	0 to 14	14 to 27	0 to 27	0 to 14	14 to 27	0 to 27	0 to 14	14 to 27	0 to 27
Diet x Challenge												
P ≤	0.712	0.078	0.237	0.238	0.754	0.891	0.288	0.359	0.427	1.000	0.649	0.250
SEM[6]	0.004	0.020	0.026	0.005	0.037	0.053	0.008	0.032	0.016	0.008	0.024	0.028
Diet Main Effect												
Control	0.442	**0.949**	1.400	0.524	1.467	1.996	1.185	**1.525**	**1.412**	0.006	0.017	0.022
L-DDGS	0.441	**0.897**	1.368	0.522	1.481	2.038	1.183	**1.612**	**1.464**	0.006	0.050	0.056
P ≤	0.920	**0.013***	0.221	0.667	0.697	0.441	0.737	**0.010***	**0.003***	1.000	0.178	0.250
SEM	0.003	0.014	0.018	0.003	0.026	0.038	0.006	0.022	0.012	0.006	0.017	0.020
Challenge Main Effect												
Challenged	0.440	0.927	1.384	0.521	1.470	2.011	1.186	1.573	1.443	0.000	0.033	0.033
Unchallenged	0.444	0.920	1.384	0.525	1.478	2.023	1.182	1.564	1.443	0.011	0.033	0.044
P ≤	0.292	0.727	0.979	0.370	0.822	0.828	0.642	0.778	0.557	0.167	1.000	0.699
SEM	0.003	0.014	0.018	0.003	0.026	0.038	0.006	0.022	0.012	0.006	0.017	0.020

[1]Each value represents means of 9 replicate pens having 10 chicks at placement.
[2]*Eimeria spp.* challenge (d 18): ADVENT® (Huvepharma) coccidiosis vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. tenella* administered at a 10 x recommended dose per challenged bird; *C. perfringens* challenge (d 21, 22, 23): Inoculated brain heart infusion broth was diluted 1:100 to produce an approximately 10^7 CFU/mL inoculum containing an isolate from a bird diagnosed with necrotic enteritis.
[3]L-DDGS = Low-oil DDGS (4.29% crude fat); 5% inclusion in the starter diet, 7.5% inclusion in the grower diet.
[4]AFCR = Mortality corrected feed conversion ratio
[5]Mortality data underwent square-root arcsine transformation before analysis.
[6]SEM= Pooled standard error.

Table 3.7 Necrotic Enteritis (NE) intestinal lesion[1,2] incidence, severity and average lesion scores of broilers challenged[3] or unchallenged with *C. perfringens* and *Eimeria* spp. fed a control diet (corn-soy) or low-oil distillers dried grains with solubles (L-DDGS)[4] diet from 0 to 27 d of age

27 d	NE Incidence[5] (%)	NE Severity[6] (%)	NE Average Lesion Score[7]
Diet x challenge			
P ≤	0.442	0.774	0.767
SEM[8]	0.072	0.039	0.130
Diet Main Effect			
Control	0.389	0.078	0.511
L-DDGS	0.356	0.067	0.417
P ≤	0.645	0.774	0.473
SEM	0.051	0.027	0.092
Challenge Main Effect			
Challenged	0.433	**0.111**	0.572
Unchallenged	0.311	**0.033**	0.356
P ≤	0.092	**0.045***	0.105
SEM	0.051	0.027	0.092

[1]Necrotic enteritis (NE) lesions were scored in the duodenum, jejunum, and ileum using a modified scale of Prescott *et al.* (1978
[2]Each value represents mean of 9 replicate pens, where 5 birds were randomly chosen per pen.
[3]*Eimeria spp.* challenge (d 18): ADVENT® (Huvepharma) coccidiosis vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. tenella* administered at a 10 x recommended dose per challenged bird; *C. perfringens* challenge (d 21, 22, 23): Inoculated brain heart infusion broth was diluted 1:100 to produce an approximately 10^7 CFU/mL inoculum containing an isolate from a bird diagnosed with necrotic enteritis.
[4]L-DDGS = Low-oil DDGS (4.29% crude fat); 5% inclusion in the starter diet, 7.5% inclusion in the grower diet.
[5]NE Incidence = Percentage of birds a lesion score ≥ 1.
[6]NE Severity = Percentage of birds a lesion score ≥ 2.
[7]NE Average Lesion Score =Mean of scores between 0 and 4.
[8]SEM = Pooled standard error.

Table 3.8 Alpha-diversity indices[1,2] of 16S rRNA gene sequences from jejunum tissue[3] of broilers challenged[4] or unchallenged with *C. perfringens* and *Eimeria* spp. fed a control diet (corn-soy) or low-oil distillers dried grains with solubles (L-DDGS)[5] diet from 0 to 27 d of age

Sample DNA	Species Richness (Margalef)[6]	SEM[8]	Shannon Index (H')[7]	SEM
27 d				
Treatments				
Challenged Control	3.616	0.375	1.909	0.280
Unchallenged Control	3.317	0.267	1.406	0.189
Challenged L-DDGS	3.579	0.350	1.937	0.191
Unchallenged L-DDGS	4.220	0.215	2.173	0.160

[1]Alpha-diversity indices were calculated from the operational taxonomic unit (OTU) table derived from sequences with a 97% similarity threshold, using QIIME (San Diego, CA) software package.
[2]Each value represents treatment means: Challenged Control (n = 6), Unchallenged Control (n = 8), Challenged L-DDGS (n = 9), Unchallenged L-DDGS (n = 7).
[3]Jejunum samples (5 birds/pen, each 2 cm in length, 15 cm proximal from Meckel's diverticulum) collected on d 27; 16S rRNA gene sequences were rarified to 400 sequences per sample due to high host-DNA presence.
[4]*Eimeria spp.* challenge (d 18): ADVENT® (Huvepharma) coccidiosis vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. tenella* administered at a 10 x recommended dose per challenged bird; *C. perfringens* challenge (d 21, 22, 23): Inoculated brain heart infusion broth was diluted 1:100 to produce an approximately 10^7 CFU/mL inoculum containing an isolate from a bird diagnosed with necrotic enteritis.
[5]L-DDGS = Low-oil DDGS (4.29% crude fat).
[6]Shannon Index: $H' = -\sum p_i \ln p_i$, where p_i is the proportion of individuals found in species i.
[7]Species richness (Margalef): $R_1 = (S\text{-}1)/Log\ N$, where S is the number of taxa and N is the number of individuals.
[8]SEM = Pooled standard error.

Table 3.9 Pairwise comparisons of treatment alpha-diversity indices[1,2] of 16S rRNA gene sequences from jejunum tissue[3] of broilers challenged[4] or unchallenged with *C. perfringens* and *Eimeria* spp. fed a control diet (corn-soy) or low-oil distillers dried grains with solubles (L-DDGS)[5] diet from 0 to 27 d of age

Sample DNA	Species Richness (Margalef)[6]	Shannon Index (H')[7]
Mann-Whitney U Test		
P-value		
Challenged L-DDGS vs. Unchallenged Control	0.333	0.054
Challenged L-DDGS vs. Unchallenged L-DDGS	0.184	0.266
Challenged L-DDGS vs. Challenged Control	0.859	0.627
Unchallenged Control vs. Unchallenged L-DDGS	**0.027***	**0.015***
Unchallenged Control vs. Challenged Control	0.746	0.156
Unchallenged L-DDGS vs. Challenged Control	0.252	0.568

[1]Values represent pairwise-comparisons between non-parametric treatments: Challenged Control (n = 6), Unchallenged Control (n = 8), Challenged L-DDGS (n = 9), Unchallenged L-DDGS (n = 7).

[2]Alpha-diversity indices were calculated from the operational taxonomic unit (OTU) table derived from sequences with a 97% similarity threshold, using QIIME (San Diego, CA) software package.

[3]Jejunum samples (5 birds/pen, each 2 cm in length, 15 cm proximal from Meckel's diverticulum) collected on d 27; 16S rRNA gene sequences were rarified to 400 sequences per sample due to high host-DNA presence.

[4]*Eimeria spp.* challenge (d 18): ADVENT® (Huvepharma) coccidiosis vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. tenella* administered at a 10 x recommended dose per challenged bird; *C. perfringens* challenge (d 21, 22, 23): Inoculated brain heart infusion broth was diluted 1:100 to produce an approximately 10^7 CFU/mL inoculum containing an isolate from a bird diagnosed with necrotic enteritis.

[5]L-DDGS = Low-oil DDGS (4.29% crude fat).

[6]Shannon Index*:* $H' = -\sum p_i \ln p_i$, where p_i is the proportion of individuals found in species *i*.

[7]Species richness (Margalef): $R_1 = (S\text{-}1)/Log\ N$, where *S* is the number of taxa and *N* is the number of individuals.

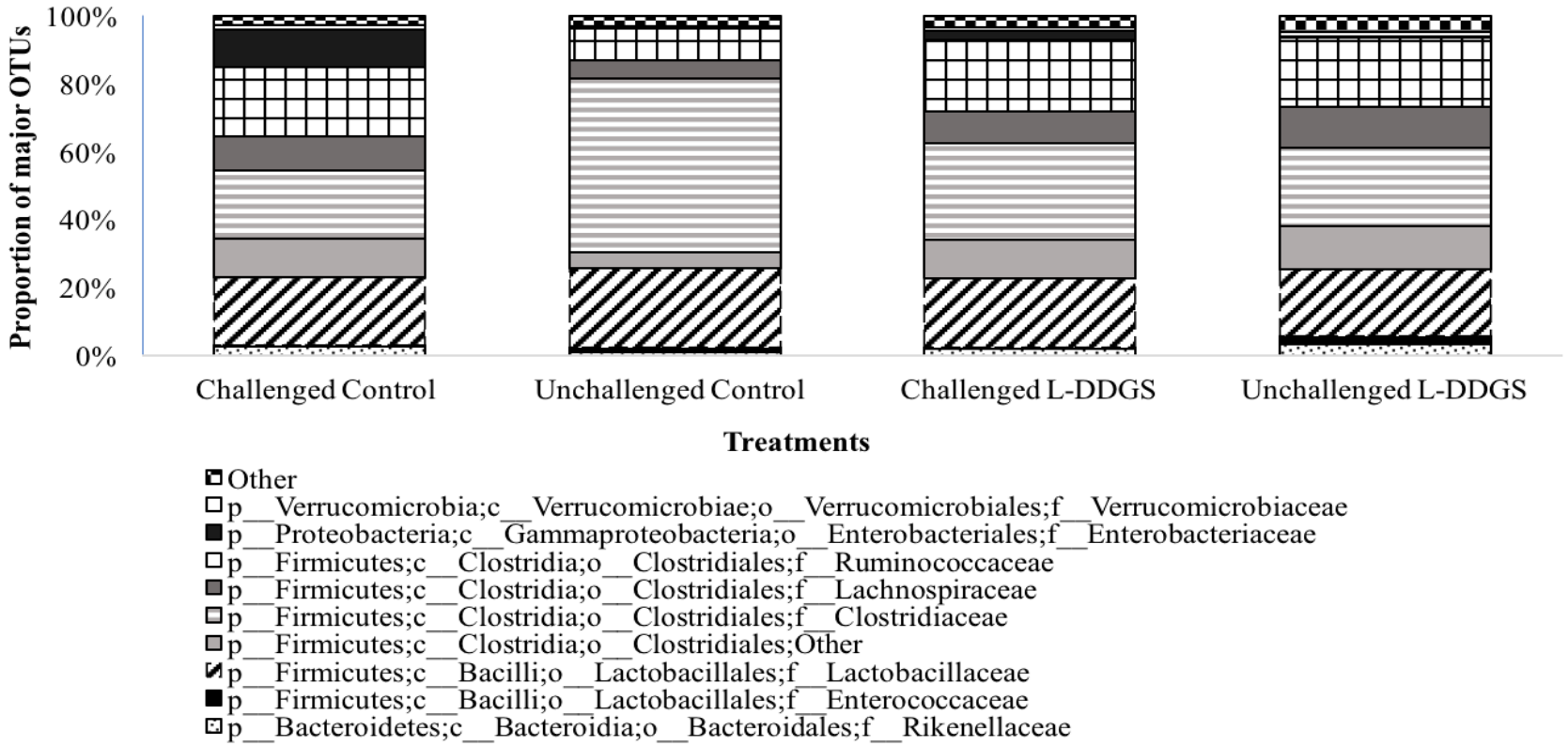

Figure 3.1 Bacterial community composition of 16S rRNA gene sequences of jejunum tissue of 27 d broilers challenged or unchallenged with *C. perfringens* and *Eimeria* spp., fed either a control diet (corn-soy diet) or low-oil distillers dried grains with solubles (L-DDGS; 4.29% crude fat) diet from 0 to 27 d of age. 16S rRNA gene sequences were rarified to 400 sequences per sample due to high host-DNA presence. To further reduce noise within data, less abundant OTU's which represented <1% in all treatment averages were categorized as "Other". OTU table (in BIOM format) was generated based on sequences with a 97% similarity threshold, using QIIME software package. Each value represents treatment means: Challenged Control (n = 6), Unchallenged Control (n = 8), Challenged L-DDGS (n = 9), Unchallenged L-DDGS (n = 7).

Chapter 4.0 Effects of feeding medium-oil distillers dried grains with solubles (M-DDGS) to broilers from 0 to 28 days post-hatch when challenged with *C. perfringens* and *Eimeria* spp. on necrotic enteritis and intestinal microbiome.

Introduction:

Increased prevalence of *Clostridium perfringens*-associated necrotic enteritis (NE) has been observed alongside the removal of antibiotic growth promoters (AGPs; Mcdevitt *et al.*, 2006) due to consumer demands. Consequently, this has elicited interest from the scientific community in understanding the pathogenicity of this enteric disease, in order to seek alternative methods for control. In order to develop NE, predisposing factors that facilitate proliferation of *Clostridium perfringens* are required. It has been proven that dietary factors, among others, strongly influences the incidence of NE in broilers (Annett *et al.*, 2002). Therefore, a refined nutritional formula that makes the broiler gut environment less susceptible, is critical for maintaining broiler optimal health and live performance (Croom *et al.*, 2000; Dahiya, 2006; Moore, 2016; Van Immerseel *et al.*, 2009).

C. perfringens lacks many genes necessary for amino acid biosynthesis. Consequently, this bacterium is incapable of growing in an environment where amino acid supply is limited. Therefore, dietary protein sources (Palliyeguru *et al.*, 2010) and amino acid balance (Wilkie *et al.*, 2005), may result in proliferation of *C. perfringens* when low protein digestibility is observed (Jia *et al.*, 2009a). Distillers dried grains with solubles (DDGS) is a co-product of ethanol production that has become a common feed ingredient in poultry diets due to its competitive price. As distiller grains undergo heating to produce the dried product, concern exists over amino acid digestibility (Fontaine *et al.*, 2007). In addition, over 80% of dry-grind ethanol companies have recently implemented oil-extraction technologies, resulting in reduced-oil (R-DDGS) products varying in oil content from 4 to 12% (CEPA, 2012). Appropriate use of these R-DDGS products requires information of nutrient utilization and their influence on intestinal microbiome.

Research involving the effects of dietary DDGS on disease challenges are extremely limited, as a greater extent of this research has been conducted in swine (Whitney *et al.*, 2006;

Perez *et al.*, 2009). The first experiment demonstrated that feeding low-oil DDGS (L-DDGS; 4.29% crude fat) at 5% of the starter diet and 7.5% of the grower diet did not onset NE development in broilers up to 27 d of age. However, this advantage was at the expense of broiler live performance. Purdum *et al.* (2011) concluded that L-DDGS products are more likely to fit in laying hen diets than that of broilers, due to their lower metabolizable energy. Therefore, the objective of this experiment was to determine the effects of medium-oil DDGS (M-DDGS; 6.6% crude fat) on broiler performance, intestinal microbiome, and incidence and severity of intestinal lesions after a NE challenge.

Materials and Methods:

Chicks and Management

Day-old broiler chicks were obtained from a commercial hatchery and transported to the Auburn University Poultry Research Farm. The chicks were unsexed and unvaccinated against coccidiosis. Upon arrival, the chicks were weighed and randomly distributed throughout four Petersime wire batteries (36 pens total, 10 birds per cage) in a temperature controlled room maintained at 35 ± 2°C during the first week and reduced 2-3°C weekly, for four weeks. The birds were fed a two-phase feeding program, consisting of a crumble starter diet (d 0 to 14) and a pelleted grower diet (d 14 to 28). Diets did not contain antibiotics. Birds were allowed *ad libitum* access to both feed and water throughout the duration of the experiment (28 d total). The DDGS source was provided by a commercial ethanol manufacturing plant in the US. Provided nutrient profiles were used in dietary formulation.

Experimental Design

The experiment consisted of a block randomized design with a 2 (diet: control or M-DDGS) × 2 (challenge: challenged or unchallenged) factorial arrangement of treatments. The control diet consisted of a corn-soy diet without M-DDGS. The M-DDGS diet consisted of a corn-soy diet, containing the M-DDGS source at 5% of the starter diet and 7.5% of the grower diet. (Table 4.2). Challenged birds were housed separate from unchallenged birds. Each of the 4 treatments were represented by 9 replicate pens, containing 10 birds each.

Overall Challenge Scheme

At d 18 of age, broilers were given a 1 mL oral gavage of an *Eimeria* spp. solution. From d 21 to 23, a 1 mL oral gavage of *C. perfringens* was administered. Intestinal lesion scoring and jejunum tissue sample collection were performed during necropsy, 10 d post-*Eimeria* challenge as described below.

Eimeria Challenge

ADVENT® (Huvepharma) coccidiosis vaccine was diluted with sterile water and administered at a 10 x recommended dose per bird. The ADVENT® coccidiosis vaccine contains live oocysts of *E. acervulina*, *E. maxima*, *E. tenella*. It contains gentamicin and amphotericin B as preservatives. One mL of this *Eimeria* spp. solution was given to the challenged birds on d 18, via an oral gavage using a 1 mL Tuberculin syringe (Thermo Fisher Scientific).

Clostridium perfringens Challenge.

A *C. perfringens* strain, with NetB toxin producing ability, was isolated from a bird that had been diagnosed with NE (Bailey *et al.* 2013). The strain was maintained as a frozen stock in glycerol at -80°C until further application. The frozen bacterial isolate was revived by streaking a loop-full of frozen culture on reduced tryptic soy agar (rTSA) containing 5% sheep blood, incubated under anaerobic conditions (5%CO_2, 5%H and 90%N_2) at 37°C for 24 hours. After 24 hours, each plate was checked for purity. A single colony with typical *C. perfringens* morphology was picked, inoculated in brain heart infusion broth (100 mL) and incubated anaerobically for 24 hours at 37°C. After incubation, the culture was diluted (1:100) to produce an approximately 10^7 CFU/mL inoculum. The inoculum was orally administered to the broilers under the challenged treatment group on d 21, 22, and 23. Fresh inoculum was prepared for each challenge day. Inoculum levels were verified by serially diluting and spread plating the inoculum on duplicate rTSA containing 5% sheep blood plates, incubated anaerobically for 24 hours at 37°C and viable colonies showing typical colony morphology were counted.

Measurements and Sample Collection

Body weight (BW) and feed intake (FI) were measured on d 0, 14, and 28 on a per pen basis. Morbid birds were removed, euthanized, and BW recorded daily; mortalities were also removed daily and recorded. Mortality corrected feed conversion ratio (AFCR), body weight gain (BWG) per bird and FI per bird were calculated based off of the removed body weights

where appropriate. On d 28, five birds were randomly selected from each pen and euthanized by CO_2 gas inhalation, followed by cervical dislocation. A jejunum sample (2 cm in length) was collected 15 cm proximal from the Meckel's diverticulum per bird, prior to lesion scoring for sterility. Samples were then pooled by pen and stored at -80°C until DNA extraction, as described below. NE lesions were scored in the duodenum, jejunum, and ileum using a modified scale of Prescott *et al.* (1978). Lesions were scored on a scale of 0 to 4, where 0 = no apparent lesions, 1 = thin friable intestines; 2 = focal necrosis, ulceration or both; 3= patchy necrosis; and 4= severe extensive mucosal necrosis. Coccidiosis lesions were scored according to the methods described by Johnson and Reid (2007). The duodenum, jejunum, and ileum were scored for *E. acervulina* and *E. maxima* on a scale of 0 to 4.

Diet and Mycotoxin Analyses

Prior to formulation, corn was analyzed for mycotoxin concentrations (Midwest Laboratories, Inc.; Table 4.3). Diet samples were analyzed for moisture, dry matter, crude protein (CP), crude fat, acid detergent fiber (ADF), neutral detergent fiber (NDF) and crude fiber (CF) (Midwest Laboratories, Inc.; Table 4.2). Following termination of the experiment, a sample of the M-DDGS source was analyzed for CP, CF, ADF and NDF (Midwest Laboratories Inc.; Table 4.1). Geometric particle size was determined by a Tyler Ro-Tap shaker (Auburn, AL; ASAE Standard S319.4, 2009; Table 4.1).

DNA Extraction, Amplification, and 16S rRNA Gene Sequencing Data Analysis

Jejunum tissue samples were removed from the -80C freezer, thawed, and bacterial DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI) according to manufacturer instructions. Extracted DNA was tested for concentration and purity with a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific) according to manufacturer instructions. The standard for purity was a 260nm/280nm ratio between 1.8 - 2.0. Extracted DNA was stored in -20°C until further analysis.

Stage 1 of PCR amplification was performed at Auburn University using an iQ5 thermocycler (Bio-Rad, Hercules, CA), with reaction conditions described in Table 4.4. Eurofins Genomics® 16S rRNA gene, universal target primers (CS1/515F and CS2/926R) were utilized for amplification (Eurofins Genomics LLC). A touchdown protocol was performed, as described

in Table 4.5. The DNA denaturation step was chosen based on the half-life of Taq (Innis and Gelfand, 2012). Annealing conditions were dependent on the base composition and length of the primers, where the primer extension parameters were based on the optimal temperature of Taq and length of the target sequence. Amplification of Stage 1 PCR yields were verified by gel electrophoresis in a 2% agarose gel. Running buffer and gels were made using 1x concentration of AccuGENE TBE buffer (Lonza Group, Basel, Switzerland). Electrophoresis was performed at 75v for ~1 hour or until sufficient separation between products was obtained. Lonza® 100 bp Extended Range DNA Ladder was used as a DNA size standard. Stage 1 PCR yields were stored at 4°C until further analysis.

Stage 1 PCR yields were sent to University of Illinois Chicago DNA Services Facility (UIC DNAS) facility for targeted amplicon sequencing (TAS). An 8 cycle Stage 2 PCR reaction (Table 4.5) was performed. The variable regions V3-V4 of the 16S rRNA gene were amplified using Fluidigm's Access Array™ Barcoded primers (0.4 μM concentration), in preparation for Illumina MiSeq sequencing (Illumina, Inc.) (Table 4.4). After sample amplification, purification and normalization were performed using SequalPrep plates (Thermo Fisher Scientific). The final pooled libraries were quantified using a formula based on Qubit 3.0 Fluorometer quantification (ThermoFisher Cat#Q33217), and diluted to 2 nM. The libraries were then denatured with 0.2 NaOH, according to Illumina MiSeq protocol. The volume of the final sample loading mixture is 600 μl, including the appropriate library concentration and spike in phiX control (generally 20%).

Forward and reverse reads were merged using the software package PEAR (Zhang *et al.*, 2014). Ambiguous nucleotides were trimmed from the ends and reads with internal ambiguous nucleotides were discarded. Primer sequences were identified using Smith-Watermann alignment, where reads that lacked either primer sequence were discarded. Sequences were then trimmed based on quality scores using a modified Mott algorithm with PHRED quality threshold of $P = 0.01$. After trimming, any sequences < 400 bases were discarded. Chimeric sequences were identified using the USEARCH algorithm with GreenGenes 13_8 reference sequences (Edgar, 2010; McDonald *et al.*, 2012).

The software package QIIME was then used to generate operational taxonomic unit (OTU) clusters in a *de novo* manner using UCLUST algorithm with a 97% similarity threshold (Edgar, 2010; Caporaso *et al.*, 2010). Taxonomic annotations for each OTU were generated using the UCLUST algorithm and the GreenGenes 13_8 reference database with a minimum similarity threshold of 90% (Edgar, 2010; McDonald *et al.*, 2012). Taxonomic and OTU abundance data were merged into a single OTU table, as a biological observation matrix (BIOM) (McDonald, 2012). Species alpha-diversity indices (Shannon index (H') and Margalef Species Richness) were calculated from the OTU table, in BIOM format. 100% stacked column histograms of experimental treatment averages based on phylogenic classification were used to assess bacterial community composition (Figure 4.1). In order to further reduce noise within the data, less abundant OTUs which represented < 1% in all treatment averages, were categorized as "Other".

Data Analysis

All statistical analyses, outside of 16S rRNA gene sequencing, were facilitated using SPSS version 22 software. Significant differences were reported at $P \leq 0.05$. A two-way ANOVA was used to determine main effect and interactions, with diet and challenge as main effects when analyzing the performance and NE results. A Mann-Whitney U Test was used to determine pairwise comparisons of treatment microbiome alpha-diversity indices.

Results and Discussion:

Provided nutrient values, as well as those values that were analyzed following termination of the experiment, are demonstrated in Table 4.1. The crude fat value of the R-DDGS source was what determined it as a medium-oil DDGS. The provided crude fat value was 6.60%, which was comparable to the M-DDGS (7.3%) utilized in a study conducted by Purdum *et al.* (2014). Provided values were included in dietary formulation and were determined to differ from those values that were analyzed (Midwest Laboratories Inc.; Table 4.1). Traditionally, animal diets (particularly swine and poultry) are formulated on metabolizable energy (ME) and digestible amino acid basis. In the current experiment, diets were formulated using a least cost formula, on a total amino acid basis (Creative Formulation Concepts, LLC; Table 4.2).

The feeding value of DDGS may be largely based on its energy content, where changing the oil content of DDGS may affect growth performance (Graham *et al.*, 2014). Hence, when utilizing the M-DDGS source in a corn-soy diet requires additional supplementation of fat (poultry oil) to meet target ME. Diet alone, resulted in no significant effects on performance during any period ($P > 0.05$; Table 4.5). However, a trend of diet and challenge interaction was observed on FI at 0 to 14 days of age ($P = 0.061$). In addition, a significant interaction of diet and challenge was observed with mortality at 0 to 14 days of age ($P = 0.028$). To reiterate, birds had not been challenged until 18 d of age with *Eimeria* spp. and 21 to 23 d of age with *C. perfringens*. Although DDGS has been found to be an acceptable ingredient in poultry diets, variation in amino acid content and digestibility has become a concern. Apparent amino acid digestibility coefficients for lysine, methionine, threonine, tryptophan, and arginine are reduced as the amount of oil in DDGS is decreased (Dozier *et al.*, 2015). Hence, broiler chick performance appeared to be effected by amino acid variation, as a result of oil-extraction. Mycotoxin concentrations of the M-DDGS sample were determined to be low and therefore did not influence broiler live performance (Midwest Laboratories Inc.; Table 4.3).

Necrotic enteritis intestinal lesion incidence, severity and average scores are demonstrated in Table 4.7. The M-DDGS diet resulted in increased NE incidence ($P = 0.012$) and NE average lesion score ($P = 0.037$; Table 4.7). Results support the findings by Macklin *et al.* (2011) implying that higher DDGS inclusion rates (7.5% vs. 15.0%) increased the incidence of NE. Based upon the day 0-14 observations (table 4.5) it can be assumed that the chicks may have had an unstable microbiome, which would have led to an increase in susceptibility (Mead, 1989). Additionally, amino acid variation in the diets could have resulted in further disruptions to the intestinal microbiome further increasing susceptibility to challenge. Furthermore, diets with imbalanced amino acid profiles reduce the digestibility of these compounds in the upper part of the digestive tract (McDevitt *et al.*, 2006), acting as a substrate for the gut microflora (Timbermont *et al.*, 2011). Fermentation of proteins results in an increased pH in the lower part of the GIT, encouraging *C. perfringens* proliferation (Juskiewicz *et al.*, 2004; Lan *et al.*, 2005). As previously mentioned, diets were formulated on a total versus digestible amino acid basis. Subsequently, pathogenicity of *C. perfringens* may have been enhanced as indicated by the

interaction effect of diet and challenge observed with NE severity ($P = 0.045$) and NE average lesion score ($P = 0.037$). Challenge also had an effect on NE incidence ($P < 0.001$) and average lesion score ($P < 0.001$), as expected. Mycotoxin concentrations of the M-DDGS source were determined (Midwest Laboratories Inc.) to be low (Table 4.3) and therefore did not increase susceptibility to disease. M-DDGS provided no evidence of exacerbating coccidiosis, as was observed with that of NE. Differences in colonization and growth characteristics explain these results, as *Eimeria* spp. are internal parasites of enterocytes, while *C. perfringens* inhabits the lumen. Therefore, diet would have a more prominent effect on *C. perfringens*, as described above. These results support the findings of Perez *et al.* (2011) who concluded that concentrations up to 20% of the diet did not prevent *E. acervulina* infection or promote faster recovery in young chicks.

Microbes of the GIT can generally be divided into potentially pathogenic and beneficial groups. The interaction between the microflora and diet can affect intestinal development (Apajalahti *et al.*, 2004) and its ability to impair bacterial adhesion. Therefore, Species Richness (Margalef) and Shannon Index (H') values were analyzed in order to provide information on broiler jejunum bacteria community composition. The jejunum was the intestinal segment chosen for collection, as this is where NE lesions have been found to be most common and severe (Truscott & Al-Sheikhly, 1977; Fukata *et al.*, 1991; Long *et al.*, 1973). In addition, it's involvement in absorption of nutrients and minerals, and monitoring of luminal content (Bar-Shira *et al.*, 2003; Shokker *et al.*, 2009) allowed the effects of dietary M-DDGS to be assessed. No significant pairwise comparisons between treatments were observed for either Species Richness or Shannon Index ($P > 0.05$; Table 4.9). Species richness (Margelf) implies no particular regularity in distribution, in which the number of individuals in all the species are taken into account (Margalef, 1969). The Shannon index accounts for both abundance and evenness of the species present (Beals *et al.*, 2000). When looking at average treatment values of the following alpha-diversity indices, the challenged M-DDGS treatment appeared to be less diverse, dominated by one or a few species (Table 4.8). However, the unchallenged M-DDGS treatment was a highly diverse and evenly distributed system, in which an unknown individual can belong to any species. This was similar to that of the first experiment, in which the

unchallenged L-DDGS resulted in similar results. However, it important to note that a direct comparison of OTUs and taxonomic composition between reported and present study may not be accurate due to differences in experimental approaches (Cisek and Binek, 2014; Lumpkins *et al.*, 2010). Data rarefication to 400 sequences per sample may also influence results, as this may lead to a more conservative view of the abundances of uncommon taxa across samples (Goodrich *et al.*, 2014).

Bacterial community composition per treatment was assessed according to higher-level taxonomic groups, as members of the same bacterial genus are not necessarily each other's closest relatives (Goodrich *et al.*, 2014). 16S rRNA gene sequences were rarified to 400 sequences per sample due to high host-DNA presence, where those samples that contained < 400 sequences were excluded from data analysis. The OTU table, in BIOM format, revealed 9 phyla, 17 classes, 30 orders and 54 families of known bacteria present in total rarefied samples (n = 35). In order to further reduce noise within the data, less abundant OTUs which represented < 1% in all treatment averages, were classified as "Other". The four most common (> 1% in at least one treatment average) phyla were *Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria* (Figure 4.1). When Wei *et al.* (2013) sampled and analyzed the global diversity of intestinal microbiome sampled from chickens, *Firmicutes, Bacteroidete*s, and *Proteobacteria* were also found to be the three most predominant phyla. *Actinobacteria* has been found to be dominant in the ileum, followed by the jejunum, duodenum and colon of broilers, where it is nearly absent in the ceca (Xiao *et al.*, 2017). Other studies have found *Actinobacteria* to be a predominant minor phylum in the intestinal tract (Wei *et al.*, 2013). *Firmicutes* was the predominant phylum found in all treatment birds at 28 d of age, accounting on average, for more than 87% of all major OTUs. This supports the findings from other studies that have found *Firmicutes* to account for 60-70% all bacterial sequence of chicken intestinal microbiome (Pan and Yu, 2013; Xiao *et al.*, 2016).

Within the phylum *Actinobacteria*, the family *Brevibacteriaceae* was intriguingly prominent in the unchallenged M-DDGS treatment. *Brevibacteriaceae* have been isolated from various habitats including milk products, soil, insects, brown algae, the human body (Batt, 2014) and chicken fecal microbiota (Videnska *et al.*, 2014). Strains isolated from poultry have had a

higher optimum growth temperature of 37°C (Batt, 2014). Within the phylum *Bacteroidetes*, the family *Rikenellaceae* was present in all treatments, with the greatest proportion found in the unchallenged M-DDGS treatment. *Rikenellaceae* has been found to most abundant in the cecum, where it has been found to sharply increase from 2% (d 15) to 24% (d 22) and then to 36% (d 29) (Ranjitkar *et al.*, 2016). The increased presence of *Brevibacteriaceae* and *Rikenellaceae* in the unchallenged M-DDGS treatment remains unclear; however, its contribution to increased alpha-diversity was noticed. The family *Enterobacteriacea* (phylum *Proteobacteria*) was also present in all treatments, in which this family includes the genera *Salmonella* and *Escherichia*, contributing to a number of important diseases (Pattison, 2008).

In the phylum *Firmicutes*, the families *Staphylococcaceae*, *Enterococcaceae*, *Lactobacillaceae, Clostridiaceae, Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae* were most common in different proportions. *Lactobacillaceae* were the most prominent in all treatments (Figure 4.1). At the genus level, *Lactobacillus* has been found to be more common in the small intestine, indicating its contribution towards intestinal functions related to nutrient absorption. It is considered one of the most important bacterial groups that maintains the equilibrium of the microbial ecosystem (Pyrde *et al.*, 1999). In addition, its interactions with epithelial cells can result in protection against pathogens (Sengupta *et al.*, 2013). *Lactobacillaceae* proportions were highest in the challenged M-DDGS treatment. When observing its relationship with *Clostridiaceae*, the family that contains *C. perfringens*, it appears to have an inverse effect. This differs from observations of the first experiment, where *Lactobacillaceae* proportions tended to increase with that of *Clostridiaceae*.

Some strains of *Ruminococcaceae* have been suspected to contribute to cytokine production that support small intestine integrity by affecting tight junction expression and epithelial cell proliferation (Furness *et al.*, 2013). Butyrate-producing strains of the *Ruminococcaceae* family have been shown to decrease by *Eimeria* administration, and are decreased in broilers that develop NE (Wu *et al.*, 2014). Similar observations were made in the current experiment, where the challenged M-DDGS treatment birds had the least proportion of *Ruminococcaceae* in comparison to all other treatments. Results imply a beneficial effect of

Ruminococcaceae in NE formation, in which its relationship with lactate-producing bacteria remains unclear.

Conclusion:

When feeding M-DDGS (6.6% crude fat; 5% of starter and 7.5% of grower diets) to broilers up to 28 d of age, bioassays of M-DDGS samples should be considered to determine nutrient content prior to diets being formulated on a digestible amino acid basis. In addition, undigested amino acids can result in *C. perfringens* proliferation subsequently enhancing pathogenicity of NE. Although alpha-diversity indices were similar between treatments, bacterial community composition implied a suppression of butyrate-producing strains in broilers that develop NE. Their relationship with lactate-producing bacteria should be further studied as the commercial industry moves towards the removal of AGPs, as these bacteria seem to effect *C. perfringens* colonization and disease induction.

Table 4.1 Proximate nutrient composition of medium-oil distillers dried grains with solubles (M-DDGS)[1]

Item	Value	Method of Analysis[2,3]
Protein, fat, fiber (%)		
Moisture	10.90	
Dry matter	89.01	
Crude protein	30.30	
Analyzed	28.30	AOAC official method 990.03
Crude fat	6.60	
Acid detergent fiber, *Analyzed*	9.80	AOCS Ba 6a-05
Neutral detergent fiber, *Analyzed*	24.3	ANKOM Tech. Method
Crude fiber, *Analyzed*	5.86	ANKOM Tech./AOAC official method 2001.11
Glucose	0.40	
Color (L*)	57.54	
Particle Size (microns)	249	ASAE Standard S319.4

[1]Medium-oil DDGS (6.60% crude fat) on an as-fed basis.
[2]All method of analysis were determined by Midwest Laboratories, Inc. (Omaha, NE).
[3]AOAC = AOAC International (Gaithersburg, MD); ANKOM = ANKOM Technology (Macedon, NY); AOCS = The American Oil Chemists' Society (Urbana, IL); ASAE = American Society of Agricultural Engineers (St. Joseph, MI).

Table 4.2 Ingredient and nutrient composition of diets fed to broilers from 0 to 28 d of age

	Starter Diets		Grower Diets		
Ingredient, %	Control	M-DDGS	Control	M-DDGS	Method of Analysis[4]
Corn	62.361	58.513	66.540	53.401	
Soybean Meal 48	28.929	27.398	24.830	29.320	
M-DDGS (6.60% crude fat)[1]	0.000	5.000	0.000	7.500	
Poultry Meal 65	4.000	4.000	4.000	3.636	
Poultry Oil	1.043	1.519	1.112	3.101	
Salt	0.404	0.371	0.405	0.382	
Dicalcium Phosphate	1.405	1.267	1.111	0.889	
Limestone	1.127	1.210	1.079	1.203	
DL-Methionine	0.313	0.292	0.314	0.269	
L-Lysine 98	0.168	0.180	0.246	0.049	
L-Threonine	0.000	0.000	0.112	0.000	
Vitamin Premix[2]	0.050	0.050	0.050	0.050	
Choline Chloride Dry	0.100	0.100	0.100	0.100	
Trace Minerals Premix[3]	0.100	0.100	0.100	0.100	
Calculated Nutrient Content (% unless otherwise indicated)					
ME (kcal/kg)	3,053	3,053	3,109	3,109	
Crude Protein	21.500	21.991	20.00	22.916	
Calcium	0.930	0.930	0.840	0.840	
Phosphorous, Available	0.450	0.450	0.390	0.390	
Sodium	0.200	0.200	0.200	0.209	
Proximate Analysis Nutrient Content (% unless otherwise indicated)					
Dry Matter	88.380	87.870	87.700	87.860	Calculation
Crude Protein	20.500	21.400	19.300	23.100	AOAC official method 990.03
Crude Fat	3.710	3.870	4.970	6.320	AOAC official method 945.16
Acid Detergent Fiber	3.600	3.700	3.000	3.800	ANKOM Tech. Method
Ash	4.880	4.830	4.510	4.820	AOAC official method 942.05
Crude Fiber	1.800	1.940	1.310	2.120	AOCS Ba 6a-05
Neutral Detergent Fiber	7.500	6.400	6.700	7.600	Ankom Tech./AOAC official method 2001.11

[1]M-DDGS = medium-oil DDGS (6.60% crude fat) on an as fed-basis.
[2]Vitamin premix includes per kg of diet: Vitamin A (Vitamin A acetate), 18,739 IU; Vitamin D (cholecalciferol), 6,614 IU; Vitamin E (DL-alpha tocopherol acetate), 66 IU; menadione (menadione sodium bisulfate complex), 4 mg; Vitamin B12 (cyanocobalamin), 0.03 mg; folacin (folic acid), 2.7 mg: D-pantothenic acid (calcium pantothenate), 31 mg; riboflavin (riboflavin), 22.1 mg; niacin (niacinamide), 88.2 mg; thiamin (thiamin mononitrate), 5.5 mg; D-biotin (biotin), 0.18 mg; and pyridoxine (pyridoxine hydrochloride), 7.7 mg.
[3]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, cypress excel Se yeast), 0.3 mg
[4]All methods of analysis were determined by the Midwest Laboratories Inc. (Omaha, NE).

[4]AOAC = AOAC International (Gaithersburg, MD); ANKOM = ANKOM Technology (Macedon, NY); AOCS = The American Oil Chemists' Society (Urbana, IL); ASAE = American Society of Agricultural Engineers (St. Joseph, MI).

Table 4.3 Mycotoxin concentrations of medium-oil distillers dried grains with solubles (M-DDGS) and ground corn included in diets fed to broiler 0 to 28 d of age

Analysis[1,2]	Results	Method[3]
Experimental Corn (ppb, unless otherwise indicated)		
AF B1	ND[4]	Internal SOP-14-168
AF B2	ND	Internal SOP-14-168
AF G1	ND	Internal SOP-14-168
AF G2	ND	Internal SOP-14-168
DON, ppm	ND	Internal SOP-14-168
FUM B1, ppm	1.80	AOAC 999.15 with modifications
FUM B2, ppm	0.50	AOAC 999.15 with modifications
FUM B3, ppm	ND	AOAC 999.15 with modifications
OTA	ND	AOAC 2000.03 with modifications
T-2 Toxin	ND	Internal SOP-14-168
ZEN	ND	Internal SOP-14-168
M-DDGS[5] (ppb, unless otherwise indicated)		
Total AF	ND	Internal SOP-14-168
Total FUM, ppm	0.90	AOAC 999.15 with modifications
OTA	ND	AOAC 2000.03 with modifications
DON, ppm	3.7	Internal SOP-14-168
T-2 Toxin, ppm	ND	Internal SOP-14-168
ZEN	144	Internal SOP-14-168

[1]All methods of analysis were determined by Trilogy Analytical Laboratory, Inc. (Washington, MO).
[2]AF = Alfatoxin; DON = Deoxynivalenol; FUM = Fumonisin; OTA = Ochratoxin A; ZEN = Zearalenone; ppb = Parts per billion; ppm = Parts per million.
[3]SOP = Standard operating procedure; AOAC = AOAC International (Gaithersburg, MD).
[4]ND = None detected.
[5]M-DDGS = Medium-oil DDGS (6.60% crude fat).

Table 4.4 Reaction conditions and primers for amplification of bacterial populations of jejunum tissue collected from broilers at 28 d of age by polymerase chain reaction (PCR)

Reaction conditions	1 x Rxn volume (μL)
Stage 1[1]	
Master Mix[2]	12.5
DdH_2O[3]	10.75
DNA Template[4]	1.0
Forward Primer[5]	0.25
Reverse Primer[6]	0.25
BSA[7]	0.25
Stage 2[8]	
Primers[9]	0.4
Stage 1 PCR Yield	1.0

[1]Performed at Auburn University Laboratory (Auburn, AL).
[2]Master Mix = Lucigen EconoTaq Plus 2x Master Mix (Middleton, WI): 0.1 units/μL of EconoTaq DNA Polymerase, Reaction Buffer (pH 9.0), 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dATP, 3 mM MgCl2, and a proprietary mix of PCR Enhancer/Stabilizer and blue and yellow tracking dyes.
[3]DdH_2O = double distilled H_2O.
[4]DNA Template = pooled jejunum tissue sample (5 birds/pen, each 2 cm in length, 15 cm proximal from Meckel's diverticulum) bacterial DNA extracted using Wizard Genomic Purification Kit (Madison, WI).
[5]Forward Primer = Eurofins Genomics CS1/515F Universal Target 16S rRNA Gene (Louisville, KY): 5'-GTGYCAGCMGCCGCGGTAA-3', expected amplicon size with CS1 456, annealing temperature 50°C.
[6]Reverse primer = Eurofins Genomics® CS2/926R Universal Target 16S rRNA Gene (Louisville, KY): 5'-CCGYCAATTYMTTTRAGTTT-3', expected amplicon size with CS2 456, annealing temperature 50°C.
[7]BSA (Bovine Serum Albumin) = Purified BSA 100x (Lawrenceville, GA).
[8]Performed at University of Illinois Chicago DNA Services Facility (Chicago, IL).
[9]Primers = Fluidigm's AccessArray Barcoded Primers (San Francisco, CA): Read 1: Standard CS1: A+CA+CTG+ACGACATGGTTCTACA; Index Read: CS2rc: A+GAC+CA+AGTCTCTGCTACCGTA; Read 2: Standard CS2: T+AC+GGT+AGCAGAGACTTGGTCT (A "+" preceding a base indicates a Locked Nucleic Acids(LNA)-base).

Table 4.5 Polymerase chain reaction (PCR) conditions for amplification of bacterial populations of jejunum tissue collected from broilers at 28 d of age

Cycle Step	Temperature	Time	Cycles
Stage 1[1]			
Initial Denaturation	94°C	5 min	1x
Variable Temperature Annealing			15x, reduce 1°C/cycle
Denaturation	94°C	30 sec	
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Constant Temperature Annealing	94°C		30x
Denaturation	94°C	30 sec	
Annealing	55°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	7 min	1x
Hold	4°C	∞	
Stage 2[2]			
Initial Denaturation	95°C	5 min	1x
Variable Temperature Annealing			28x
Denaturation	95°C	30 sec	
Annealing	60°C	30 sec	
Extension	72°C	30 sec	

[1]Performed at Auburn University Laboratory (Auburn, AL) using a Bio-Rad IQ5 thermocycler (Hercules, CA).
[2]Performed at University of Illinois Chicago DNA Services Facility (Chicago, IL) in preparation for Illumina MiSeq (San Diego, CA).

Table 4.6 Growth performance[1] of broilers challenged[2] or unchallenged with *C. perfringens* and *Eimeria* spp. fed a control diet (corn-soy) or medium-oil distillers dried grains with solubles (M-DDGS)[3] diet from 0 to 28 d of age

	BW Gain (kg/bird)			Feed Intake (kg/bird)			AFCR[4] (kg/kg)			Mortality[5] (%)		
	0 to 14	14 to 28	0 to 28	0 to 14	14 to 28	0 to 28	0 to 14	14 to 28	0 to 28	0 to 14	14 to 28	0 to 28
Diet x Challenge												
P ≤	0.542	0.665	0.733	0.061	0.984	0.615	0.289	0.876	0.475	**0.028***	1.000	0.221
SEM[6]	0.005	0.022	0.015	0.014	0.040	0.063	0.027	0.021	0.018	0.017	0.029	0.031
Diet Main Effect												
Control	0.439	1.058	1.520	0.566	1.689	2.286	1.283	1.523	1.454	0.028	0.044	0.072
M-DDGS	0.446	1.041	1.526	0.584	1.673	2.309	1.290	1.494	1.437	0.033	0.067	0.100
P ≤	0.215	0.459	0.719	0.233	0.690	0.713	0.804	0.161	0.363	0.744	0.453	0.380
SEM	0.004	0.016	0.011	0.010	0.028	0.044	0.019	0.015	0.013	0.012	0.021	0.022
Challenge Main Effect												
Challenged	0.446	**1.022**	1.512	**0.591**	1.683	2.333	1.306	1.501	1.447	0.033	0.078	0.111
Unchallenged	0.439	**1.077**	1.534	**0.559**	1.680	2.262	1.268	1.516	1.445	0.028	0.033	0.061
P ≤	0.197	**0.020***	0.158	**0.034***	0.935	0.266	0.171	1.484	0.908	0.744	0.139	0.119
SEM	0.004	0.016	0.011	0.010	0.028	0.044	0.019	0.015	0.013	0.012	0.021	0.022

[1]Each value represents means of 9 replicate pens having 10 chicks at placement.
[2]*Eimeria spp.* challenge (d 18): ADVENT® (Huvepharma) coccidiosis vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. tenella* administered at a 10 x recommended dose per challenged bird; *C. perfringens* challenge (d 21, 22, 23): Inoculated brain heart infusion broth was diluted 1:100 to produce an approximately 10^7 CFU/mL inoculum containing an isolate from a bird diagnosed with necrotic enteritis.
[3]M-DDGS = Medium-oil DDGS (6.60% crude fat); 5% inclusion in the starter diet, 7.5% inclusion in the grower diet.
[4]AFCR = Feed conversion ratio corrected for mortality.
[5]Mortality data underwent square-root arcsine transformation before analysis.
[6]SEM= Pooled standard error.

Table 4.7 Necrotic Enteritis (NE) intestinal lesion[1,2] incidence, severity and average lesion scores of broilers challenged[3] or unchallenged with *C. perfringens* and *Eimeria* spp. fed a control diet (corn-soy) or medium-oil distillers dried grains with solubles (M-DDGS)[4] diet from 0 to 28 d of age

28 d	NE Incidence[5] (%)	NE Severity[6] (%)	NE Average Lesion Score[7]
Diet x Challenge			
P ≤	0.115	**0.045**	**0.037**
SEM[8]	0.070	0.038	0.090
Diet Main Effect			
Control	0.344	0.067	0.411
M-DDGS	0.522	0.078	0.600
P ≤	**0.012***	0.388	**0.037***
SEM	0.050	0.027	0.063
Challenge Main Effect			
Challenged	0.567	0.089	0.656
Unchallenged	0.300	0.056	0.356
P ≤	**< 0.001***	0.773	**< 0.001***
SEM	0.050	0.027	0.063

[1]Necrotic enteritis (NE) lesions were scored in the duodenum, jejunum, and ileum using a modified scale of Prescott *et al.* (1978

[2]Each value represents mean of 9 replicate pens, where 5 birds were randomly chosen per pen.

[3]*Eimeria spp.* challenge (d 18): ADVENT® (Huvepharma) coccidiosis vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. tenella* administered at a 10 x recommended dose per challenged bird; *C. perfringens* challenge (d 21, 22, 23): Inoculated brain heart infusion broth was diluted 1:100 to produce an approximately 10^7 CFU/mL inoculum containing an isolate from a bird diagnosed with necrotic enteritis.

[4]M-DDGS = Medium-oil DDGS (6.60% crude fat); 5% inclusion in the starter diet, 7.5% inclusion in the grower diet.

[5]NE Incidence = Percentage of birds a lesion score ≥ 1.

[6]NE Severity = Percentage of birds a lesion score ≥ 2.

[7]NE Average Lesion Score =Mean of scores between 0 and 4.

[8]SEM = Pooled standard error.

Table 4.8 Alpha-diversity indices[1,2] of 16S rRNA gene sequences from jejunum tissue[3] of broilers challenged[4] or unchallenged with *C. perfringens* and *Eimeria* spp. fed a control diet (corn-soy) or medium-oil distillers dried grains with solubles (M-DDGS)[5] diet from 0 to 28 d of age

Sample DNA	Species Richness (Margalef)[6]	SEM[8]	Shannon Index (H')[7]	SEM
28 d				
Treatments				
Challenged Control	3.025	0.255	1.487	0.192
Unchallenged Control	3.283	0.303	1.578	0.235
Challenged M-DDGS	2.837	0.337	1.316	0.215
Unchallenged M-DDGS	3.443	0.197	1.645	0.193

[1]Alpha-diversity indices were calculated from the operational taxonomic unit (OTU) table derived from sequences with a 97% similarity threshold, using QIIME (San Diego, CA) software package.
[2]Each value represents treatment means: Challenged Control (n = 8), Unchallenged Control (n = 9), Challenged L-DDGS (n = 9), Unchallenged L-DDGS (n = 8).
[3]Jejunum samples (5 birds/pen, each 2 cm in length, 15 cm proximal from Meckel's diverticulum) collected on d 27; 16S rRNA gene sequences were rarified to 400 sequences per sample due to high host-DNA presence.
[4]*Eimeria spp.* challenge (d 18): ADVENT® (Huvepharma) coccidiosis vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. tenella* administered at a 10 x recommended dose per challenged bird; *C. perfringens* challenge (d 21, 22, 23): Inoculated brain heart infusion broth was diluted 1:100 to produce an approximately 10^7 CFU/mL inoculum containing an isolate from a bird diagnosed with necrotic enteritis.
[5]M-DDGS = Medium-oil DDGS (6.60% crude fat).
[6]Shannon Index*:* $H' = -\sum p_i \ln p_i$, where p_i is the proportion of individuals found in species *i*.
[7]Species richness (Margalef): $R_1 = (S\text{-}1)/Log\ N$, where *S* is the number of taxa and *N* is the number of individuals.
[8]SEM = Pooled standard error.

Table 4.9 Pairwise comparisons of treatment alpha-diversity indices[1,2] of 16S rRNA gene sequences from jejunum tissue[3] of broilers challenged[4] or unchallenged with *C. perfringens* and *Eimeria* spp. fed a control diet (corn-soy) or medium-oil distillers dried grains with solubles (M-DDGS)[5] diet from 0 to 28 d of age

Sample DNA	Species Richness (Margalef)[6]	Shannon Index (H')[7]
Mann-Whitney U Test		
P-value		
Challenged L-DDGS vs. Unchallenged Control	0.350	0.480
Challenged L-DDGS vs. Unchallenged L-DDGS	0.192	0.336
Challenged L-DDGS vs. Challenged Control	0.771	0.564
Unchallenged Control vs. Unchallenged L-DDGS	0.961	0.810
Unchallenged Control vs. Challenged Control	0.358	0.700
Unchallenged L-DDGS vs. Challenged Control	0.204	0.753

[1]Values represent pairwise-comparisons between non-parametric treatments: Challenged Control (n = 8), Unchallenged Control (n = 9), Challenged L-DDGS (n = 9), Unchallenged L-DDGS (n = 8).

[2]Alpha-diversity indices were calculated from the operational taxonomic unit (OTU) table derived from sequences with a 97% similarity threshold, using QIIME (San Diego, CA) software package.

[3]Jejunum samples (5 birds/pen, each 2 cm in length, 15 cm proximal from Meckel's diverticulum) collected on d 27; 16S rRNA gene sequences were rarified to 400 sequences per sample due to high host-DNA presence.

[4]*Eimeria spp.* challenge (d 18): ADVENT® (Huvepharma) coccidiosis vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. tenella* administered at a 10 x recommended dose per challenged bird; *C. perfringens* challenge (d 21, 22, 23): Inoculated brain heart infusion broth was diluted 1:100 to produce an approximately 10^7 CFU/mL inoculum containing an isolate from a bird diagnosed with necrotic enteritis.

[5]M-DDGS = Medium-oil DDGS (6.60% crude fat).

[6]Shannon Index*:* $H' = -\sum p_i \ln p_i$, where p_i is the proportion of individuals found in species *i*.

[7]Species richness (Margalef): $R_1 = (S\text{-}1)/Log\ N$, where *S* is the number of taxa and *N* is the number of individuals.

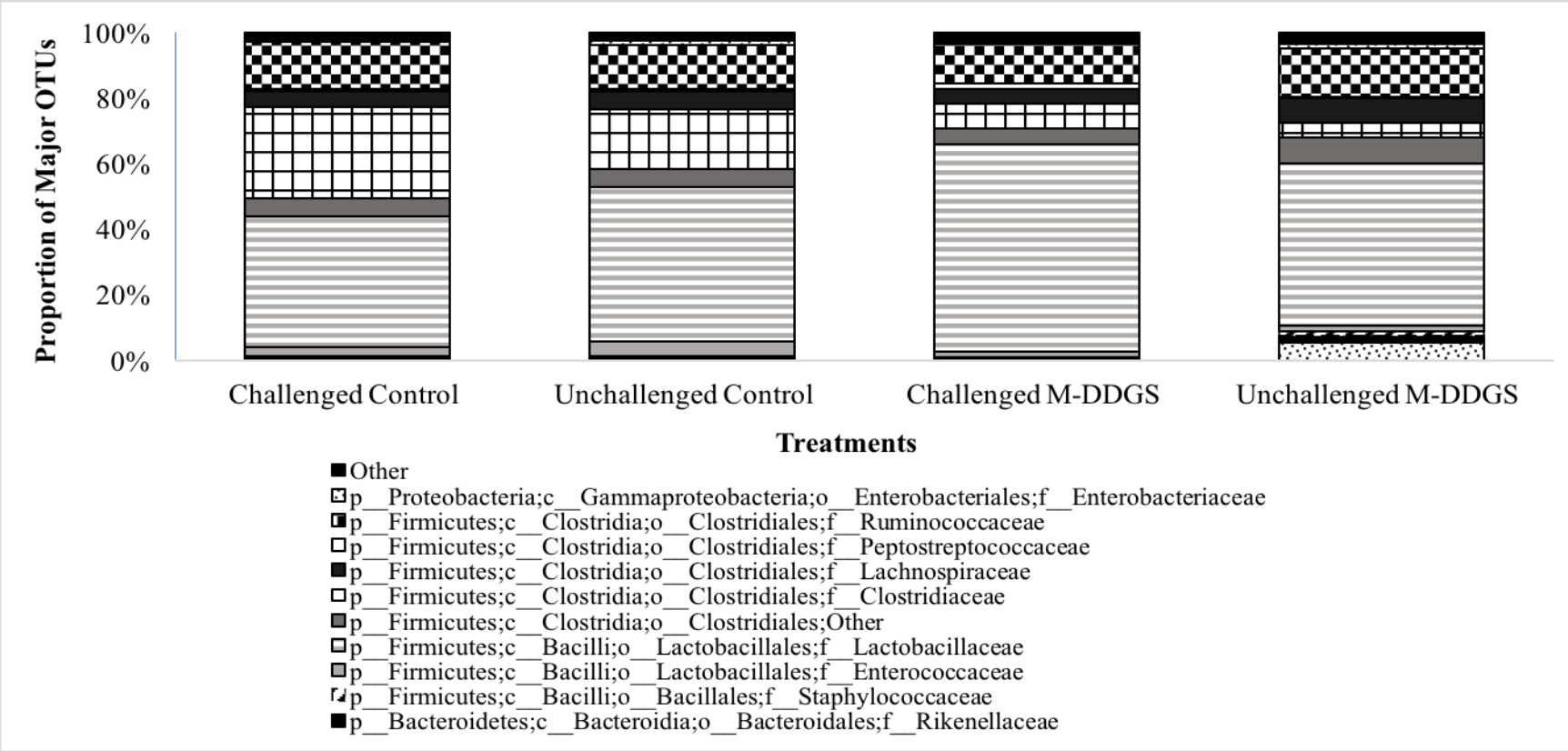

Figure 4.1 Bacterial community composition according to phyla, class, order and family of 16S rRNA sequences of jejunum tissue samples from 28 d broilers challenged or unchallenged with *C. perfringens* and *Eimeria* spp., provided *at libitum* access to either a control diet (corn-soy diet) or M-DDGS diet (5% M-DDGS in the starter, 7.5% in the grower) from 0 to 28 d of age. 16S rRNA gene sequences were rarified to 400 sequences per sample due to high host-DNA presence. To further reduce noise within data, less abundant OTU's which represented <1% in all treatment averages were categorized as "Other". OTU table (in BIOM format) was generated based on sequences with a 97% similarity threshold, using QIIME software package. Each value represents treatment means: Challenged Control (n = 8), Unchallenged Control (n = 9), Challenged M-DDGS (n = 9), and Unchallenged M-DDGS (n = 8)

Chapter 5.0 Effects of medium-oil distillers dried grains with solubles (M-DDGS) endogenous fiber composition on broilers challenged with *C. perfringens* and *Eimeria* spp. on necrotic enteritis and intestinal microbiome from 0 to 28 days post-hatch

Introduction:

Clostridium perfringens normally inhabits the small intestine of broilers at low concentrations (Kondo, 1988). However, disturbances to the intestinal microflora can increase numbers of this Gram-positive, spore-forming, anaerobic bacterium to 10^7 to 10^9 CFU/g of the digesta (Kondo, 1988). Subsequently, this can result in the development of subclinical or clinical necrotic enteritis (NE; Kondo, 1988). Although the clinical form results in increased flock mortality, the subclinical form is more economically detrimental due to its lack of premonitory signs. Overall, NE has been estimated to cost the international poultry industry up to $6 billion annually (Wade and Keyburn, 2015).

Antibiotic growth promoters (AGPs) have long suppressed NE incidence (Mcdevitt *et al.*, 2006). However, increasing demands to reduce the use of AGPs in feedstuffs by consumers, has warranted alternative methods for control. Dietary components have the ability to alter the gastrointestinal tract (GIT), influencing NE incidence (Annett *et al.*, 2002). Distillers dried grains with solubles (DDGS) has become an economical source of nutrients in poultry diets over the past decade. However, over 80% of dry-grind ethanol plants have recently implemented oil extraction technologies, resulting in the production of reduced-oil DDGS (R-DDGS) sources (CEPA, 2012). Extracting oil from DDGS not only results in fluctuation of fat content, but the concentration of fiber and protein (Loar *et al.*, 2012). The fiber composition of traditional DDGS is predominately insoluble (42%) with a smaller amount being soluble (0.7%) (Whitney *et al.*, 2006). However, appropriate use of R-DDGS sources requires reassessment of their nutrient values and influence on the broiler intestinal microbiome.

It has been proposed that inclusion of moderate amounts of fiber in the diet may have positive effects on the broiler GIT. Furthermore, fiber influences the viscosity of the digesta and

can impair bacterial adhesion (Smith and Halls, 1968; Lawrence, 1972). However, positive and negative factors appear to be associated with feeding R-DDGS sources. It has been suspected that chicks need certain levels of fiber present in DDGS, but higher levels can have detrimental effects (Guney *et al.*, 2013). In addition, the onset of NE is associated with a shift in microbiota present within the GIT (Antonissen *et al.*, 2015). Whether this shift is a predisposing factor or consequence of *C. perfringens* proliferation and necrosis remains unclear. Therefore, the objective of this experiment was to examine the effects of dietary M-DDGS endogenous fiber composition on broiler performance, intestinal microbiome, and the incidence and severity of intestinal lesions after an NE challenge.

Materials and Methods:

Chicks and Management

Day-old broiler chicks were obtained from a commercial hatchery and transported to the Auburn University Poultry Research Farm. The chicks were unsexed and unvaccinated against coccidiosis. Upon arrival, the chicks were weighed and randomly distributed throughout four Petersime wire batteries (36 pens total, 10 birds per cage) in a temperature controlled room maintained at 35 ± 2°C during the first week and reduced 2-3°C weekly, for four weeks. The birds were fed a two-phase feeding program, consisting of a crumble starter diet (d 0 to 14) and a pelleted grower diet (d 14 to 28). Diets did not contain antibiotics. Birds were allowed *ad libitum* access to both feed and water throughout the duration of the experiment (28 d total). The DDGS utilized was provided by a commercial ethanol manufacturing plant in the US. Provided nutrient profiles were used in dietary formulation.

Experimental Design

The experiment consisted of a block randomized design with a 2 (diet: control or M-DDGS) × 2 (challenge: challenged or unchallenged) factorial arrangement of treatments. The control diet consisted of a corn-soy diet without L-DDGS. The M-DDGS diet consisted of a corn-soy diet, containing the M-DDGS source at 5% of the starter diet and 7.5% of the grower diet. (Table 5.2). Challenged birds were housed separate from unchallenged birds. Each of the 4 treatments were represented by 9 replicate pens, containing 10 birds each.

Overall Challenge Scheme

At d 18 of age, broilers were given a 1 mL oral gavage of an *Eimeria* spp. solution. From d 21 to 23, a 1 mL oral gavage of *C. perfringens* was administered. Intestinal lesion scoring and jejunum tissue sample collection were performed during necropsy, 10 d post-*Eimeria* challenge as described below.

Eimeria Challenge

ADVENT® (Huvepharma) coccidiosis vaccine was diluted with sterile water and administered at a 10 x recommended dose per bird. The ADVENT® coccidiosis vaccine contains live oocysts of *E. acervulina*, *E. maxima*, *E. tenella*. It contains gentamicin and amphotericin B as preservatives. One mL of this *Eimeria* spp. solution was given to the challenged birds on d 18, via an oral gavage using a 1 mL Tuberculin syringe (Thermo Fisher Scientific).

Clostridium perfringens Challenge.

A *C. perfringens* strain, with NetB toxin producing ability, was isolated from a bird that had been diagnosed with NE (Bailey *et al.* 2013). The strain was maintained as a frozen stock in glycerol at -80°C until further application. The frozen bacterial isolate was revived by streaking a loop-full of frozen culture on reduced tryptic soy agar (rTSA) containing 5% sheep blood, incubated under anaerobic conditions (5%CO_2, 5%H and 90%N_2) at 37°C for 24 hours. After 24 hours, each plate was checked for purity. A single colony with typical *C. perfringens* morphology was picked, inoculated in brain heart infusion broth (100 mL) and incubated anaerobically for 24 hours at 37°C. After incubation, the culture was diluted (1:100) to produce an approximately 10^7 CFU/mL inoculum. The inoculum was orally administered to broilers of the challenged treatments groups on d 21, 22, and 23. Fresh inoculum was prepared for each challenge day. Inoculum levels were verified by serially diluting and spread plating the inoculum on duplicate rTSA containing 5% sheep blood plates, incubated anaerobically for 24 hours at 37°C and viable colonies showing typical colony morphology were counted.

Measurements and Sample Collection

Body weight (BW) and feed intake (FI) were measured on d 0, 14, and 28 on a per pen basis. Morbid birds were removed, euthanized, and BW recorded daily; mortalities were also removed daily and recorded. Mortality corrected feed conversion ratio (AFCR), body weight

gain (BWG) per bird and FI per bird were calculated based off of the removed body weights where appropriate. On d 28, five birds were randomly selected from each pen and euthanized by CO_2 gas inhalation, followed by cervical dislocation. A jejunum sample (2 cm in length) was collected 15 cm proximal from the Meckel's diverticulum per bird, prior to lesion scoring for sterility. Samples were then pooled by pen and stored at -80°C until DNA extraction, as described below. NE lesions were scored in the duodenum, jejunum, and ileum using a modified scale of Prescott *et al.* (1978). Lesions were scored on a scale of 0 to 4, where 0 = no apparent lesions, 1 = thin friable intestines; 2 = focal necrosis, ulceration or both; 3= patchy necrosis; and 4= severe extensive mucosal necrosis. Coccidiosis lesions were scored according to the methods described by Johnson and Reid (2007). The duodenum, jejunum, and ileum were scored for *E. acervulina* and *E. maxima* on a scale of 0 to 4.

Diet and Mycotoxin Analyses

Prior to formulation, corn was analyzed for mycotoxin concentrations (Midwest Laboratories, Inc.; Table 5.3). Diet samples were analyzed for moisture, dry matter, crude protein (CP), crude fat, acid detergent fiber (ADF), neutral detergent fiber (NDF) and crude fiber (CF) (Midwest Laboratories, Inc.; Table 5.2). Following termination of the experiment, a sample of the M-DDGS source was analyzed for CP, CF, ADF and NDF (Midwest Laboratories Inc.; Table 5.1). Geometric particle size was determined by a Tyler Ro-Tap shaker (Auburn, AL; ASAE Standard S319.4, 2009; Table 5.1).

DNA Extraction, Amplification, and 16S rRNA Gene Sequencing Data Analysis

Jejunum tissue samples were removed from a -80C freezer, thawed, and bacterial DNA extracted using the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI) according to manufacturer instructions. Extracted DNA was tested for concentration and purity with a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific) according to manufacturer instructions. The standard for purity was a 260nm/280nm ratio between 1.8 - 2.0. Extracted DNA was stored in -20°C until further analysis.

Stage 1 of PCR amplification was performed at Auburn University using an iQ5 thermocycler (Bio-Rad, Hercules, CA), with reaction conditions described in Table 5.5. Eurofins Genomics® 16S rRNA gene, universal target primers (CS1/515F and CS2/926R) were utilized

for amplification (Eurofins Genomics LLC). A touchdown protocol was performed, as described in Table 5.6. The DNA denaturation step was chosen based on the half-life of Taq (Innis and Gelfand, 2012). Annealing conditions were dependent on the base composition and length of the primers, where the primer extension parameters were based on the optimal temperature of Taq and length of the target sequence. Amplification of Stage 1 PCR yields were verified by gel electrophoresis in a 2% agarose gel. Running buffer and gels were made using 1x concentration of AccuGENE TBE buffer (Lonza Group, Basel, Switzerland). Electrophoresis was performed at 75v for ~1 hour or until sufficient separation between products was obtained. Lonza® 100 bp Extended Range DNA Ladder was used as a DNA size standard. Stage 1 PCR yields were stored at 4°C until further analysis.

Stage 1 PCR yields were sent to University of Illinois Chicago DNA Services Facility (UIC DNAS) facility for targeted amplicon sequencing (TAS). An 8 cycle Stage 2 PCR reaction (Table 5.6) was performed. The variable regions V3-V4 of the 16S rRNA gene were amplified using Fluidigm's Access Array™ Barcoded primers (0.4 μM concentration), in preparation for Illumina MiSeq sequencing (Illumina, Inc.) (Table 5.5). After sample amplification, purification and normalization were performed using SequalPrep plates (Thermo Fisher Scientific). The final pooled libraries were quantified using a formula based on Qubit 3.0 Fluorometer quantification (ThermoFisher Cat#Q33217), and diluted to 2 nM. The libraries were then denatured with 0.2 NaOH, according to Illumina MiSeq protocol. The volume of the final sample loading mixture is 600 μl, including the appropriate library concentration and spike in phiX control (generally 20%).

Forward and reverse reads were merged using the software package PEAR (Zhang *et al.*, 2014). Ambiguous nucleotides were trimmed from the ends and reads with internal ambiguous nucleotides were discarded. Primer sequences were identified using Smith-Watermann alignment, where reads that lacked either primer sequence were discarded. Sequences were then trimmed based on quality scores using a modified Mott algorithm with PHRED quality threshold of $P = 0.01$. After trimming, any sequences < 400 bases were discarded. Chimeric sequences were identified using the USEARCH algorithm with GreenGenes 13_8 reference sequences (Edgar, 2010; McDonald *et al.*, 2012).

The software package QIIME was then used to generate operational taxonomic unit (OTU) clusters in a *de novo* manner using UCLUST algorithm with a 97% similarity threshold (Edgar, 2010; Caporaso *et al.*, 2010). Taxonomic annotations for each OTU were generated using the UCLUST algorithm and the GreenGenes 13_8 reference database with a minimum similarity threshold of 90% (Edgar, 2010; McDonald *et al.*, 2012). Taxonomic and OTU abundance data were merged into a single OTU table, as a biological observation matrix (BIOM) (McDonald, 2012). Species alpha-diversity indices (Shannon index (H') and Margalef Species Richness) were calculated from the OTU table, in BIOM format. 100% stacked column histograms of experimental treatment averages based on phylogenic classification were used to assess bacterial community composition (Figure 5.1). In order to further reduce noise within the data, less abundant OTUs which represented < 1% in all treatment averages, were categorized as "Other".

Data Analysis

All statistical analyses, outside of 16S rRNA gene sequencing, were facilitated using SPSS version 22 software. Significant differences were reported at $P \leq 0.05$. A two-way ANOVA was used to determine main effect and interactions, with diet and challenge as main effects when analyzing the performance and NE results. A Mann-Whitney U Test was used to determine pairwise comparisons of treatment microbiome alpha-diversity indices.

Results and Discussion:

The provided proximate nutrient composition of the M-DDGS source, as well was those values that were analyzed following termination of the experiment, are displayed in Table 5.1. Limited research has analyzed the effects of feeding R-DDGS to poultry. However, the crude fat value (7.07%) was found to be similar to that of a M-DDGS product (7.30%) used by Purdum *et al.*, (2014). The swine NRC (2012) considers a M-DDGS product to include 6 to 9% oil, in which this source additionally corresponds. Provided nutrient profiles of the M-DDGS source were used in diet formulation. However, provided values were determined to differ from those values that were analyzed (Midwest Laboratories Inc.; Table 5.1). Diets were formulation

(Creative Formulations Concepts Inc.) using a least cost formula, on a total-amino acid basis (Table 5.2).

Oil-extraction of DDGS may affect growth performance, due to its influence on energy content (Graham *et al.*, 2014). Hence, the addition of the M-DDGS source to a corn-soy diet required additional supplementation of fat (poultry oil) to meet target ME for expectations of equal performance. When M-DDGS (7.3% crude fat) was fed to laying hens at the rate of 20% of the ration, Purdum *et al.* (2014) observed no significant effects on performance. However, in the current experiment when feeding M-DDGS (7.07%) at 5% of the starter diet and 7.5% of the grower diet, significant effects on performance were observed (Table 5.6). Diet resulted in significant effects on BWG at 0 to 28 d of age ($P = 0.039$), with no effect of challenge or interaction observed ($P > 0.05$). However, a diet and challenge interaction effect was observed on FI at 0 to 14 d of age ($P = 0.038$). Attempts were made to formulate diets to balance all known nutrients and expected performances to be equal. Mycotoxin concentrations of the M-DDGS source were determined by Midwest Labs (Midwest Laboratories Inc.) to be low and therefore did not influence performance results. Challenge alone, resulted in significant effects at 14 to 28 d and 0 to 28 d of age on FI, AFCR and mortality ($P < 0.05$); however, this was expected. As seen in the second experiment, amino acid variation due to oil-extraction appears to be the contributing factor. If diets had been formulated on a digestible amino acid basis and analyzed M-DDGS values been accounted for, performance results may have differed.

Table 5.7 demonstrates NE intestinal lesion incidence, severity and average scores. An effect of challenge was observed on NE incidence ($P = 0.015$), as well as an effect of diet ($P = 0.040$). However, no significant interaction was present ($P = 0.189$). In addition, diet alone had an effect on NE average lesion score ($P = 0.027$), without an effect of challenge or interaction observed ($P > 0.05$). Those birds consuming the M-DDGS diet experienced greater NE average lesion scores than those birds consuming the control diets (0.344 vs. 0.156). Including M-DDGS in the diet did not exacerbate NE development (NE incidence, severity or average lesion score), as no interaction effects of diet and challenge were observed ($P > 0.05$). The significant interaction effect of diet and challenge on FI during the two-week post-hatch period, imply a disturbance in the GIT before a stable microbiome could be established. Therefore, broiler chicks

may have experienced increased susceptibility to *Eimeria* spp. and *C. perfringens* challenge. Subsequently, resulting in diet and challenge separately impacting NE development negatively.

In order to analyze how the intestinal microbiome shifts and interacts when birds consume dietary M-DDGS, samples of jejunum tissue were collected from broilers at 28 d of age. The jejunum is an intestinal segment in which majority of NE lesions have been found to be most common and severe (Truscott & AL-Sheikhly, 1977; Fukata *et al.*, 1991, Long *et al.*, 1974). In addition, it functions in nutrient absorption and monitoring of luminal content (Bar-Shira *et al.*, 2003; Shokker *et al.*, 2009) allowing the inclusion of M-DDGS in the diet to be assessed. Targeted amplicon sequencing (TAS) was used to uncover bacterial communities of the jejunum tissue samples of broilers collected at 28 d of age. Higher-level taxonomic groups were used to assess bacterial community composition per treatment, as members of the same bacterial genus are not necessarily each other's closest relatives (Goodrich *et al.*, 2014). However, to account for species diversity, alpha-diversity indices were analyzed (Goodrich *et al.*, 2014).

Alpha-diversity was analyzed, according to species richness (Margalef) and Shannon index (H'), to provide information about the rarity and commonness of species in a community in order to better understand its structure (Table 5.9). Species richness (Margalef) implies no particular regularity in distribution, in which the number of individuals of all species are taken into account. The Shannon index accounts for both abundance and evenness of the species present (Beals *et al.*, 2000). No significant pairwise comparisons between treatments were observed of either Species Richness or Shannon Index ($P > 0.05$; Table 5.9). When looking at average treatment values of the following alpha-diversity indices, birds consuming dietary M-DDGS appeared to be comprised of a less diverse system, dominated by one or a few species. Whereas, those birds consuming the control diet appeared to be comprised of a highly diverse system.

Bacterial community composition is displayed in Figure 5.1. The OTU table, in BIOM format, revealed 9 phyla, 17 classes, 31 orders, and 64 families of known bacteria presented in rarified samples ($n = 34$). To further reduce noise within the data, less abundant OTUs, which represented <1% in all treatment averages, were classified as "Other". The four most common (>

1% in at least one treatment average) phyla were *Bacteroidetes, Firmicutes, Proteobacteria* and *Tenericutes*. Previous studies have also shown *Bacteroidetes, Firmicutes*, and *Proteobacteria* to be the three most predominant phyla of the intestinal microbiome of chickens (Wei *et al.*, 2003; Oakley *et al.*, 2014). Awad *et al.* (2016) observed a more diverse bacterial community of jejunal mucosa by 28 d of age. *Proteobacteria* were found to be significantly more present during the first day of life and decreased thereafter. Whereas, *Firmicutes* was the predominant phylum of birds at 28 d of age. Results from this experiment support these findings, as *Firmicutes* accounted for at least 86% of major OTUs at birds 28 d of age and *Proteobacteria* accounted for at most, 2.4%. In birds infected with *Campylobacter jejuni*, *Bacteroidetes* has been found to be more abundant in the jejunal mucosa than that of the control (non-infected) birds (Awad *et al.*, 2016). Results support these findings as *Bacteroidetes* was more present in challenged treatments versus unchallenged treatments, regardless of diet. Previous studies have also found *Tenericutes* present in the chicken intestinal microbiome of chickens, specifically in the jejunal mucosa (Awad *et al.*, 2016), but at a lower abundance (Wei *et al.*, 2013; Waite and Taylor, 2014).

Within the phylum *Firmicutes*, the families *Lactobacillaceae, Clostridiaceae, Lachnospiraceae*, *Peptostreptococcaceae* and *Ruminococcaceae* were most common with different proportions in each treatment. *Lactobacillaceae* were the most prominent family across all treatments, with greater presence in challenged treatments versus unchallenged treatments. *Lactobacillaceae Lactobacillus* assists in maintaining the equilibrium of the microbial ecosystem (Pyrde *et al.*, 1999), in which its interactions with epithelial cells can result in protection against pathogens (Sengupta *et al.*, 2013). Therefore, previous studies involving a *C. perfringens* challenge have also found *Lactobacillaceae* to dominate the intestinal bacterial assemblage (Fasina *et al.*, 2015; Feng *et al.*, 2010). *Clostridiaceae* has been found to be dominant in birds 28 d of age (Awad *et al.*, 2016), whereas studies involving a *C. perfringens* challenge have found increased abundances (Fasina *et al.*, 2015. Higher proportions of *Clostridiaceae* were detected in M-DDGS diet treatments compared to control diet treatments. Furthermore, this supports NE lesion score results previously described. Dietary M-DDGS treatments were also found to have reduced proportions of *Lactobacillaceae*, further supporting their beneficial contributions against disease development.

Butyrate-producing members of the *Lachnospiraceae* and *Ruminococcaceae* family have been isolated from cecal content of chickens (Eeckhaut *et al.*, 2011), where some strains have been shown to reduce the severity of NE when given to broilers (Antonissen *et al.*, 2017). Results from this experiment support these findings, as greatest severity of NE was observed in the challenged M-DDGS treatment. Subsequently, this treatment had also experienced reduced proportions of *Ruminococcaceae* and *Lachnospiraceae*. *Ruminococcaceae* presence was additionally reduced in challenged treatments in comparison to unchallenged treatments. An indirect effect of lactate-producing bacteria to cross-feed lactate-utilizing and butyrate-producing bacteria has been suspected to be of importance (De Maesschalck *et al.*, 2015). However, results from this experiment provide no further clarity, as varying proportions were found.

Within the phylum *Proteobacteria*, the family *Enterobacteriaceae* existed within treatments at different proportions. An inverse relationship has been observed between the phylum *Firmicutes* and *Proteobacteria* (Awad *et al.*, 2016), as described above. *Enterobacteriaceae* were observed in low proportions across treatments at 28 d of age, as the phylum *Proteobacteria* has been found to be more dominant during the first day of life than in that of older birds (Liu *et al.*, 2003). As a consequence, the gut is first colonized by facultative anaerobes which are later in life substituted by anaerobes, altering the gut ecosystem, facilitating subsequent growth and colonization of obligate anaerobes (Wise and Siragusa, 2007), such as *C. perfringens*. Within the phylum *Bacteroidetes*, the families *Bacteroidaceae* and *Rikenellaceae* were additionally present. *Rikenellaceae* has been noticed to sharply increase as birds get older from 2% (d 15) to 24% (d 22) and then to 36% (d 29).

Conclusion:

Including M-DDGS (7.07% crude fat) at 5% of the starter diet and 7.5% of the grower diet did not exacerbate the effects of NE development. Significant main effects of challenge and diet were noticed on NE development, alone. However, this is believed to be derived from amino acid variation versus that of endogenous fiber composition, negatively influencing broilers before a stable microbiome was established (starter phase). Therefore, when feeding M-DDGS sources to broilers up to 28 d of age, nutrient values should be analyzed prior to formulating diets

on a digestible amino acid basis. Within the phylum Firmicutes, *Lachnospiraceae* and *Ruminococcaceae* appear to have beneficial against *C. perfringens* colonization and disease induction. However, further research is needed to provide further clarity on their indirect effects on each other and on NE development.

Table 5.1 Proximate nutrient composition of medium-oil distillers dried grains with solubles (M-DDGS)[1]

Item	Value	Method of Analysis[2,3]
Protein, fat, fiber (%)		
Moisture	8.43	
Dry matter	91.57	
Crude protein	28.03	
Analyzed	24.60	AOAC official method 990.03
Crude fat	7.07	
Crude fiber	8.53	
Analyzed	7.07	ANKOM Tech./AOAC official method 2001.11
ADF	10.66	
Analyzed	15.4	AOCS Ba 6a-05
NDF	32.13	
Analyzed	37.3	ANKOM Tech. Method
Minerals (%, unless otherwise indicated)		
Calcium	0.02	
Phosphorous	0.76	
Sodium	0.19	
Potassium	1.02	
Magnesium	0.28	
Sulfur	0.48	
Copper (ppm)	4.42	
Iron (ppm)	58.78	
Manganese (ppm)	11.90	
Zinc (ppm)	44.38	
Particle size (microns)	578	ASAE Standard S319.4
Hunter L Color (L*)	48.96	

[1]Medium-oil DDGS (7.07% crude fat) on an as-fed basis.
[2]All method of analysis were determined by Midwest Laboratories, Inc. (Omaha, NE).
[3]AOAC = AOAC International (Gaithersburg, MD); ANKOM = ANKOM Technology (Macedon, NY); AOCS = The American Oil Chemists' Society (Urbana, IL); ASAE = American Society of Agricultural Engineers (St. Joseph, MI).

Table 5.2 Ingredient and nutrient composition of diets fed to broilers from 0 to 28 d of age

	Starter Diets		Grower Diets		
Ingredient, %	Control	M-DDGS	Control	M-DDGS	Method of Analysis[4]
Corn	62.361	60.899	66.540	53.315	
Soybean Meal 48	28.929	25.749	24.830	28.493	
M-DDGS (7.07% crude fat)[1]	0.000	5.000	0.000	7.500	
Poultry Meal 65	4.000	4.000	4.000	2.000	
Poultry Oil	1.043	0.980	1.112	2.606	
Salt	0.404	0.222	0.405	0.392	
Dicalcium Phosphate	1.405	1.196	1.111	0.942	
Limestone	1.127	1.235	1.079	1.280	
DL-Methionine	0.313	0.289	0.314	0.217	
L-Lysine 98	0.168	0.180	0.246	0.004	
L-Threonine	0.000	0.000	0.112	0.000	
Vitamin Premix[2]	0.050	0.050	0.050	0.050	
Choline Chloride Dry	0.100	0.100	0.100	0.100	
Trace Minerals Premix[3]	0.100	0.100	0.100	0.100	
Calculated Nutrient Content (% unless otherwise indicated)					
ME (kcal/kg)	3,053	3,053	3,109	3,109	
Crude Protein	21.500	22.180	20.000	22.307	
Calcium	0.930	0.930	0.840	0.840	
Phosphorous, Available	0.450	0.450	0.390	0.390	
Sodium	0.200	0.139	0.200	0.200	
Proximate Analysis Nutrient Content (% unless otherwise indicated)					
Dry Matter	86.770	86.210	87.640	88.150	Calculation
Crude Protein	20.40	22.100	19.900	21.200	AOAC official method 990.03
Crude Fat	7.750	4.100	3.4600	5.3200	AOAC official method 945.16
Acid Detergent Fiber	3.60	2.900	2.700	3.800	ANKOM Tech. Method
Ash	4.760	4.640	4.370	4.640	AOAC official method 942.05
Crude Fiber	1.990	2.820	2.210	2.820	AOCS Ba 6a-05
Neutral Detergent Fiber	6.600	7.600	6.000	8.000	Ankom Tech./AOAC official method 2001.11

[1]M-DDGS = medium-oil DDGS (7.07% crude fat) on an as fed-basis.
[2]Vitamin premix includes per kg of diet: Vitamin A (Vitamin A acetate), 18,739 IU; Vitamin D (cholecalciferol), 6,614 IU; Vitamin E (DL-alpha tocopherol acetate), 66 IU; menadione (menadione sodium bisulfate complex), 4 mg; Vitamin B12 (cyanocobalamin), 0.03 mg; folacin (folic acid), 2.7 mg: D-pantothenic acid (calcium pantothenate), 31 mg; riboflavin (riboflavin), 22.1 mg; niacin (niacinamide), 88.2 mg; thiamin (thiamin mononitrate), 5.5 mg; D-biotin (biotin), 0.18 mg; and pyridoxine (pyridoxine hydrochloride), 7.7 mg.
[3]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, cypress excel Se yeast), 0.3 mg
[4]All methods of analysis were determined by the Midwest Laboratories Inc. (Omaha, NE).

[4]AOAC = AOAC International (Gaithersburg, MD); ANKOM = ANKOM Technology (Macedon, NY); AOCS = The American Oil Chemists' Society (Urbana, IL); ASAE = American Society of Agricultural Engineers (St. Joseph, MI).

Table 5.3 Mycotoxin concentrations of medium-oil distillers dried grains with solubles (M-DDGS) and ground corn included in diets fed to broiler 0 to 28 d of age

Analysis[1,2]	Results	Method[3]
Experimental Corn (ppb, unless otherwise indicated)		
AF B1	3.45	Internal SOP-14-168
AF B2	ND	Internal SOP-14-168
AF G1	ND	Internal SOP-14-168
AF G2	ND	Internal SOP-14-168
DON, ppm	ND	Internal SOP-14-168
FUM B1, ppm	4.6	AOAC 999.15 with modifications
FUM B2, ppm	1.3	AOAC 999.15 with modifications
FUM B3, ppm	0.2	AOAC 999.15 with modifications
OTA	ND	AOAC 2000.03 with modifications
T-2 Toxin	ND	Internal SOP-14-168
ZEN	ND	Internal SOP-14-168
M-DDGS[5] (ppb, unless otherwise indicated)		
Total AF	ND	Internal SOP-14-168
Total FUM, ppm	0.20	AOAC 999.15 with modifications
OTA	ND	AOAC 2000.03 with modifications
DON, ppm	2.5	Internal SOP-14-168
T-2 Toxin, ppm	ND	Internal SOP-14-168
ZEN	116	Internal SOP-14-168

[1]All methods of analysis were determined by Trilogy Analytical Laboratory, Inc. (Washington, MO).
[2]AF = Alfatoxin; DON = Deoxynivalenol; FUM = Fumonisin; OTA = Ochratoxin A; ZEN = Zearalenone; ppb = Parts per billion; ppm = Parts per million.
[3]SOP = Standard operating procedure; AOAC = AOAC International (Gaithersburg, MD).
[4]ND = None detected.
[5]M-DDGS = Medium-oil DDGS (7.07% crude fat).

Table 5.4 Reaction conditions and primers for amplification of bacterial populations of jejunum tissue collected from broilers at 28 d of age by polymerase chain reaction (PCR)

Reaction conditions	1 x Rxn volume (µL)
Stage 1[1]	
Master Mix[2]	12.5
DdH_2O[3]	10.75
DNA Template[4]	1.0
Forward Primer[5]	0.25
Reverse Primer[6]	0.25
BSA[7]	0.25
Stage 2[8]	
Primers[9]	0.4
Stage 1 PCR Yield	1.0

[1]Performed at Auburn University Laboratory (Auburn, AL).
[2]Master Mix = Lucigen EconoTaq Plus 2x Master Mix (Middleton, WI): 0.1 units/µL of EconoTaq DNA Polymerase, Reaction Buffer (pH 9.0), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dATP, 3 mM MgCl2, and a proprietary mix of PCR Enhancer/Stabilizer and blue and yellow tracking dyes.
[3]DdH_2O = double distilled H_2O.
[4]DNA Template = pooled jejunum tissue sample (5 birds/pen, each 2 cm in length, 15 cm proximal from Meckel's diverticulum) bacterial DNA extracted using Wizard Genomic Purification Kit (Madison, WI).
[5]Forward Primer = Eurofins Genomics CS1/515F Universal Target 16S rRNA Gene (Louisville, KY): 5'-GTGYCAGCMGCCGCGGTAA-3', expected amplicon size with CS1 456, annealing temperature 50°C.
[6]Reverse primer = Eurofins Genomics® CS2/926R Universal Target 16S rRNA Gene (Louisville, KY): 5'-CCGYCAATTYMTTTRAGTTT-3', expected amplicon size with CS2 456, annealing temperature 50°C.
[7]BSA (Bovine Serum Albumin) = Purified BSA 100x (Lawrenceville, GA).
[8]Performed at University of Illinois Chicago DNA Services Facility (Chicago, IL).
[9]Primers = Fluidigm's AccessArray Barcoded Primers (San Francisco, CA): Read 1: Standard CS1: A+CA+CTG+ACGACATGGTTCTACA; Index Read: CS2rc: A+GAC+CA+AGTCTCTGCTACCGTA; Read 2: Standard CS2: T+AC+GGT+AGCAGAGACTTGGTCT (A "+" preceding a base indicates a Locked Nucleic Acids(LNA)-base).

Table 5.5 Polymerase chain reaction (PCR) conditions for amplification of bacterial populations of jejunum tissue collected from broilers at 28 d of age

Cycle Step	Temperature	Time	Cycles
Stage 1[1]			
Initial Denaturation	94°C	5 min	1x
Variable Temperature Annealing			15x, reduce 1°C/cycle
Denaturation	94°C	30 sec	
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Constant Temperature Annealing	94°C		30x
Denaturation	94°C	30 sec	
Annealing	55°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	7 min	1x
Hold	4°C	∞	
Stage 2[2]			
Initial Denaturation	95°C	5 min	1x
Variable Temperature Annealing			28x
Denaturation	95°C	30 sec	
Annealing	60°C	30 sec	
Extension	72°C	30 sec	

[1]Performed at Auburn University Laboratory (Auburn, AL) using a Bio-Rad IQ5 thermocycler (Hercules, CA).
[2]Performed at University of Illinois Chicago DNA Services Facility (Chicago, IL) in preparation for Illumina MiSeq (San Diego, CA).

Table 5.6 Growth performance[1] of broilers challenged[2] or unchallenged with *C. perfringens* and *Eimeria* spp. fed a control diet (corn-soy) or medium-oil distillers dried grains with solubles (M-DDGS)[3] diet from 0 to 28 d of age

	BW Gain (kg/bird)			Feed Intake (kg/bird)			AFCR[4] (kg/kg)			Mortality[5] (%)		
Day	0 to 14	14 to 28	0 to 28	0 to 14	14 to 28	0 to 28	0 to 14	14 to 28	0 to 28	0 to 14	14 to 28	0 to 28
Diet x Challenge												
P ≤	0.386	0.120	0.242	**0.038***	0.337	0.355	0.515	0.241	0.111	0.083	0.143	0.566
SEM	0.007	0.032	0.030	0.005	0.051	0.068	0.017	0.029	0.017	0.012	0.026	0.029
Diet Main Effect												
Control	0.427	1.008	**1.492**	0.518	1.780	2.366	1.196	1.544	1.439	0.017	.106	0.122
M-DDGS	0.423	0.957	**1.427**	0.519	1.682	2.258	1.208	1.584	1.465	0.017	.089	0.106
P ≤	0.521	0.115	**0.039***	0.894	0.064	0.123	0.497	0.176	0.130	1.000	0.525	0.566
SEM	0.005	0.023	0.022	0.003	0.036	0.048	0.012	0.020	0.012	0.009	0.018	0.020
Challenge Main Effect												
Challenged	0.429	**0.924**	1.435	0.521	**1.803**	**2.424**	1.206	**1.599**	**1.474**	0.011	**0.150**	**0.161**
Unchallenged	0.421	**1.041**	1.484	0.515	**1.658**	**2.201**	1.198	**1.529**	**1.430**	0.022	**0.044**	**0.067**
P ≤	0.244	**<0.001***	0.118	0.226	**0.008***	**0.003***	0.667	**0.019***	**0.017***	0.378	**<0.001***	**0.002***
SEM	0.005	0.023	0.022	0.003	0.036	0.048	0.012	0.020	0.012	0.009	0.018	0.020

[1]Each value represents means of 9 replicate pens having 10 chicks at placement.
[2]*Eimeria spp.* challenge (d 18): ADVENT® (Huvepharma) coccidiosis vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. tenella* administered at a 10 x recommended dose per challenged bird; *C. perfringens* challenge (d 21, 22, 23): Inoculated brain heart infusion broth was diluted 1:100 to produce an approximately 10^7 CFU/mL inoculum containing an isolate from a bird diagnosed with necrotic enteritis.
[3]M-DDGS = Medium-oil DDGS (7.07% crude fat); 5% inclusion in the starter diet, 7.5% inclusion in the grower diet.
[4]AFCR = Feed conversion ratio corrected for mortality.
[5]Mortality data underwent square-root arcsine transformation before analysis.
[6]SEM= Pooled standard error.

Table 5.7 Necrotic Enteritis (NE) intestinal lesion[1,2] incidence, severity and average lesion scores of broilers challenged[3] or unchallenged with *C. perfringens* and *Eimeria* spp. fed a control diet (corn-soy) or medium-oil distillers dried grains with solubles (M-DDGS)[4] diet from 0 to 28 d of age

28 d	NE Incidence[5] (%)	NE Severity[6] (%)	NE Average Lesion Score[7]
Diet x Challenge			
P ≤	0.189	0.316	0.239
SEM[8]	0.059	0.022	0.085
Diet Main Effect			
Control	**0.144**	0.011	**0.156**
M-DDGS	**0.267**	0.033	**0.344**
P ≤	**0.040***	0.316	**0.027***
SEM	0.042	0.016	0.060
Challenge Main Effect			
Challenged	**0.278**	0.022	0.322
Unchallenged	**0.133**	0.022	0.178
P ≤	**0.015***	1.000	0.090
SEM	0.042	0.016	0.060

[1]Necrotic enteritis (NE) lesions were scored in the duodenum, jejunum, and ileum using a modified scale of Prescott *et al.* (1978

[2]Each value represents mean of 9 replicate pens, where 5 birds were randomly chosen per pen.

[3]*Eimeria spp.* challenge (d 18): ADVENT® (Huvepharma) coccidiosis vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. tenella* administered at a 10 x recommended dose per challenged bird; *C. perfringens* challenge (d 21, 22, 23): Inoculated brain heart infusion broth was diluted 1:100 to produce an approximately 10^7 CFU/mL inoculum containing an isolate from a bird diagnosed with necrotic enteritis.

[4]M-DDGS = Medium-oil DDGS (7.07% crude fat); 5% inclusion in the starter diet, 7.5% inclusion in the grower diet.

[5]NE Incidence = Percentage of birds a lesion score ≥ 1.

[6]NE Severity = Percentage of birds a lesion score ≥ 2.

[7]NE Average Lesion Score =Mean of scores between 0 and 4.

[8]SEM = Pooled standard error

Table 5.8 Alpha-diversity indices[1,2] of 16S rRNA gene sequences from jejunum tissue[3] of broilers challenged[4] or unchallenged with *C. perfringens* and *Eimeria* spp. fed a control diet (corn-soy) or medium-oil distillers dried grains with solubles (M-DDGS)[5] diet from 0 to 28 d of age

Sample DNA	Species Richness (Margalef)[6]	SEM[8]	Shannon Index (H')[7]	SEM
28 d				
Treatments				
Challenged Control	3.375	0.289	1.855	0.226
Unchallenged Control	3.524	0.166	2.057	0.137
Challenged M-DDGS	3.338	0.286	1.909	0.241
Unchallenged M-DDGS	3.171	0.187	1.930	0.201

[1]Alpha-diversity indices were calculated from the operational taxonomic unit (OTU) table derived from sequences with a 97% similarity threshold, using QIIME (San Diego, CA) software package.
[2]Each value represents treatment means: Challenged Control (n = 9), Unchallenged Control (n = 9), Challenged L-DDGS (n = 9), Unchallenged L-DDGS (n = 8).
[3]Jejunum samples (5 birds/pen, each 2 cm in length, 15 cm proximal from Meckel's diverticulum) collected on d 28; 16S rRNA gene sequences were rarified to 400 sequences per sample due to high host-DNA presence.
[4]*Eimeria spp.* challenge (d 18): ADVENT® (Huvepharma) coccidiosis vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. tenella* administered at a 10 x recommended dose per challenged bird; *C. perfringens* challenge (d 21, 22, 23): Inoculated brain heart infusion broth was diluted 1:100 to produce an approximately 10^7 CFU/mL inoculum containing an isolate from a bird diagnosed with necrotic enteritis.
[5]M-DDGS = Medium-oil DDGS (7.07% crude fat).
[6]Shannon Index*:* $H' = -\sum p_i \ln p_i$, where p_i is the proportion of individuals found in species *i*.
[7]Species richness (Margalef): $R_1 = (S\text{-}1)/Log\ N$, where *S* is the number of taxa and *N* is the number of individuals.
[8]SEM = Pooled standard error.

Table 5.9 Pairwise comparisons of treatment alpha-diversity indices[1,2] of 16S rRNA gene sequences from jejunum tissue[3] of broilers challenged[4] or unchallenged with *C. perfringens* and *Eimeria* spp. fed a control diet (corn-soy) or medium-oil distillers dried grains with solubles (M-DDGS)[5] diet from 0 to 28 d of age

Sample DNA	Species Richness (Margalef)[6]	Shannon Index (H')[7]
Mann-Whitney U Test		
P-value		
Challenged M-DDGS vs. Unchallenged Control	0.755	0.965
Challenged M-DDGS vs. Unchallenged DDGS	0.498	0.700
Challenged M-DDGS vs. Challenged Control	0.964	0.627
Unchallenged Control vs. Unchallenged M-DDGS	0.463	0.700
Unchallenged Control vs. Challenged Control	0.929	0.566
Unchallenged M-DDGS vs. Challenged Control	0.190	1.000

[1]Values represent pairwise-comparisons between non-parametric treatments: Challenged Control (n = 9), Unchallenged Control (n = 9), Challenged L-DDGS (n = 9), Unchallenged L-DDGS (n = 8).
[2]Alpha-diversity indices were calculated from the operational taxonomic unit (OTU) table derived from sequences with a 97% similarity threshold, using QIIME (San Diego, CA) software package.
[3]Jejunum samples (5 birds/pen, each 2 cm in length, 15 cm proximal from Meckel's diverticulum) collected on d 28; 16S rRNA gene sequences were rarified to 400 sequences per sample due to high host-DNA presence.
[4]*Eimeria spp.* challenge (d 18): ADVENT® (Huvepharma) coccidiosis vaccine containing live oocysts of *E. acervulina*, *E. maxima*, *E. tenella* administered at a 10 x recommended dose per challenged bird; *C. perfringens* challenge (d 21, 22, 23): Inoculated brain heart infusion broth was diluted 1:100 to produce an approximately 10^7 CFU/mL inoculum containing an isolate from a bird diagnosed with necrotic enteritis.
[5]M-DDGS = Medium-oil DDGS (7.07% crude fat).
[6]Shannon Index*:* $H' = -\sum p_i \, ln \, p_i$, where p_i is the proportion of individuals found in species *i*.
[7]Species richness (Margalef): $R_1 = (S\text{-}1)/Log\ N$, where *S* is the number of taxa and *N* is the number of individuals.

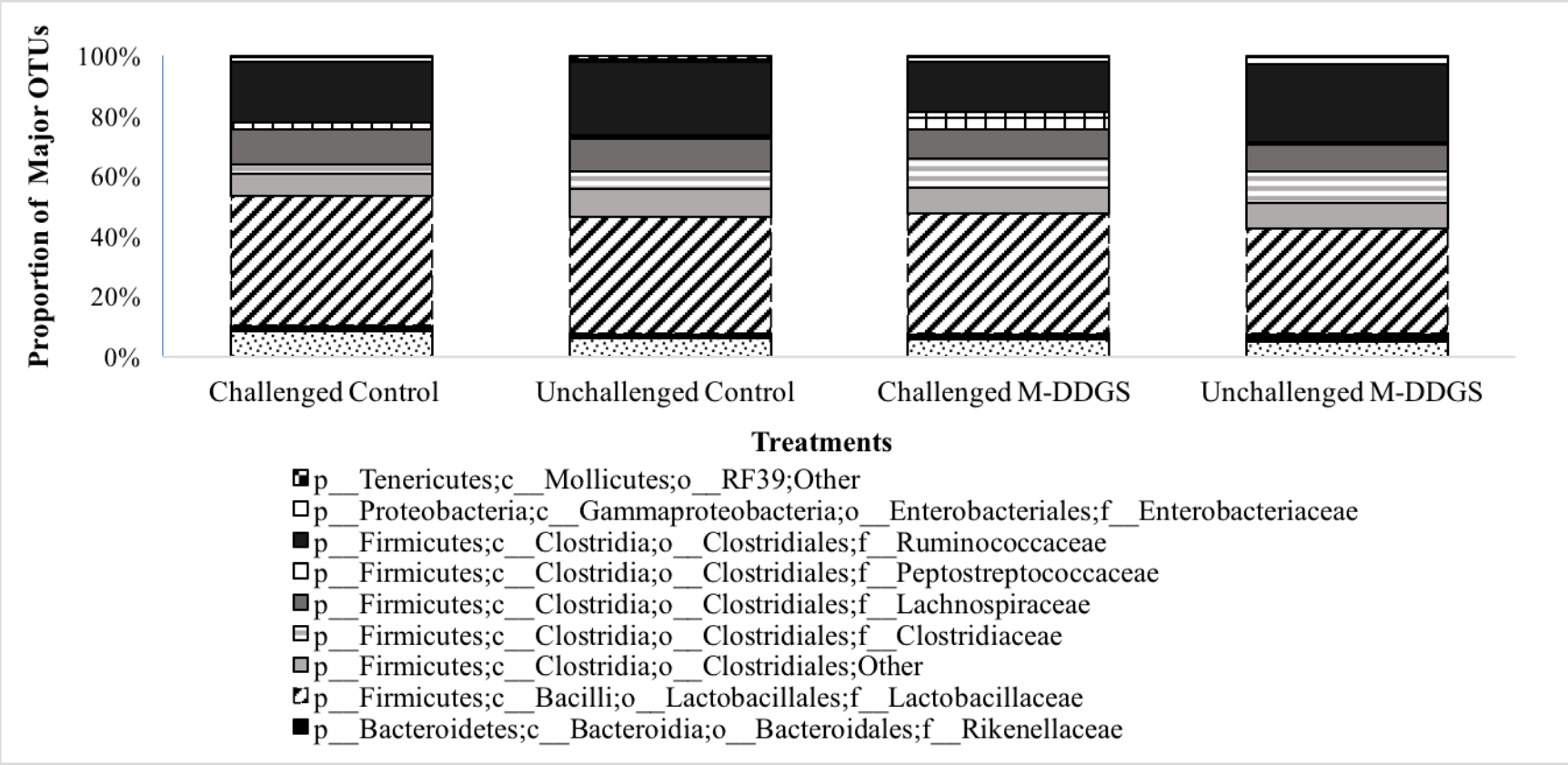

Figure 5.1 Bacterial community composition according to phyla, class, order and family of 16S rRNA sequences of jejunum tissue samples from 28 d broilers challenged or unchallenged with *C. perfringens* and *Eimeria* spp., provided *at libitum* access to either a control diet (corn-soy diet) or M-DDGS diet (5% M-DDGS in the starter, 7.5% in the grower) from 0 to 28 d of age. 16S rRNA gene sequences were rarified to 400 sequences per sample due to high host-DNA presence. To further reduce noise within data, less abundant OTU's which represented <1% in all treatment averages were categorized as "Other". OTU table (in BIOM format) was generated based on sequences with a 97% similarity threshold, using QIIME software package. Each value represents treatment means: Challenged Control (n = 9), Unchallenged Control (n = 9), Challenged L-DDGS (n = 9), Unchallenged L-DDGS (n = 8).

Chapter 6.0 Summary and Conclusion

Future success of the poultry industry will ultimately depend on how well the industry meets U.S. consumer demands, and emerging food trends (Richard Kottmeyer, 2016). Hence, the industry is increasingly moving towards the production of antibiotic-free poultry (CSR Wire, 2007). However, necrotic enteritis (NE) incidence in broilers has increased in countries and commercial companies that have stopped using antibiotic growth promotors (AGPs; McReynolds *et al.*, 2007). Therefore, alternative methods for control and prevention are being warranted. Certain dietary components have been established as predisposing factors for NE, due to their effects on the microbiota present in the gastrointestinal tract (GIT). Reduced-oil distillers dried grains with solubles (R-DDGS) sources are becoming increasingly more available to the poultry industry, as a result of recent implementation of oil-extraction technology by ethanol plants. However, appropriate inclusion of R-DDGS sources poultry diets requires information of nutrient utilization and their influence on GIT microbiome.

Loss in broiler live performance can result in substantial economic losses to the poultry industry. Low-oil DDGS (L-DDGS) has been determined to more likely fit into laying hen diets than that of broilers, due to their lower metabolizable energy (Purdum *et al.*, 2014). However, if L-DDGS (4.29% crude fat) nutrient composition is analyzed prior to diets being formulation on a digestible amino acid basis, its inclusion (5% starter, 7.5% grower diets) in broiler diets can be economically advantageous. When considered, similar broiler live performance is suspected to be observed with that of a corn-soy diet. Low-oil DDGS inclusion in broiler diets did not shift the jejunum microbiome of challenged birds and no significant main effects or interactions were observed on NE development (incidence, severity, or average lesion score). Therefore, results imply that L-DDGS sources can be fed to antibiotic-free broilers without concerns of predisposing to NE. However, research extending beyond 27 d with broilers housed in floor pens, should be assessed prior to inclusion in commercial broiler diets.

When feeding M-DDGS (6.60% or 7.07% crude fat) sources, emphasis should be placed on amino acid content and quality during diet formulation. When unaccounted for, amino acid

variation can reduce broiler live performance and increase susceptibility to NE development. Furthermore, undigested amino acids can result in *C. perfringens* proliferation subsequently enhancing pathogenicity of NE. Results from the second experiment (M-DDGS, 6.60% crude fat) support this, as significant interaction effects of diet and challenge on NE severity and NE average lesion score were observed. However, when broilers were fed M-DDGS with 7.07% crude fat content significant main effects of diet and challenge were observed separately, but there was no significant interaction. Therefore, results could imply that increased endogenous fiber composition of M-DDGS (7.07% crude fat) can have beneficial effects against *Eimeria* spp. and *C. perfringens* colonization. Although, the more prominent effect of amino acid variation did not allow for a clear assessment.

When including R-DDGS sources in poultry diets, amino acid content and quality of the source must be considered. Diets should be formulated on a digestible versus total amino acid basis, in order to limit the amount of undigested amino acids available to *C. perfringens*. In all experiments, lactate- and butyrate-producing bacteria appeared to have beneficial effects against *C. perfringens* colonization and disease induction. Whether this is a pre-disposing factor, or consequence of NE formation remains unclear. Future research should focus on the indirect effects of these bacteria, as well as the effects of endogenous fiber composition of R-DDGS sources, on NE development. Knowledge gained in these areas can benefit the poultry industry, as antibiotic-free broilers are increasingly produced.

Chapter 7.0 References

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