Effects of dietary enzyme supplementation on performance, bone ash, small intestinal morphology, and apparent ileal amino acid digestibility of broilers exposed to a live coccidia oocyst vaccine

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ABSTRACT

A series of experiments were conducted to evaluate the effects of dietary enzyme supplementation in broilers exposed to a live coccidia oocyst vaccine. In each experiment, Cobb 500 broilers were obtained at day of hatch from a commercial hatchery. Half of the chicks were sprayed with Coccivac B^{TM} and housed in battery brooders in experiment (EXP) 1 and 2 or floor pens (EXP 3). Experimental diets were formulated according to Cobb 500 nutrient recommendations (positive control; PC) with the exception of Ca and available P (aP), which were reduced in the negative control (NC) diets approximately 0.1% (EXP 1), 0.11 and 0.13%, respectively (EXP 2), and 0.13% (EXP 3). Negative control diets in EXP 1 were supplemented with phytase (PHY), protease (PRO), xylanase (XYL), and the combination of PHY+PRO, PHY+XYL, and PHY+PRO+XYL. Negative control diets in EXP 2 were supplemented with PHY A, PHY B, and PHY C. In EXP 3, PC diets were supplemented with PHY at 1000 FTU/kg, and NC diets were supplemented with PHY at 1000 or 5000 FTU/kg. In all three experiments PHY supplementation generally improved ($P \leq 0.05$) broiler performance and bone ash. Vaccination reduced ($P \leq 0.05$) broiler performance in EXP 1 and 3, but increased ($P \leq 0.05$) broiler performance in EXP 2. Xylanase and/or PHY supplementation tended to improve ($P = 0.10$) ileal amino acid digestibility (IAAD) in vaccinated broilers in EXP 1 and EXP 3. Phytase supplementation improved ($P \leq 0.05$) IAAD and vaccination reduced ($P \leq 0.05$) IAAD in EXP 3.

Small intestinal morphology and goblet cell numbers were affected by enzyme supplementation and vaccination, which resulted in significant ($P \leq 0.05$) interactions. In general, vaccination increased ($P \leq 0.05$) small intestinal crypt depth and reduced ($P \leq 0.05$) goblet cell numbers in EXP 3. Phytase supplementation of the NC diets fed to vaccinated broilers ameliorated the reduction in ileal goblet cells associated with vaccination (EXP 3). Enzyme supplementation tended to improve nutrient digestibility and altered small intestinal morphology in vaccinated broilers. Vaccination reduced broiler performance but nutrient digestion and dietary enzymes may improve nutrient utilization during a coccidia vaccination.

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CHAPTER I

Literature Review

COCCIDIOSIS

Coccidiosis is a devastating disease in poultry caused by protozoan parasites of the *Eimeria spp*. There are eight coccidia species known to infect chickens. Each species invades the intestine in a site specific manner and causes a different degree of pathogenicity (Williams, 2005). For the purposes of this review, only *Eimeria acervulina, E. maxima,* and *E. tenella* will be considered. Treatment and prevention of coccidiosis costs the U.S. poultry industry an estimated $700 million dollars annually (Lillehoj et al., 2007). Feed intake (FI) and body weight gain (BWG) are severely depressed during coccidia infections. Broilers orally inoculated with *E. acervulina* oocysts had reduced BWG compared to non-inoculated pair-fed broilers (Adams et al., 1996). Reduced FI is not the only factor that results in reductions in BWG during a coccidial infection. Decreased digestive enzyme activities, reduced absorptive capacity of the intestinal tract (Adams et al., 1996), and reduced digestion of nutrients such as amino acids and metabolizable energy (ME; Persia et al., 2006) may also contribute to reductions in BWG and feed efficiency. According to a review article by Williams (2005), other pathophysiological effects associated with coccidiosis include, but are not limited to, poor feed efficiency, reduced water intake, increased intestinal passage time, decreased digesta viscosity, intestinal malabsorption, villus atrophy, intestinal leakage of plasma proteins, and increased intestinal acidity.

Eimeria spp. possess a complex lifecycle, comprising both asexual and sexual reproduction and intracellular and extracellular development (Yun et al., 2000). Broilers become infected with coccidia upon ingestion of oocysts in contaminated litter, feed, or during preening.

Sporulation of the oocyst occurs within the host due to natural action of the gizzard, digestive enzymes, and/or carbon dioxide (Lotze and Leek, 1968; Hibbert et al., 1969; Wang and Stotish, 1975). The oocyst releases four sporocysts, each containing two sporozoites (Lillehoj and Trout, 1996). The sporozoites invade the intestinal epithelium in a site specific manner (Dalloul and Lillehoj, 2006) and form trophozoites. The mechanism by which coccidia select their specific host cells remains unknown, but may involve recognition of carbohydrate moieties (Baba et al., 1996). After invasion, trophozoites form immature meronts following nuclear division. The meronts reproduce asexually to form numerous merozoites within the epithelium in a process called merogony. The epithelial cells rupture, releasing the merozoites, which may undergo several more processes of merogony. The merozoites released re-invade the epithelial cells and develop into microgamonts or macrogamonts and undergo sexual reproduction to produce oocysts. The host cell then ruptures and releases the oocyst into the intestinal lumen and out in the feces, resulting in re-cycling of the parasitic infection. Pre-patent periods may range from 4 to 5 days, with a maximum of 6 to 9 days post infection (Allen and Fetterer, 2002).

Due to the complex lifecycle of the *Eimeria spp.*, natural infection induces both antibody and cell mediated immune responses (Lillehoj and Trout, 1996). Cell mediated immunity plays a major role in disease resistance (Lillehoj and Trout, 1996), and cell mediated immune responses include both antigen specific and non-specific activation of T-lymphocytes, natural killer (NK) cells, and macrophages (Yun et al., 2000). Additionally, circulating IgY and IgA specific for coccidial parasites are usually detected one week after the initial infection (Yun et al., 2000). A mixed *Eimeria spp*. infection composed of *E. acervulina, E. maxima,* and *E. tenella* resulted in significant changes in $CD4^+$ cells and macrophages in the duodenum, jejunum, and cecum of broilers and increased the frequency of $CD8^+$ cells in the duodenum and cecum (Cornelissen et

al., 2009). The same authors also found the host response to the *Eimeria spp*. was limited to the site of infection; and the severity of infection can be correlated to the number of oocysts ingested. The early endogenous stages of the parasite lifecycle are considered to be more immunogenic than the later sexual stages (Yun et al., 2000). The level of susceptibility to coccidiosis also depends on the *Eimeria spp.*, prior host exposure, nutritional status of the infected host, and genetic makeup of the host (Lillehoj et al., 2004). The development of novel strategies to control coccidiosis will be contingent upon the use of molecular and cellular tools to further characterize host-parasitic interactions and immune activation (Lillehoj et al., 2007).

COCCIDIOSIS VACCINES

Anticoccidial vaccines have been available since 1952 (Williams, 2002b) but did not see common use in the US broiler industry due to the availability of antibiotics and ionophores. However, coccidia have developed a resistance to all of the available anticoccidial drugs (Chapman, 1997), development of new chemotherapeutic drugs is expensive and time consuming (Lillehoj et al., 2007), and consumer preference for a more "natural" poultry product has encouraged the use of anti-coccidial vaccines. Research focusing on specific antigens or DNA subunit vaccines is promising (Wu et al., 2004; Lillehoj et al., 2005), but the complex lifecycle and immune response associated with coccidiosis has created issues with definitive vaccination strategies. Currently, at least 10 live anti-coccidial vaccines are commercially available for use in poultry (Williams, 2002a; Dalloul and Lillehoj, 2005). Live oocyst vaccines differ in many ways, such as the type of *Eimeria spp.* (virulent or attenuated), the drug resistance, the species composition of the product, and the delivery method (Dalloul and Lillehoj, 2005). Nonattenuated live vaccines utilize low levels of field or laboratory strains of *Eimeria*

spp. oocysts and attenuated oocyst vaccines utilize parasites of artificially reduced virulence (Williams, 2002a; Dalloul and Lillehoj, 2005).

Vaccination with live coccidia oocysts induces protective immunity to a secondary exposure of the same *Eimeria spp.* (Pierson et al., 1997; Yi et al., 2005). Unfortunately, the utilization of live oocyst vaccines is not common in the broiler industry due to reductions in bird performance and feed efficiency associated with the initial infection (Danforth, 1998; Yi et al., 2005; Parker et al., 2007; Lehman et al., 2009) and concerns regarding necrotic enteritis (NE) outbreaks associated with coccidia infections. Other problems associated with the use of nonattenuated live oocyst vaccines include: lack of sufficient numbers of oocysts to induce protective immunity, introduction of new *Eimeria spp.* into the poultry flock, and the large number of parasites utilized in the vaccine increases the labor and production costs (Dalloul and Lillehoj, 2005). Attenuated vaccines may reduce the potential concerns associated with the introduction of new species and the negative affects on performance, but higher production costs (Dalloul and Lillehoj, 2005), failure to produce attenuated species, and an unsatisfactory combination of immunogenicity and attenuation of virulence (Williams, 2002a) may over-rule the known advantages. Several new vaccines utilize non-attenuated, ionophore resistant *Eimeria spp*. Therefore, ionophores may be present in the diet to reduce natural coccidia infections and control NE (Williams, 2002a). However, non-attenuated vaccines utilize killed organisms and/or parasitic sub-units that may not induce protective immunity similar to that of live vaccines due to the inability to activate cell mediated immunity (Yun et al., 2000).

Immunity to coccidia is species specific and is stimulated and boosted by multiple re-infections from oocysts in the litter (Williams, 2002a). There are two separate mechanisms associated with protective immunity following exposure to coccidiosis: innate immunity during

the initial pathogen exposure and acquired immunity following secondary infection (Lillehoj et al., 2007). The innate defense system consists of three components: mechanical, chemical, and cellular (Pearson and Brownlee, 2005). The mechanical component is the epithelial cells and their secretions, i.e. mucus and cellular sloughing (Pearson and Brownlee, 2005). Antimicrobial proteins, peptides, and glycoproteins, such as cytokines and chemokines, and cells that secrete them (M cells, lymphocytes, epithelial cells, macrophages) compose the chemical and cellular components of the innate immune system (Pearson and Brownlee, 2005). Mucosal surfaces within the gastrointestinal tract contain the digestive epithelium, mucin, and the gut associated lymphoid tissues (GALT; Montagne et al., 2004). Mucins are polymeric glycoproteins that comprise the main component of the mucus layer that covers the epithelium of the gastrointestinal tract (Corfield et al., 2000; Uni et al., 2003; Montagne et al., 2004). The main function of mucin is to protect the epithelium from chemical, enzymatic, physical, and bacterial aggressors (Montagne et al., 2004). Mucins are produced and secreted by goblet cells within the intestinal epithelium (Uni et al., 2003) and are composed of a protein backbone rich in serine, threonine, proline, alanine, glycine, and cysteine (Montagne et al., 2004). Approximately 80% of the mucin molecule is composed of monosaccharides such as N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose, and sialic acids (Uni et al., 2003; Montagne et al., 2004; Pearson and Brownlee, 2005). Acidic containing goblet cells were observed in the small intestine of broiler chicks as early as three days prior to hatch (Uni et al., 2003). After hatch, goblet cell numbers increase at a constant proportion to enterocytes, with the density of goblet cells increasing distally (Uni et al., 2003). Mucin in mature goblet cells accumulates in the theca of the cell (Collier et al., 2008). Coccidiosis increases goblet cell theca area (a reflection of mucus accumulation) and goblet cell numbers (Collier et al., 2008). An increase in villi cell

proliferation will reduce the age and maturity of goblet cells and the quality of mucin they produce and increase the energy requirement of the intestinal tract (Choct, 2009). Pathogen exposure will increase crypt cell depth, indicating rapid cellular turnover, and reduce absorptive surface area by shortening the villi (Choct, 2009). Mucin and pathogen interactions are extremely important in terms of gut health. For example, an inflammatory immune response associated with coccidiosis increases mucin production and the mucolytic bacteria *Clostridium perfringens* in the small intestine of broilers (Collier et al., 2008). Many commensal and pathogenic bacteria adhere to the complex carbohydrate chains attached to mucins resulting in bacterial colonization of the intestinal tract and/or protection from invasion (Montagne et al., 2004). Pathogens may also utilize the carbohydrates attached to mucin for production of short chain fatty acids (SCFA), which can be readily absorbed by the intestinal mucosa (Montagne et al., 2004). Further characterization of the host inflammatory response and mucin production may facilitate better understanding of pathogenic bacterial colonization within the intestinal tract.

Protection of the epithelium from pathogen invasion requires an intact and functional mucin layer. However, certain pathogens such as *Eimeria spp*. require enterocyte invasion to continue their lifecycle. In chickens, the GALT is the first line of defense on mucosal surfaces against invading pathogens (Lillehoj and Trout, 1996). Gut associated lymphoid tissues comprise a variety of lymphoid organs (Peyer's patches, cecal tonsils, Meckel's diverticulum, and the bursa of Fabricius) and lymphocytes, antigen presenting cells, and NK cells within the lamina propria and submucosa (Yun et al., 2000). There are three general functions of the GALT in host defense against coccidiosis: processing and presentation of antigens, production of intestinal antibodies, and activation of cell mediated immunity (Yun et al., 2000). Shortly after infection, some coccidia species invade cells of the intestinal or cecal surface epithelium, while

others develop in the endothelial cells of the villi, lamina propria, or crypts (Lillehoj and Trout, 1996). Tissue permeability, cellular infiltration, crypt cell proliferation, and mucin production increases, and complex interactions with lymphocytes, macrophages, and epithelial cells create an inhospitable environment for pathogens (Lillehoj and Trout, 1996) and may result in host tissue damage. Inflammation results from an increased blood supply to the infected area, increased capillary permeability due to retraction of endothelial cells lining the vessels, and migration of heterophils, monocytes, lymphocytes, and macrophages into the surrounding tissues (Male et al., 2006). Sporozoites are detected in macrophages, dendritic cells, and epithelial cells, and they are processed by these cells to activate antigen specific responses involving B and T cells. Binding of an antigen to B cells stimulates secretion of IgA and IgM with identical antigen specificity (Lillehoj and Trout, 1996). Secretory IgA functions to prevent invasion of microbes into the intestine (Lillehoj and Trout, 1996). Binding of IgA to the coccidial surface may prevent epithelial binding by direct blocking, steric hindrance, induction of conformational changes, and/or reduction of motility (Yun et al., 2000). However, the ability of circulating antibodies to limit coccidia infection is minimal, and high levels are usually detected in the bile but not intestinal sections after coccidia infection (Lillehoj and Trout, 1996; Yun et al., 2000). The importance of T cells in the immune response to coccidia has been well documented (Lillehoj and Trout, 1996). T cells can be classified as $CD4^+$ or $CD8^+$ cells. $CD4^+$ cells, also known as T helper cells, induce immune responses by recognizing specific antigens, while $CD8^+$ cells are predominantly cytotoxic (Male et al., 2006). Both $CD4^+$ cells and the cytokines they produce are critical to control parasite replication during primary infection (Lillehoj and Trout, 1996; Yun et al., 2000). However, it appears that $CD8^+$ T cells mediate host immunity to secondary coccidia infections (Lillehoj and Trout, 1996; Yun et al., 2000). Macrophages and T cells stimulate the

production of cytokines, namely interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α; Yun et al., 2000). Interleukin-1 is a potent inducer of fever and appetite suppression, while TNF-α promotes cellular migration and adhesion and induces apoptosis (Male et al., 2006). Understanding the host immune cell and parasite interactions in the gut is crucial to understanding coccidia resistance (Lillehoj and Trout, 1996), pathologies associated with the disease, and development of effective vaccination strategies.

NECROTIC ENTERITIS

In addition to the direct impact of coccidiosis, the resulting damage to the gut and nutrient passage to the lower intestine can often predispose the bird to another costly disease known as necrotic enteritis (NE). Necrotic enteritis is caused by the Gram-positive, anaerobic, spore-forming bacterium *Clostridium perfringens* and may present as acute clinical disease, which causes increased mortality, or subclinical disease (Van Immerseel et al., 2004). *Clostridium perfringens* are common inhabitants of soil, sewage, and the broiler intestine and are classified into five types (A, B, C, D, and E), with type A producing a chromosomal-encoded α-toxin (Petit et al., 1999) that is absorbed into the blood stream causing toxemia (Porter, 1998). Enterocytes and cellular membranes are surrounded by phospholipids. Alpha-toxin is a phospholipase C sphingomyelinase that hydrolyzes phospholipids and promotes membrane disorganization (Titball et al., 1999). The toxin causes contraction of small intestinal tissue, activates the arachidonic acid cascade, leading to increased vascular permeability and edema, and induces platelet aggregation factors (Sakurai et al., 2004).

In a recent review article, Williams (2005) reported NE most commonly affects chickens from two to six weeks of age and mortalities may reach approximately 1% per day. Lesions occur in the jejunum and ileum and may extend to the duodenum and cecum (Porter, 1998).

Clinical signs of NE include: depression, ruffled feathers, diarrhea, huddling, and anorexia (Porter, 1998). In severe cases, the intestinal epithelium is eroded and detached with a diphtheritic membrane of dead mucosal enterocytes and lymphocytes trapped in fibrin covering the mucosa. The gut is fragile, dilated, and gas-filled with foul-smelling brown liquid contents (Porter, 1998; Williams, 2005). Recent evidence suggests α-toxin may not be the cause of the pathology associated with NE, and the toxic agent may still be unknown (Van Immerseel et al., 2009). It is possible that other virulence factors such as hydrolytic enzymes and unknown toxins may play a role in the pathogenesis associated with NE (Van Immerseel et al., 2009). Only *C. perfringens* isolated from field outbreaks of NE was able to induce intestinal lesions in poultry, suggesting the presence of host-specific virulence factors (Timbermont et al., 2008). Further research utilizing sensitive molecular techniques may provide valuable information regarding the causative toxins associated with *C. perfringens* and NE.

Necrotic enteritis is a multi-factorial disease process incorporating various pre-disposing factors (Dahiya et al., 2006) such as: dietary ingredients, intestinal disease, intestinal stasis, and immunosuppression. Dietary composition has been well characterized as a factor that influences the growth of *C. perfringens*. Birds fed fish meal and highly viscous grains such as wheat, rye, and barley suffer from NE more severely than birds fed corn diets (Porter, 1998). Viscous grains may cause wet and sticky litter conditions, which may encourage bacterial and coccidial growth in the environment. Interestingly, high dietary levels of Zn and Ca have also been implicated as pre-disposing factors for NE due to mineral usage in the α-toxin pathway (Titball et al., 1999). Rye supplementation to corn and soy diets significantly increases *C. perfringens* numbers in the ileum, ceca, and feces of broilers (Craven, 2000). Highly viscous grains such as rye may induce an inflammatory immune response and promote mucin production, thereby stimulating

proliferation of *C. perfringens*. Electron microscopy revealed *C. perfringens* was intimately associated with inflammation, but the microorganisms did not invade viable tissue (Porter, 1998). Coccidiosis is known to cause intestinal villus atrophy, plasma protein leakage, and reduced intestinal transit time, and it is often reported along with NE (Williams, 2005; Van Immerseel et al., 2009). Immunosuppression may also increase the incidence of NE in broilers. Potential methods to control NE include, dietary modifications utilizing different feed ingredients or dietary enzymes, low animal protein diets, and anticlostridial drugs. Clostridial vaccines are available for humans, goats, swine, calves (Williams, 2005) and conditionally available for use in broilers in the US.

PHYTATE AND PHYTASE

Phytate or phytic acid (myo-inositol hexakisphosphate) is a complex salt of Ca, Mg, K, and proteins within single-membrane particles (globoids, aleurone) in grains and seeds (Shamsuddin, 1999; Angel et al., 2002; Loewus, 2002; Sathe and Reddy, 2002; Bohn et al., 2008). It is the primary storage form of P (Angel et al., 2002; Sathe and Reddy, 2002; Wu et al., 2004b), an energy store, competitor for ATP, immobilizer of divalent cations, and regulator of available P (aP) in seeds (Angel et al., 2002). The phytate molecule contains approximately 28.2% P and is the main source of P in seed based poultry diets (Angel et al., 2002). Estimates suggest, approximately 60 – 80% of the P present in seeds and grains is in the form of phytate P (Lott et al., 2000; Pirgozliev et al., 2007; Manangi and Coon, 2008). Phytate is a strong acid, with a net charge of -3 at pH 1.5 and rising to -8 at pH 7.5 (Selle et al., 2000), and a chelator of divalent minerals such as Cu, Ca, Mg, Zn, and Fe. The ionic charge of phytic acid allows it to act directly with charged groups of proteins, mediated by cations such as Ca (Sathe and Reddy, 2002). Binary protein-phytate complexes are present at acidic pH, and tertiary protein-mineral-

phytate complexes are formed as pH approaches neutrality (Selle et al., 2000). Many of these phytate complexes are soluble at pH less than 3.5. As the pH reaches 4 to 7, the phytate molecule becomes insoluble and unavailable for absorption or hydrolysis (Selle et al., 2000; Sathe and Reddy, 2002). Phytate may also reduce the availability of carbohydrates and apparent metabolizable energy (AME) digestibility (Ravindran et al., 2006) by direct interaction with digestive enzymes or interactions with proteins (Pirgozliev et al., 2007). Phytate can lead to mineral deficiencies in human populations where staples like wheat, rice, and corn are the only source of nutrition (Bohn et al., 2008). In poultry diets, high dietary phytate reduced performance (Liu et al., 2008) and increased the excretion of sialic acid by approximately 185% (Cowieson et al., 2004). Sialic acids are distributed in nature as components of glycoproteins and mucins (Jourdian et al., 1971). An increased concentration of sialic acid is often associated with cellular senescence, bacterial infections, pathological conditions, and osmotic fragility (Pirgozliev et al., 2007). Phytate also increased the secretion of essential and non-essential amino acids and certain minerals such as Ca, Fe, Na, and S (Cowieson et al., 2004; Cowieson et al., 2006c; Ravindran et al., 2006; Cowieson et al., 2008). The increased endogenous secretion associated with phytate may be a result of increased amino acid (Cowieson and Ravindran, 2007; Cowieson et al., 2008) and mucin excretion (Cowieson et al., 2004) or the binding of proteins and digestive enzymes, thereby altering their solubility or function and reducing nutrient utilization (Cowieson et al., 2006c). Interestingly, while phytate may inhibit digestion of vital nutrients, it may have anti-tumor-like properties and is vital in cell signaling and trafficking. Uptake of pure phytate within the gastrointestinal tract is accomplished via high affinity binding proteins (Shamsuddin, 1999). Phytate and lower inositol phosphates have been detected as intracellular molecules in most mammalian cells and act as important regulators of signal

transduction, such as mitosis via mobilization of intracellular Ca (Shamsuddin, 1999), and as phosphate donors/acceptors (Bohn et al., 2008). Phytate may also have antioxidant properties by binding to and inactivating iron ions in the Fenton reaction (Bohn et al., 2008). As a chelator of minerals, pure phytate may also have antimicrobial properties. For example, Ca and Zn are necessary for α-toxin production associated with *C. perfringens*. Therefore, binding of Ca and Zn to phytate may reduce the pathology associated with *C. perfringens*. Inositol phosphates reduce cellular proliferation and are associated with cellular senescence and loss of function (Shamsuddin, 1999). However, the presence of dietary components and endogenous phytase may inhibit absorption of large quantities of phytate by enterocytes (Shamsuddin, 1999), thereby altering dietary phytate uptake and effects on intracellular functions.

Phytase (myo-inositol hexaphosphate phosphohydrolase) enzymes of endogenous or exogenous origins have the capacity to dephosphorylate phytate in a step-wise manner to a series of lower inositol phosphate esters (Selle et al., 2000). Monogastric animals lack significant amounts of endogenous phytase, therefore phytate and its bound components are largely unavailable for utilization in poultry (Selle et al., 2000; Wu et al., 2004b; Cowieson et al., 2006c; Pirgozliev et al., 2007). The International Union of Biochemistry recognizes two general classes of phytases, 3- phytase and 6-phytase, based on the location of the phosphate group that is hydrolyzed first (Selle et al., 2000; Augspurger et al., 2003; Applegate and Angel, 2004; Haefner et al., 2005). Forty years ago, Nelson et al. (1968, 1971) published the first experiments evaluating an *Aspergillus ficuum* phytase in broiler diets and the first commercially introduced phytase (*Aspergillus niger*) was available in 1991 (Haefner et al., 2005; Selle and Ravindran, 2007). Recently, phytases derived from *Escherichia coli* have been developed, and they exhibit a higher resistance to pepsin and superior efficacy compared to fungal phytases (Augspurger et

al., 2003; Augspurger and Baker, 2004; Pillai et al., 2006). However, *E. coli* phytases are more susceptible to trypsin (Rodriguez et al., 1999) and have a single pH optimum of 2.5 to 4.5 (Simon and Igbasan, 2002; Augspurger et al., 2003), suggesting the highest activity within the gizzard and proventriculus of the chicken (Applegate and Angel, 2004). Initially, phytase was utilized to reduce P excretion and subsequent eutrophication of rivers, lakes, and streams from land application of poultry litter and swine manure. However, supplementation of phytase in monogastric nutrition has exceeded earlier predictions with further understanding of the anti-nutrient effects of phytate and confirmed benefits of utilizing phytase.

Approximately 20 years after Nelson et al. (1968, 1971) published their articles evaluating phytase Simons et al. (1990) evaluated the effect of phytase supplementation in low Ca and aP diets on broiler performance and P excretion. Since then numerous peer-reviewed articles have demonstrated the beneficial effects of supplementing phytase in poultry diets on growth performance, feed efficiency, bone ash, and/or mineral retention (Viveros et al., 2002; Augspurger et al., 2003; Augspurger and Baker, 2004; Dilger et al., 2004; Onyango et al., 2004; Silversides et al., 2004; Wu et al., 2004b; Zyla et al., 2004; Onyango et al., 2005; Cowieson et al., 2006d; Jendza et al., 2006; Pillai et al., 2006; Watson et al., 2006; Pirgozliev et al., 2007; Liu et al., 2008; Manangi and Coon, 2008; Pirgozliev et al., 2008; Ravindran et al., 2008). More recently, research evaluating anti-nutrient effects of phytate provided further evidence of the beneficial effects of supplementing phytase on AME and/or amino acid digestibility (Ravindran et al., 1999b; Rutherfurd et al., 2004; Silversides et al., 2004; Wu et al., 2004c; Cowieson et al., 2006c; Pirgozliev et al., 2007; Ravindran et al., 2008). Interestingly, although amino acid digestibility may be improved by phytase supplementation, the response is highly variable depending on the amino acid analyzed (Rutherfurd et al., 2004; Cowieson et al., 2006c) and the

age of the broiler (Huang et al., 2005). For example, the digestibilities of valine, threonine, isoleucine, asparginine, alanine (Namkung and Leeson, 1999; Rutherfurd et al., 2002; Cowieson et al., 2006d), arginine, histidine, tryptophan, tyrosine, phenylalanine, cysteine, glutamate, glycine, and proline were improved with phytase supplementation (Jendza et al., 2006). However, other reports suggest amino acid digestibility was not affected by phytase supplementation (Rutherfurd et al., 2002).

Dietary composition may also influence the efficacy of phytase. In particular, phytase supplementation to diets composed primarily of soybean meal resulted in no significant improvements in true ileal amino acid digestibility (except Ile) compared to diets composed primarily of wheat and corn (Rutherfurd et al., 2002). Soybean meal is considered a highly digestible source of protein. Therefore, phytase may be more efficacious in diets with less available protein. Wheat-based diets were more responsive to phytase supplementation in regards to amino acid digestibility (Selle et al., 2006), and AME and ileal N digestibility compared to corn-soy diets (Wu et al., 2003). Phytate is likely to complex more readily with protein from wheat than from corn (Selle et al., 2006). Phytase may be disrupting the cell wall matrix and enhancing the interaction between digestive enzymes and cell contents (Ravindran et al., 1999a). The overall beneficial effects of phytase supplementation on performance, and AME and amino acid digestibilities may also be mediated by both a reduction in endogenous losses and improvements in the absorption and retention of nutrients (Selle et al., 2000; Cowieson et al., 2004; Cowieson et al., 2008; Wyatt et al., 2008). Improving nutrient absorption and reducing the anti-nutrient effects of phytate may also improve intestinal integrity. Phytase supplementation increases villus heights and crypt depths in the jejunum and ileum of broilers (Smulikowska et al., 2008).

Other researchers report no additional benefits on energy, amino acid, mineral, and/or morphological parameters in broilers fed diets supplemented with phytase (Zhang et al., 1999; Peter and Baker, 2001; Augspurger and Baker, 2004; Dilger et al., 2004; Onyango et al., 2005; Leslie et al., 2007; Pirgozliev et al., 2007, 2008). Sialic acid excretion was also not affected by phytase supplementation (Pirgozliev et al., 2007). However, the effect of phytase supplementation is variable depending on the dietary ingredients (Peter and Baker, 2001; Rutherfurd et al., 2002), age of the poultry species (Selle et al., 2000; Pirgozliev et al., 2008), microbial fermentation in the hindgut (Pirgozliev et al., 2008), and the health of the intestinal tract. Phytate occlusion with other tissue components, limited solubility in various regions of the intestinal tract, and short reaction time in poultry intestines are other major factors that reduce the efficacy of phytase (Zyla et al., 2004). The influence of phytase supplementation may also be affected by the Ca:aP ratio and level in the diet (Tamim et al., 2004; Zyla et al., 2004) and/or dietary electrolyte balance (Ravindran et al., 2008). Phytase elicits a greater response on performance and/or bone ash at higher Ca levels and lower aP levels (Driver et al., 2005; Watson et al., 2006; Manangi and Coon, 2008). The efficacy of any enzyme as a feed additive depends on the biochemical characteristics of the enzyme (Simon and Igbasan, 2002) and the limit of the nutrient spared in the diet (Wyatt et al., 2008).

NON-STARCH POLYSACCHARIDES AND CARBOHYDRASES

Poor performance associated with diets containing highly viscous grains (rye, wheat, and barley) can be attributed to the gel-forming capacity of the water soluble, non-starch polysaccharides (NSP) present in these grains (Silva and Smithard, 2002). An increase in digesta viscosity may reduce nutrient digestion by limiting the access of endogenous enzymes to substrates within the digesta (Jaroni et al., 1999). Additionally, movement of smaller molecules

to the mucosal surface for absorption may be impeded (Jaroni et al., 1999). Broiler weight gain has been reported to decrease, and feed conversion (FC) ratio and digesta viscosity increase by increasing the dietary inclusion levels of carboxymethyl cellulose (CMC), an indigestible but highly soluble polysaccharide (Waldenstedt et al., 2000). Non-starch polysaccharides may also result in an increase in digesta transit time, which may in turn facilitate pathogenic bacterial growth (Wyatt et al., 2004). Harmful bacteria may be involved in localized or systematic infections, intestinal putrefaction, or toxin formation (Yegani and Korver, 2008). Compared to wheat and barley, corn and soybean meal are not known to contain high levels of soluble NSPs. However, the nutritional value of these ingredients may vary widely based on growing conditions and location (Iji et al., 2003; Wyatt et al., 2004; Wyatt et al., 2008). Undigested starch at the terminal ileum is known as resistant or insoluble starch, and approximately 15% of the starch in corn is resistant to digestion (Iji et al., 2003). Resistant starch may also encapsulate nutrients such as starch and protein, which can bypass digestion and be broken down by bacteria in the lower gut (Wyatt et al., 2008). Carbohydrase (NSPase) supplementation may improve nutrient digestion and absorption by breaking down cell walls in corn and soy diets and indirectly improving the digestion processes such as motility, endogenous enzyme access, and by reducing digesta transit time, which may stimulate FI and subsequent growth (Garcia et al., 2003; Wyatt et al., 2004).

Common NSPases utilized in the poultry industry include: xylanase, mannanase, cellulases, and glucanases (Wyatt et al., 2008). Xylan is the principle type of hemicellulose in wheat, barley, and rye (Polizeli et al., 2005). Xylanases cleave the 1 – 4 β-glycosidic bonds present along the xylan backbone (Polizeli et al., 2005). Inclusion of an enzyme cocktail contaiing predominantly xylanase (Avizyme 1300) improved broiler performance, reduced

digesta viscosity, and increased the apparent digestibilities of dry matter, fat and N, and ME in rye/soybean meal diets (Silva and Smithard, 2002). The same xylanase cocktail improves protein digestibility and increases jejunum villus height in laying hens fed diets containing various levels of wheat midds (Jaroni et al., 1999). Crypt cell proliferation rate is significantly increased in the small intestine of birds fed high levels of xylanase (10 and 30 times the industry recommendation), but there is no effect when birds are fed standard levels of xylanase (Silva and Smithard, 2002). Xylanase supplementation of corn and soy diets may also stimulate beneficial bacteria proliferation in the cecum by altering the composition of fiber to short chain oligosaccharides (Bedford, 2000; Wyatt et al., 2008). Beneficial bacteria may facilitate vitamin production, stimulate the immune system, or inhibit pathogenic bacteria colonization through competitive exclusion. Jia et al. (2009) did not report any differences in lactic acid bacteria numbers in broilers fed corn/ soy diets supplemented with a carbohydrase cocktail. However, the authors did report reductions in lactic acid bacteria numbers in broilers fed wheat diets supplemented with the same enzyme cocktail (Jia et al., 2009). Other authors report an increase in lactic acid bacteria in broilers fed wheat diets supplemented with xylanase (Engberg et al., 2004).

Experiments evaluating xylanase supplementation in corn and soy diets are limited. Most published data involve xylanase supplementation together with amylase and protease, as discussed in the section below. Supplementing corn and soybean meal diets with α-galactosidase significantly improved broiler performance, AME, and crude protein (CP) retention (Ao et al., 2009). Average daily gain and total tract energy digestibility in pigs (Fang et al., 2007) and AME and amino acid digestibility in broilers (Rutherfurd et al., 2007) were improved in corn/soy diets supplemented with carbohydrase enzyme cocktails containing predominantly xylanase.

Ileal digestible energy was improved in pure corn and pure soybean meal diets supplemented with glucanase (Leslie et al., 2007). The combination of phytase with glucanase side enzyme activity and xylanase supplementation significantly increased AME, affected intestinal morphology in the jejunum and ileum, and reduced goblet cell numbers, but did not affect broiler performance (Wu et al., 2004a). The combination of xylanase and β-glucanase supplemented in wheat diets reduced small intestine weight and length and altered volatile fatty acid profiles (Wang et al., 2005), and cellulase, xylanase, and α-amylase supplementation reduced the secretion of sialic acid (Cowieson et al., 2003). Improving digestion in the upper intestine and lowering endogenous secretions by the gut villi reduces the maintenance requirement spent on digestion and improves nutrient retention (Wyatt et al., 2008). The response associated with NSPases can vary depending on the enzyme or enzyme cocktail supplemented, the quality of the feed ingredients, substrate availability, and thermo-tolerant status of the enzyme (Wyatt et al., 2008). The nature of the substrate molecule, chain length, and degree of branching, may also affect the efficacy of xylanase (Polizeli et al., 2005). Most xylanases are active at a pH range of 4.5 to 6.0 (Polizeli et al., 2005). Xylanase activity in the small intestine is found to be approximately 15 to 20% of the total xylanase reported in the diet, indicating xylanase can survive acidic and proteolytic activities and act throughout the digestive tract (Silva and Smithard, 2002). The age of the bird may also influence the effect of carbohydrase supplementation. For example, the benefit of supplemental enzymes is likely mediated through proliferation of beneficial bacteria in older birds, while younger birds have a poorly developed digestive system and may benefit more from the direct effects of the enzymes on nutrient availability (Bedford, 2000). Furthermore, digesta viscosity decreases as the bird ages (Petersen et al., 1999), which facilitates intestinal motility, enzyme activity, and substrate availability.

Carbohydrase supplementation may also reduce the encapsulation of proteins, amino acids, or minerals with resistant starches, thereby improvements in broiler performance can be more than just improvements in energy digestibility (Wyatt et al., 2004).

PROTEASE

Experimental data evaluating dietary protease supplementation are limited in comparison to phytase and xylanase. Most experiments conducted have evaluated exogenous protease supplementation to reduce anti-nutrients present in soybean meal, such as protease inhibitors, lectins, and antigenic proteins (Ghazi et al., 2002) or involve the addition of protease together with amylase, phytase, and/ or xylanase in an enzyme cocktail. Commercially available protease enzymes are derived from *Bacillus* or *Aspergillus spp*. *Bacillus* proteases are active at neutral to alkaline pH, and *Aspergillus* proteases are active at neutral to acidic pH (Ghazi et al., 2002). In a series of experiments, Odetallah et al., (2003, 2005) evaluated the use of a keratinase produced from *Bacillus licheformis* and known to hydrolyze a variety of proteins including casein, collagen, elastin, and keratin, in corn and soy diets of broilers during the starter period. The authors found protease supplementation had either no effect or improved BW in broilers fed diets formulated to contain 18% CP, but these improvements were not comparable to the PC diet with 21% CP (Odetallah et al., 2003). In a later experiment, Odetallah et al. (2005) reported keratinase supplementation to corn and soybean meal diets formulated to contain 17.78% CP and 95% of the amino acid requirements according to the NRC, improved BWG and FC compared to diets without keratinase supplementation. These improvements in BWG were present up to market age, even though enzyme supplementation was discontinued at day 21. In an earlier study, Ghazi et al. (2002) evaluated the use of two types of proteases produced from *Bacillus* and *Aspergillus*. To reduce the proteinaceous anti-nutrient factors, soybean meal was pretreated in

alkaline (*Bacillus*) or acidic (*Aspergillus*) conditions with and without protease. The authors reported improvements in chick FI and body weight (BW), but only when birds were fed diets containing soybean meal treated with protease produced from *Aspergillus*. They speculated improvements resulted from increases in FI due to improvements in N digestibility and retention and increased digesta passage rate (Ghazi et al., 2002). In a follow-up series of experiments, Ghazi et al. (2003) reported improvements in total N digestibility, live weight gain, and FC in broilers fed soybean meal pre-treated or mixed with protease produced from *Aspergillus niger*. Protease supplementation increased FI and BW but did not have an effect on intestinal morphology in weanling piglets (Rooke et al., 1998).

In 2002, Yu et al. evaluated a plant derived protease called bromelain in poultry diets formulated using different protein sources. These authors reported enzyme supplementation did not affect ileal protein digestibility or protein characteristics within the gastrointestinal tract. In vitro analysis of the protease revealed enzyme activity approached zero at pH 2.0. However, reactivity remained at 80% at pH 3.2 to 4.0. This suggests other factors may be influencing the effect of the supplementation of certain proteases in broiler diets such as endogenous enzymes, or pelleting conditions. Other researchers did not report improvements in performance or protein digestibility in broilers fed diets supplemented with protease (Simbaya et al., 1996; Marsman et al., 1997).

ENZYME COCKTAILS

Enzyme cocktails, or the combined use of multiple enzymes, may provide additional benefits to poultry producers and nutritionists. Enzymes utilized in cocktails may target different substrates and act in different locations within the intestinal tract. The complementary, additive, or synergistic actions of multiple enzymes may indirectly reduce gut maintenance (Wyatt et al.,

2008), increase nutrient availability and absorption, and reduce production costs by improving FC and maintaining broiler performance when fed diets formulated to contain marginal nutrient levels. However, careful consideration of enzyme combination and substrate availability are necessary due to antagonistic enzyme interactions (Ghazi et al., 2003; Saleh et al., 2004) and inconsistent performance and nutrient digestibility results (Iji et al., 2003; Cowieson and Adeola, 2005; Cowieson et al., 2006a, b). In broilers, the combination of protease and α-galactosidase reduced the performance response associated with the individual enzymes (Ghazi et al., 2003). *In vitro* analysis of the combined effects of various carbohydrases, phytase, and protease in corn and soybean meal suggests protease may digest other enzymes present in the matrix (Saleh et al., 2003a, b, 2004). However, other *in vitro* studies evaluating the use of various proteases do not accurately predict in vivo results (Simbaya et al., 1996; Ghazi et al., 2002; Yu et al., 2002).

In addition to antagonistic effects, discrepancies in published results may also be attributed to variations in dietary ingredients or nutrient content. For example, supplementation of a commercially available combination of xylanase, amylase, protease (Avizyme 1505), and phytase (Phyzyme XP) in corn and soybean meal diets significantly improved BWG and FC in nutritionally marginal diets, but not comparable to a nutritionally adequate positive control (PC) diet (Cowieson and Adeola, 2005). The authors concluded the enzyme cocktail was effective at improving nutrient digestibility and performance of broilers fed nutritionally marginal diets. However, ME and aP may have been limiting in those diets, altering the efficacy of the enzyme cocktail to improve performance comparable to the PC. In a series of follow up trials, Cowieson et al. (2006a, b) and Olukosi et al. (2007a) further evaluated the efficacy of the same commercially available enzyme cocktail and phytase supplementation on broiler performance and nutrient utilization in diets of differing nutrient quantities. Cowieson et al. (2006a, b)

reported Avizyme 1505 and Phyzyme XP supplementation improved broiler performance, Ca, P, isoleucine, tryptophan, asparginine, cysteine, glutamine, and proline digestibility in negative control diets (NC) formulated to allow for the expected improvements in nutritional value associated with enzyme addition, comparable to a nutritionally adequate PC diet. In this experiment, nutrient reductions in the NC diet were determined using predictive models based on broiler trials using phytase and Avizyme 1505. Olukosi et al. (2007a, 2008) reported additive effects of Avizyme 1505 and Phyzyme XP supplementation on growth performance and ME retention in broilers fed diets marginal in ME and aP. Supplementation of broiler diets with an enzyme cocktail containing xylanase, glucanase, protease, and amylase (Avizyme 1500) improved BWG, but did not have an affect on digesta transit time, organ weights, or energy and amino acid digestibility (Iji et al., 2003). Other authors reported beneficial effects on performance, apparent retention of AME and CP, jejunum villus height, and improvements in the digestibility of certain amino acids in broilers fed diets supplemented with various enzyme cocktails containing xylanase, protease, and amylase (Zanella et al., 1999; Garcia et al., 2003; Cowieson and Ravindran, 2008a, b). However, Ritz et al. (1995), Pinheiro et al. (2004), and Olukosi et al. (2007b) reported no additional benefits on broiler or turkey performance or nutrient digestibility when feeding diets supplemented with an enzyme cocktail containing protease, xylanase, amylase, phytase, or pectinase. Understanding the interactions between exogenous enzymes in enzyme cocktails and incorporation of the enzyme cocktails into broiler diets requires maintaining an adequate balance between substrate, nutrient content, and enzyme matrix. These qualities are imperative when evaluating performance parameters and digestibility assays associated with the use of enzyme cocktails (Cowieson and Ravindran, 2008a).

Minimal *in vivo* experiments have been conducted to evaluate the interactions between enzymes incorporated into enzyme cocktails. Simbaya et al. (1996) evaluated *in vitro* properties of various enzymes designed to degrade protein and carbohydrates, and incorporated the most promising enzymes in an in vivo experiment in broilers. A NC diet, deficient only in aP, was supplemented with phytase, phytase and protease, phytase and carbohydrase, or the combination of phytase, protease and carbohydrase. Supplementation of phytase, protease, and carbohydrase improved BWG and FC compared to the NC, and diets supplemented with phytase and protease alone or and phytase and carbohydrase (Simbaya et al., 1996). However, these improvements were only apparent during the first week, and overall performance was not different between the NC, NC supplemented with enzymes, or the PC. Very young chicks will likely derive greater benefit from exogenous enzyme cocktails used to target specific anti-nutrients in their diet than older chickens (Olukosi et al., 2007a) due to the immaturity of their digestive tract and the linear increase in digestive enzyme activity over time (Mahagna and Nir, 1996). The use of enzymes such as carbohydrases may release cell wall bound proteins, allowing them to be degraded by exogenous proteases in an enzyme cocktail and improve broiler performance and protein digestibility (Silva and Smithard, 2002). Supplemental enzyme cocktails are effective in corn/ soy diets and can reduce feed costs while maintaining performance, augment endogenous alpha-amylase activity, improve digestion of resistant starches, modify intestinal microbial communities, improve protein solubility and digestibility, and reduce the effects of plant derived anti-nutrient factors (Cowieson and Ravindran, 2008a, b). However, there is still a great deal of uncertainty regarding the modes of action of exogenous enzymes (Cowieson and Adeola, 2005) and interpretation of dietary enzyme supplementation. These interactions require careful

consideration of substrate availability, ingredient quality, characteristics of the enzymes supplemented, product formation, and nutrient availability (Bedford, 2000).

DIETARY MANIPULATION DURING INTESTINAL DISEASE

The digestive tract of the chicken is a potential site for pathogen exposure (Yegani and Korver, 2008). Anything that affects gut health will affect the nutrient uptake and requirements of the animal (Choct, 2009). The gut harbors more than 640 different bacterial species, contains over 20 different hormones, accounts for 20% of the body's energy expenditure, and is the largest immune organ in poultry (Choct, 2009). Nutrition plays a significant role in the development and function of the chicken immune system (Dalloul and Lillehoj, 2005; Yegani and Korver, 2008). As the growth period gets progressively shorter, the maintenance of health and nutrition in chickens is becoming more demanding (Choct, 2009). Many experiments have evaluated the abilities of various dietary ingredients to reduce pathologies associated with *Eimeria spp.* and *C. perfringens* (Allen et al., 1998; Van Immerseel et al., 2004; Williams, 2005; Dahiya et al., 2006). However, studies evaluating the total nutrient requirements of sick animals are scarce. An immune response elicits a multitude of behavioral and metabolic changes such as reduced FI and feed efficiency, huddling, ruffled feathers, and lethargy. Metabolic changes, mediated through the release of IL-1 and TNF-α from leukocytes (Klasing et al., 1987; Klasing and Barnes, 1988; Benson et al., 1993) that may influence nutritional requirements include an increased body temperature, redistribution of divalent cations, increased energy expenditure, increased skeletal muscle catabolism, increased amino acid oxidation, increased gluconeogenesis from amino acids, and decreased fatty acid uptake by adipose tissue (Klasing and Barnes, 1988). Therefore, determining nutrient requirements of sick animals is a balance between a decreased

requirement due to reduced growth, FI, and an increased requirement due to the energy utilized to mount an immune response (Klasing and Barnes, 1988).

Improvements in the availability of essential nutrients and supplementation of dietary ingredients or enzymes may provide protection against intestinal pathogens, activate an immune response, or enhance nutrient utilization to reduce the detrimental impact on performance, nutrient digestion and absorption, and intestinal integrity associated with coccidiosis and/or NE. Dietary nutrients such as amino acids are essential for maintenance of cellular functions and the immune system, growth, and skeletal muscle accumulation. For example, glutamine is considered a conditionally essential amino acid, an important energy source for immune cells, and is found largely in skeletal muscle (Calder and Yaqoob, 1999). Therefore, stimulation of skeletal muscle catabolism during an inflammatory response may act to provide a glutamine rich energy source for activated immune cells. Glutamine supplementation may have spared immune cells and reduced intestinal lesions associated with *E. maxima* infection in broilers (Yi et al., 2005). Gelatin, containing mostly glycine, serine, and proline, supplementation of corn and soybean meal diets improved BWG and feed efficiency in broilers vaccinated using live coccidia oocysts (Lehman et al., 2009). Gelatin supplementation may facilitate goblet cell proliferation and mucin production (Lehman et al., 2009). Interestingly, intestinal pathogens may also utilize dietary CP for growth and proliferation. In 1964, Britton et al. first reported an interaction between low dietary protein levels and a reduced incidence of coccidiosis in chickens. Mortality associated with *E. tenella* and *E. acervulina* oocyst shedding was higher in chicks consuming a 24% CP diet compared to those fed 16 or 20% CP diets (Sharma et al., 1973). Low protein diets may reduce endogenous trypsin secretion and subsequent oocysts excystation (Britton et al., 1964). Morbidity associated with *E. acervulina* infection may depend on the amino acid

adequacy of the diet. Willis and Baker (1981) and Izquierdo and Baker (1988) reported improvements in BWG in birds orally inoculated with *Eimeria spp*. above the non-inoculated control birds when fed diets severely deficient in sulfur amino acids or lysine. However, *E. tenella* oocyst shedding was significantly reduced in broilers orally gavaged with daily doses of L-arginine (Allen, 1999), and oocyst shedding decreased as dietary CP level increased from 19 to 23% in broilers vaccinated with live coccidia oocysts (Parker et al., 2007). The lysine and methionine requirement for growth and feed efficiency is decreased during immunologic stress and amino acid deficiencies may result in a depressed immune response, resulting in an abridged metabolic response, such as only a modest reduction in BWG and energy expenditure (Klasing and Barnes, 1988). Webel et al. (1998) reported LPS appeared to have minimal negative effects on chick performance when diets were deficient in lysine and threonine. Therefore, when evaluating dietary requirements of immunologically stimulated animals, it is imperative to consider production status and fate of the amino acid. Growing birds will have a considerably different amino acid requirement than mature birds challenged with an intestinal parasite. Non-essential amino acids such as glutamine, glycine, serine, and proline may be utilized by the immune system as an energy source and component of mucin synthesis, while essential amino acids may exacerbate coccidiosis due to normal or excessive dietary supplementation and presence in the lumen. A natural response to an inflammatory reaction is a reduction in FI and skeletal muscle break down. Therefore, nutrients essential for an immune response may need to be acquired through muscle protein rather than the diet. Further characterization of nutrient requirements of immune cells, pathogens, and intestinal cells will provide better recommendations for diet formulations and prevention or treatment of intestinal diseases.

Differences in performance responses and levels of oocyst shedding associated with dietary protein/amino acids and intestinal pathogens may be the result of genetic selection, pathogen exposure, commensal intestinal bacteria, and/or dietary composition. Many experiments evaluating the interaction between coccidiosis and dietary protein were conducted forty to fifty years ago. Genetic selection has substantially altered the broiler in regards to FI, growth, lean muscle accumulation, and immune status. Therefore, amino acid requirements and immune function in the present broiler may be quite different than broilers fifty years ago. Protein source may also affect the response associated with dietary CP and *Eimeria spp.* or *C. perfringens*. For example, *E. vermiformis* oocyst excretion was significantly higher in mice consuming diets containing soy protein compared to casein protein (Ford et al., 2001). The numbers of *C. perfringens* present in the ileum and cecum were also significantly affected by protein source (Drew et al., 2004). Birds fed diets formulated to contain fish-meal as the primary protein source had higher *C. perfringens* counts in the ileum and cecum compared to birds fed diets formulated to contain soy-protein isolates as the primary protein source (Drew et al., 2004).

In addition to altering protein and amino acid requirements, coccidiosis may also significantly reduce fat absorption and AME in broilers (Sharma and Fernando, 1975). This suggests dietary enzymes may enhance nutrient availability and absorption during an intestinal infection from coccidiosis or NE. Beta-mannanase is an enzyme that hydrolyzes β-mannans in soybean meal. Mannans are surface polysaccharides of repeating mannose units on many pathogens including bacteria, fungi, and viruses (Hsiao et al., 2006) and structural components of soybeans. The innate immune response recognizes mannans on pathogens and in soybean meal and stimulates an increase in macrophage, monocyte, and cytokine proliferation (Hsiao et al., 2006), which may result in reductions in BW. Beta-mannanase improved BWG and lesion

scores in the upper intestine, but not the lower intestine, in broilers exposed to a NE challenge model including oral inoculation with *E. acervulina*, *E. maxima*, and broth cultures of *C. perfringens* (Jackson et al., 2003). The authors theorized improvements in carbohydrate digestion would reduce nutrient shunting to the lower intestine for microbe utilization (Jackson et al., 2003). The experiment did not evaluate AME digestibility, however, β-mannanase may have improved energy utilization and digestion during a period of high energy demand. Raising the energy density in a diet with carbohydrates ameliorates the growth depressing effects of immunologic stress (Benson et al., 1993). An enzyme cocktail composed predominantly of carbohydrase enzymes significantly improves broiler BWG, but does not affect lactic acid bacteria counts, during an oral challenge with *C. perfringens* (Jia et al., 2009). Interestingly, *Salmonella spp*. prevalence in naturally infected turkeys is reduced with wheat based diets supplemented with xylanase compared to corn based diets supplemented with xylanase (Santos et al., 2008). Bacterial diversity is increased in diets supplemented with xylanase, and the authors speculate changes in fermentable carbohydrates altered the intestinal bacterial populations, which reduce the prevalence of *Salmonella* (Santos et al., 2008).

The use of other dietary enzymes has also been evaluated during intestinal pathogenesis. For example, phytase supplementation improves BWG and FI in broilers orally gavaged with *E. acervulina* (Watson et al., 2005). However, the magnitude of response to phytase is greater in healthy chickens compared to challenged birds. Not only does phytase supplementation improve nutrient availability, it has been shown to stimulate $CD4^+$ and $CD8^+$ cells (Liu et al., 2008), important modulators of the immune response against coccidiosis. Therefore, phytase may improve broiler performance during an *Eimeria spp*. infection by improving nutrient utilization and providing protection from invading parasites by activating the immune system. The

combined supplementation of xylanase, protease, and amylase in the diets of broilers vaccinated with live coccidia oocysts and challenged with mixed *Eimeria spp*. improved FC and reduces the average intestinal lesion score (Parker et al., 2007). Enzyme supplementation may improve nutrient utilization and/or contribute to a higher production of volatile fatty acids which may reduce the pathogenicity of *E. tenella* (Parker et al., 2007). Adaptive immunity in the GALT matures over a period of two weeks (Bar-Shira and Friedman, 2006). However, immune protection in the first week of life is slight and may be provided by maternal antibodies and innate effector mechanisms (Bar-Shira and Friedman, 2006). Intake of feed is accompanied by rapid development of the gastrointestinal tract and associated organs (Yegani and Korver, 2008). Immune responses within the intestine can have negative impacts on feed efficiency and are energetically expensive (Yegani and Korver, 2008). Early feeding can influence both the small intestinal and GALT development (Yegani and Korver, 2008). As a percent of intake, the resting immune system only utilizes approximately 1 – 2% lysine in growing broilers (Klasing, 2007). However, lysine use increases approximately 6-fold during an immune response (Klasing, 2007) and deficiencies in lysine may modulate the immune response, which may substantially down-regulate the metabolic changes associated with immunogenic stimulation. The costs for development and activation of the immune system are less well defined than the costs for maintenance (Klasing, 2007). Understanding the complex interactions between diet and the immune system, formulating feeds to promote intestinal integrity, and utilization of dietary enzymes to provide readily available nutrients may be imperative to maintaining intestinal integrity and broiler performance during a pathogenic challenge.

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CHAPTER II

Effects of dietary enzymes on performance, gut morphology, and apparent ileal amino acid digestibility of broilers exposed to a live coccidia oocyst vaccine

ABSTRACT

An experiment was conducted to evaluate the effects of dietary enzymes on performance, bone ash, apparent ileal amino acid digestibility, and gut morphology of broilers administered a live coccidia oocyst vaccine. At day-of-hatch (DOH), Cobb 500 straight run broilers were obtained from a commercial hatchery and half of the chicks were sprayed with Coccivac B™. Chicks were weighed and placed in battery brooders with respect to non-vaccinated and vaccinated groups according to eight dietary treatments. This resulted in a total of 16 treatments with nine replicate pens and 12 chicks/pen at DOH. Dietary treatments were positive control (PC; 0.90% Ca and 0.45% aP), negative control (NC; 0.80% Ca and 0.35% aP), NC + phytase (PHY), NC + protease (PRO), NC + xylanase (XYL), NC + phytase + protease (PHY+PRO), NC + phytase + xylanase (PHY+XYL), and NC + phytase + protease + xylanase (PHY+PRO+XYL). Performance of vaccinated birds was similarly influenced by dietary treatments resulting in no diet by vaccination interactions. PHY+XYL improved ($P = 0.0442$) feed conversion (FC) compared to the NC diet. Coccidia vaccination reduced ($P = 0.0478$) feed intake (FI), and reduced ($P = 0.0169$) body weight gain (BWG) from d 0 to 18. Percent tibia ash was reduced ($P = 0.0001$) in the NC and diets supplemented with PRO or XYL alone, and these effects were more prevalent at d 7 compared to d 18, resulting in a significant ($P = 0.0003$) diet by day interaction. Diet, vaccination, and day affected ($P \leq 0.05$) villus height, crypt depth, and villus height to crypt depth ratio (VCR) in the duodenum, jejunum, and ileum resulting in two- and three-way interactions. Proline, leucine, and phenylalanine digestibility were affected by diet,

vaccination, and day which resulted in significant ($P \leq 0.05$) three-way interactions. Mortality was not affected by diet or vaccination at any day measured. These data indicate selected dietary enzymes and a live coccidia oocyst vaccine may alter broiler performance, small intestinal morphology, and amino acid digestibility. The responses associated with performance and intestinal integrity may differ depending on the stage of infection, dietary enzyme, amino acid evaluated, and intestinal section.

Keywords: phytase, protease, xylanase, broiler, coccidia

INTRODUCTION

The poultry industry in the United States and Europe is faced with numerous challenges and opportunities. Governmental regulations have imposed strict guidelines on animal welfare, animal feeding practices, and animal feed ingredients. Overall increases in the cost to produce grains and increased prices of feed ingredients have led poultry producers to seek alternative, less commonly used dietary ingredients and dietary manipulation to reduce nutrient content without affecting performance and efficiency. By-product feeds, previously limited by inadequate supply or geographical location (Leeson, 2008) may be further incorporated into poultry diets. Dietary modification to reduce nutrients such as energy, minerals, and/ or crude protein (CP) may be enhanced by the utilization of dietary enzymes to improve feed efficiency and nutrient availability. Phytase is commonly used in poultry diets to reduce the anti-nutrient effects of phytate and improve mineral utilization. Recent research suggests dietary phytase supplementation may also improve amino acid digestibility (Cowieson et al., 2006a) and apparent metabolizable energy (AME; Cowieson et al., 2006b; Pirgozliev et al., 2007). Phytate may bind to protein at low pH, such as in the proximal digestive tract, rendering it partially unavailable to pepsin digestion (Selle et al., 2000). Phytase supplementation may reduce the

ability of phytate to bind to protein (Selle et al., 2000) or reduce endogenous amino acid losses associated with high dietary phytate (Cowieson et al., 2004; Cowieson and Ravindran, 2007) thereby improving their availability.

Dietary enzymes previously known to improve broiler performance and starch digestibility in diets composed of highly viscous grains may have benefits in corn and soy diets (Cowieson and Ravindran, 2008a), which may contain approximately 10% nonstarch polysaccharides (NSPs; Malathi and Devedowda, 2001) and 15% resistant starch (Iji et al., 2003). Nonstarch polysaccharides are known to alter digesta viscosity, bacterial colonization, nutrient digestion and absorption, and intestinal weight and length (Smits et al., 1997). Undigested starch at the terminal ileum is known as resistant starch, which may alter bacterial proliferation. Starch encapsulated by beta-glucans and mannans may also bind nutrients such as amino acids and bypass digestion for use by bacteria in the lower intestine (Wyatt et al., 2008). The combined use of enzymes may provide additional improvements in nutrient digestibility (Cowieson and Adeola, 2005; Cowieson and Ravindran, 2008a, b), reduce variation in the energy content in corn and soybean meal (Iji et al., 2003; Wyatt et al., 2004), liberate encapsulated nutrients (Wyatt et al., 2008), and reduce production costs associated with reduced feed efficiency and/or anti-nutrient effects of commonly used ingredients or by-product feed ingredients.

Another challenge to the poultry industry is based on consumer preferences for a more "natural" poultry product. Concerns regarding antibiotic resistance and antibiotic residues have created mandatory or voluntary removal of antibiotics (Leeson, 2008) and anti-coccidials in poultry diets. Reports suggest animal feeds utilize between 40 and 87% of the antibiotics produced (Gilchrist et al., 2007). However, published reports establishing correlations between

human antibiotic resistance and the usage of antibiotics in poultry production are scarce or poorly conducted. Subtherapeutic antibiotic usage in poultry diets acts as a growth promotant and may improve feed efficiency by altering gut microflora and reducing exposure to harmful pathogens. Maintaining intestinal health is vitally important in broilers due to the large immune function of the intestinal tract and the animals' rapid growth. Bans on subtherapeutic antibiotics may increase prevalence of intestinal diseases, impair intestinal health and integrity, and alter the nutrient requirements of broilers.

Coccidiosis is a common disease in poultry caused by protozoan parasites of the *Eimeria spp.* Estimates suggest chemotherapeutic treatment and production losses associated with coccidiosis cost the poultry industry in excess of $700 million annually (Lillehoj et al., 2007). Broilers become infected with coccidia upon ingestion of the oocyst in contaminated litter or feed. The parasite then excysts within the intestinal tract and invades the enterocyte where it undergoes a complex, multistage lifecycle composed of sexual, asexual, intracellular, and extracellular components (Lillehoj and Trout, 1996). The complex lifecycle of *Eimeria spp.* poses a significant problem for treatment and prevention of the disease. Coccidiosis is known to affect cellular integrity and cause an inflammatory immune response within the gut (Lillehoj and Trout, 1996) and to reduce the digestibility and absorption of nutrients (Williams, 2005). Poor nutrient utilization and an active immune response associated with coccidia infection resulted in reductions in broiler feed intake (FI) and body weight gain (BWG), poor feed efficiency, morbidity, and in severe cases mortality (Williams, 2005). Even subclinical coccidiosis has economic ramifications related to treatment expenses and loss in bird performance and feed efficiency (Williams, 2005).

Due to chemotherapeutic costs, the impending threat of antibiotic bans, and animal welfare issues associated with coccidosis, researchers are continuously evaluating alternative methods of control. Live coccidia oocyst vaccines are commercially available and provide immunity to secondary exposure of the same *Eimeria spp.* (Williams, 2002). Unfortunately, similar to other treatment and preventative methods, challenges are associated with the use of live coccidia vaccines. Protective immunity is only elicited for the species of *Eimeria* provided in the vaccine, and the large number of parasites included in the vaccine makes them labor intensive and costly to produce (Dalloul and Lillehoj, 2005). Live oocyst vaccines promote the coccidia life cycle, and slight reductions in performance or feed efficiency may be associated with the vaccination (Danforth, 1998; Parker et al., 2007). Intestinal damage from coccidia may exacerbate other intestinal diseases such as necrotic enteritis (NE; Williams, 2005). In general, the costs associated with live oocyst vaccines may out weigh the benefits. However, removal of antimicrobials from poultry diets and the continuous emergence of resistant pathogens may necessitate the use of vaccines and dietary ingredients to promote animal health.

Feed enzymes are utilized currently in the poultry industry to improve nutrient utilization and reduce anti-nutritive effects of NSPs, resistant starch, or phytate. Maintaining a homeostatic balance between intestinal growth, cellular turnover, and bacterial colonization is imperative to produce healthy broilers. Dietary ingredients can significantly affect intestinal integrity, bacterial population, immune function, and disease state in broilers. Therefore, research evaluating dietary and intestinal parasitic interactions may provide further understanding of intestinal health and disease prevention. Current research involving dietary enzymes and coccidia infection is scarce and provides inconsistent results. Watson et al. (2005) evaluated the interactive effects of *E. acervulina*, phytase, Ca, and aP in broilers. The authors found phytase

supplementation increased gain and FI in broilers regardless of the coccidial infection, though phytase was more effective in healthy chicks with regards to tibia ash and feed efficiency. The combination of amylase, protease, and xylanase supplementation to corn and soy diets did not significantly affect performance of coccidia-vaccinated broilers (Parker et al., 2007). However, dietary composition, mainly protein concentration, did affect the response of chicks vaccinated with live coccidia oocysts (Parker et al., 2007). The objective of this experiment was to evaluate the interactive effects of various dietary enzymes alone or in combination on performance, bone ash, small intestinal integrity, and apparent ileal amino acid digestibility (IAAD) of growing broilers exposed to a commercially available live coccidia oocyst vaccine.

MATERIALS AND METHODS

Animals and Husbandry

Straight run, Cobb 500 broilers (n=1728) were obtained from a commercial hatchery at day-of-hatch (DOH) and transported to the poultry research farm at Virginia Tech. Upon arrival, half of the chicks (n=864) were sprayed with a commercially available live coccidia oocyst vaccine[1] according to manufacturer's recommendations. Chicks were then randomly selected, weighed, and placed in Petersime battery brooders with respect to non-vaccinated and vaccinated groups. Non-vaccinated birds were placed in batteries separate from vaccinated birds in the same environmentally controlled room. Birds were maintained on a lighting program of 24L:0D d 0, 22L:2D d 1 to 7, and 18L:6D d 7 to 18, and they were allowed *ad libitum* access to feed and water for the 18 d trial.

Diets and Enzyme Treatments

All diets were fed in mash form and formulated on a corn/soybean meal basis according to Cobb nutrient recommendations with the exception of Ca and aP in the NC (Table 2.1).

[1] Coccivac B™, Schering Plough, Kenilworth, NJ

Dietary treatments consisted of a positive control (PC; 0.90% Ca and 0.45% aP), negative control (NC; 0.80% Ca and 0.35% aP), NC + phytase (PHY), NC + protease (PRO), NC + xylanase (XYL), NC + PHY + PRO (PHY+PRO), NC + PHY + XYL (PHY+XYL), and NC + PHY + PRO + XYL (PHY+PRO+XYL). Non-vaccinated and vaccinated birds were allowed *ad libitum* access to one of the eight dietary treatments from d 0 to 18. This resulted in a total of 16 treatments with 9 replicate pens of 12 birds per pen from d 0 to 7 and 6 birds per pen from d 7 to 18.

The enzymes used in the experiment were supplied by Danisco Animal Nutrition[2]. The phytase used was an *Escherichia coli* 6-phytase expressed in S*chizosccaromyces pombe* and formulated to provide 1,000 U/kg diet, the protease used was extracted from *Bacillus subtilis* and formulated to provide 8,000 U/kg diet, and the xylanase was a *Trichoderma reesi* endo-1, 4-beta-xylanase formulated to provide 1,200 U/kg diet. Diets were formulated to contain 0.3% titanium oxide as an indigestible marker. Enzyme activities in the experimental diets were analyzed by Danisco Animal Nutrition[2]. All experimental procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Sample Collection

Birds were weighed by pen prior to placement (d 0), d 7, and d 18 to measure mean body weight (BW) and calculate mean BWG for each period and cumulatively (d 0 to 18). Feed intake and feed conversion (FC) were also measured for each period and cumulatively. Room temperature and mortality were recorded daily and any birds removed for sampling or mortality were weighed and FI and FC were adjusted according to the number of bird days. Birds sampled were stunned by exposure to CO_2 gas and euthanized by cervical dislocation for collection of digesta and tissues for histology, digestibility assays, and bone ash.

[2] Danisco Animal Nutrition, St. Louis, MO

On d 7 and d 18, one bird/ pen (n=9 birds/diet/treatment) was euthanized to obtain tissues for morphometric and histopathological measurements. Tissues were collected from the duodenum, jejunum, and ileum for determination of villus height, crypt depth, villus height to crypt depth ratio (VCR), and goblet cell number. Intestinal segments (approximately 2 to 3 cm) were obtained and gently flushed with cold phosphate buffered saline to remove luminal contents. Collected tissues were placed in 10% neutral buffered formalin until further processing. Each fixed intestinal segment was cut into five-5 mm sections and embedded in paraffin. Embedded samples were cut at 5 μm and mounted onto slides. Slides were stained using Periodic Acid-Schiff's reagent and Alcian Blue and examined by light microscopy[3]. Measurements of villus height, crypt depth, and goblet cell number were made using Sigma Scan Pro 5[4] and digitized using Image Pro Plus[5]. Villus height (n=12) and crypt depth (n=12) were measured from 3 tissues/slide following procedures according to Sun et al. (2005). Briefly, villus height was measured from the tip of a villus to the opening of the crypt, and crypt depth was measured from the crypt opening to the crypt bottom. Villus height to crypt depth ratio was calculated and normalized using the natural log. The number of goblet cells/villus were counted, and the villus area was obtained from 4 villi/3 tissues/slide (n=12). The goblet cell number/villus area was calculated and normalized using the natural log.

On d 7 and d 18, digesta was obtained from the ileum, consisting of the section from the Meckel's diverticulum to the ileo-cecal junction, and pooled from four birds/pen (n=36 birds/diet/treatment). Ileal sub-samples (0.4 g) were obtained, homogenized, snap frozen in liquid nitrogen, and stored at -80°C until amino acid analysis. The remaining digesta samples were dried in a forced air oven at 100°C for 24 hours. Diet and digesta samples were ground to

[3] Olympus Polaroid DMC-IE camera, Polaroid Corporation, MA
[4] SPSS, Chicago, IL
[5] Media Cybernetics, Silver Springs, MD

pass through a 1 mm screen and titanium dioxide concentrations were determined according to methods by Short et al. (1996). Ileal sub-samples were freeze-dried and ground to pass through a 1 mm screen for amino acid analysis. Amino acid concentration in the diets and ileal digesta were determined using HPLC following acid hydrolysis according to modified methods of Albin et al. (2000). Briefly, 20 mg of ileal digesta was purged for 30 seconds with N_2 and hydrolyzed for 24 hours at 100°C in approximately 1 to 2 ml 6 M HCl. Hydrolyzed samples were filtered using 0.45 µm luer lock syringe filters. Filtered, hydrolyzed samples (100 µL) were added to 100 µL of the internal standard Norleucine, centrifuged, dried twice, and analyzed using a Pico-Tag column and HPLC. Amino acids were identified and integrated using Pierce hydrolyzed standards (Fisher P120088), and grams amino acid/100 g of sample was calculated. Amino acid values were then used to calculate apparent IAAD using the following equation:

$$\text{Digestibility Coefficient} = ((\text{amino acid/TiO})_d - (\text{amino acid/TiO})_i) / (\text{amino acid/TiO})_d * 100$$

Left tibias were obtained from four birds/pen (n=36 birds/diet/treatment) and pooled for determination of bone ash on d 7 and d 18. Tibias were stripped of adhering tissues, wrapped in cheese cloth, and dried overnight at 100°C. Fat was extracted from the tibias using a Soxhlet apparatus and 100% ethyl ether according to modified methods adapted from Watson et al. (2006). Fat-extracted tibias were then dried for 24 hours at 100°C and ashed in a muffle furnace for 24 hours at 600°C to determine bone ash.

Statistical Analysis

Performance data were subjected to ANOVA using the MIXED models for completely randomized design procedure of SAS[6]. Percent mortality data were arc sine transformed prior to analysis. Pen served as the experimental unit for BWG, FI, FC, percent mortality, apparent IAAD, and tibia ash. Bird served as the experimental unit for histological measurements. The statistical model included diet, vaccination, the repeated factor day, and all two and three way interactions. The statistical model for cumulative performance included diet, vaccination, and diet by vaccination interactions. Mean differences were determined using Tukey's test. Statistical significance was accepted at $P \leq 0.05$.

RESULTS

Growth Performance

Mortality was low throughout the experiment and there were no differences associated with treatment (data not shown). Dietary analysis determined the recovery of enzymes was approximately in agreement with formulated activities (Table 2.2). Xylanase activity was detected in the PC and presumed to originate from endogenous levels of the enzyme in plant ingredients or the result of sampling variation. The xylanase activity in the other diets varied or was negligible, which may suggest sampling or assay variation was quite high. The analyzed composition of dietary CP and specific amino acids were lower than anticipated, and Ca levels in the NC were higher than anticipated which increased the Ca:aP ratio to approximately 4:1 (Table 2.1). However, the diets were formulated to only contain reductions in Ca and aP, and FI and BWG were not affected by diet (Table 2.3). Phytase and xylanase supplementation improved ($P = 0.0442$) FC compared to the NC from d 0 to 18. Non-vaccinated and vaccinated birds responded similarly to dietary treatments, which resulted in no significant diet by vaccination

[6] SAS Institute, Cary, NC

interactions. Vaccination with live coccidia oocysts reduced (P = 0.0478) FI and reduced (P = 0.0169) BWG, but did not affect FC (Table 2.3).

Bone Ash

Dietary effects were more prevalent at d 7 than d 18, resulting in a significant (P = 0.0003) diet by day interaction for bone ash (Table 2.4). Particularly at d 7, the large Ca to aP ratio in the NC diet resulted in a lower tibia ash percent and phytase supplementation alone and in combination with other enzymes improved percent tibia ash. Vaccination did not affect tibia ash at either day measured (data not shown).

Small Intestinal Morphology

In the duodenum, significant differences were observed in crypt depths, VCR, and goblet cell number, but there were no differences in villus height (data not shown). Vaccination increased (P = 0.0015) duodenum crypt depth on d 18, which resulted in a vaccination by day interaction (P = 0.0077; Table 2.5), with the exception of vaccinated birds fed diets supplemented with XYL and PHY+PRO (Figure 2.1). The XYL and PHY+PRO diets fed to vaccinated birds resulted in no difference in crypt depth or shallower crypts than non-vaccinated birds fed the same diets, leading to a diet by vaccination interaction (P = 0.0057). The difference in crypt depth between non-vaccinated and vaccinated birds was greater in birds fed the PC diet than the other diets (Figure 2.1). Interestingly, on d 7, PHY+PRO resulted in the deepest crypts and on d 18 the NC diet and PHY+PRO+XYL diet resulted in the deepest crypts in the duodenum (Figure 2.2), which resulted in a diet by day interaction (P = 0.0528).

Vaccination resulted in reduced (P = 0.0040) duodenum VCR at d 18 (Table 2.5), with the exception of birds fed the NC diet and diets supplemented with XYL and PHY+PRO (Figure 2.3), which resulted in a vaccination by day interaction (P = 0.0052) and a diet by vaccination

interaction (P = 0.0228; Table 2.5). The diet by vaccination interaction indicated a relatively larger decrease in VCR in birds that were fed the PC and vaccinated as compared to the change associated with vaccinated birds fed the other diets. At d 7, birds fed the PC diet and diets supplemented with PHY had the largest VCR in the duodenum, but at d 18 birds fed diets supplemented with PHY had the lowest VCR in the duodenum with other groups having similar VCR (Figure 2.4), which resulted in a diet by day interaction (P = 0.0040).

A three-way interaction (P = 0.0007) of dietary treatment, vaccination, and day resulted in differences in goblet cells numbers (Figure 2.5). Vaccination with live coccidia oocysts increased the number of goblet cells in the duodenum of birds fed the PC, NC, and PHY+XYL at d 7. Each enzyme supplemented alone or the combination of PHY+PRO reduced goblet cell numbers in the vaccinated birds compared to the non-vaccinated birds, and the combination of all three enzymes resulted in the lowest number of goblet cells regardless of the vaccination treatment at d 7. However, at d 18, the number of goblet cells was increased in vaccinated birds fed all diets, except PHY+XYL and PHY+PRO+XYL. The biggest increase in goblet cell number in vaccinated birds compared to non-vaccinated birds was observed in birds fed the PC, XYL, or PHY+PRO diets.

In the jejunum, similar to results in the duodenum, multiple interactions resulted in differences in crypt depth, VCR, and goblet cell number but not villus height (data not shown). Vaccination resulted in reduced crypt depth at d 7 and increased crypt depth at d 18 (Figure 2.6), which indicated a vaccination by day interaction (P = 0.0464). Diet, vaccination, and day interaction (P = 0.0172) resulted in differences in VCR (Figure 2.7). While most responses to diet and vaccination were similar between d 7 and d 18, several groups had differential results. On d 7, the vaccinated birds on the NC or PHY+PRO+XYL diets had higher VCR compared to

the non-vaccinated birds fed these diets, but on d 18, VCR in the non-vaccinated birds was greater than the vaccinated birds fed these diets.

The number of goblet cells in the jejunum was affected ($P \leq 0.0001$) by interactions of diet, vaccination, and day (Figure 2.8). In general, the differences in goblet cell numbers between treatments were more evident at d 7 than d 18. This was particularly observed with birds on the PRO diet or PHY+PRO diet. Vaccination reduced the number of goblet cells in the jejunum of birds fed the PC or NC diets and diets supplemented with PHY or PRO, but it increased the number of goblet cells in birds fed diets supplemented with PHY+PRO, PHY+XYL, and PHY+PRO+XYL at d 7. On d 18, PRO, PHY+XYL, and PHY+PRO+XYL supplementation resulted in reductions in goblet cell numbers and XYL supplementation resulted an increase in the number of goblet cells in vaccinated birds compared to the non-vaccinated birds fed the same diets.

In the ileum, differences were observed in villus height, crypt depth, VCR, and goblet cell number. Vaccination resulted in increased ($P = 0.0065$) villus height in the ileum at d 7, but did not affect ileal villus height at d 18 (Table 2.6), which resulted in a vaccination by day interaction ($P = 0.0018$). Ileal crypt depth was affected by the interaction ($P = 0.0021$) of diet, vaccination, and day. At d 7, ileal crypt depth was more shallow in vaccinated broilers fed the PC, NC, and diets supplemented with PHY, PRO, or PHY+PRO compared to non-vaccinated birds fed the same diets (Figure 2.9). There was no difference in ileal crypt depth between non-vaccinated and vaccinated birds fed diets supplemented with XYL, PHY+XYL, and PHY+PRO+XYL at d 7. However, at d 18, ileal crypts were deeper in vaccinated broilers fed the PC, NC, and diets supplemented with PRO compared to non-vaccinated birds fed the same

diets. Ileal crypt depth was not affected by vaccination in broilers fed diets supplemented with PHY, XYL, PHY+XYL, and PHY+PRO+XYL at d 18.

Interactions of diet, vaccination, and day also affected VCR in the ileum (P = 0.0410; Figure 2.10). Vaccination increased VCR in the ileum regardless of diet or enzyme combination supplemented at d 7. However, at d 18, vaccinated birds had reduced VCR in the ileum when fed the PC, NC, and diets supplemented with PHY, PRO, or PHY+PRO+XYL compared to non-vaccinated birds fed the same diets. Vaccination had little effect on VCR in the ileum when birds were fed the other diets on d 18. Vaccination with live coccidia oocysts reduced the number of goblet cells in the ileum, except when birds were fed the PC or diets supplemented with PHY+PRO or PHY+PRO+XYL (Figure 2.11), which resulted in a diet by vaccination interaction (P = 0.0301).

Apparent Ileal Amino Acid Digestibility

Amino acid digestibility was not affected by diet, vaccination, or day for most amino acids analyzed with the exception of PRO, LEU, and PHE, which had significant diet, vaccination, and day interactions. In general phytase supplementation in vaccinated broilers improved PRO (P = 0.0355; Figure 2.12), LEU (P = 0.0293; Figure 2.13), and PHE (P = 0.0537; Figure 2.14), and tended to improve GLU (P = 0.1088; Figure 2.15), ARG (P = 0.0897; Figure 2.16), ALA (P = 0.0905; Figure 2.17), and TYR (P = 0.1015; Figure 2.18) digestibility at d 7 compared to non-vaccinated broilers fed diets supplemented with phytase. The phytase improved digestibility was equivalent to that of non-vaccinated birds fed the PC. Vaccinated birds fed diets supplemented with PHY+PRO had reduced PRO, LEU, and PHE and tended to result in reduced GLU, ARG, ALA, and TYR digestibility compared to non-vaccinated broilers fed diets supplemented with PHY+PRO at d 7. There were fewer and smaller changes in AA

digestibility resulting from diet and vaccination at d 18. Vaccinated birds fed the PC had improved PRO, LEU, PHE, GLU, ARG, ALA, and TYR digestibility compared to non-vaccinated broilers fed the PC, relative to the smaller changes between non-vaccinated and vaccinated birds on the other diets. Vaccination had no effect on PRO, LEU, PHE, GLU, ARG, ALA, or TYR digestibility at any day in broilers fed PHY+XYL, which resulted in no differences between the non-vaccinated and vaccinated birds. At d 18, PHY+XYL supplementation resulted in PRO, LEU, PHE, GLU, ARG, ALA, and TYR digestibility above the PC and all other dietary treatments, except vaccinated birds fed the PC.

DISCUSSION

The objective of this experiment was to evaluate the effects of various dietary enzymes on performance, bone ash, intestinal morphology, and apparent IAAD in young broilers exposed to a live coccidia oocyst vaccine. The combination of phytase and xylanase improved FC compared to the NC diet, but FI and BWG were not affected by dietary treatments. Selle et al. (2003) reported beneficial effects of supplementing wheat diets with a combination of phytase and xylanase on broiler BWG and feed efficiency. Cowieson and Adeola (2005) and Olukosi et al. (2007a) reported improvements in performance and nutrient digestibility in chicks fed nutritionally marginal corn/soy diets supplemented with phytase and an enzyme combination of xylanase, amylase, and protease. In the current experiment, the NC diet was formulated to meet or exceed Cobb nutrient requirements, with the exception of Ca and aP, which were reduced to facilitate adequate phytase efficiency (Qian et al., 1997; Manangi and Coon, 2008). The analyzed Ca levels in the NC diet were substantially different than formulated, which resulted in a Ca:aP ratio of approximately 4:1 (Table 2.1). While the imbalance of Ca to aP in the NC diet did not appear to affect performance, mineral absorption was reduced, which affected tibia ash in

the NC. Phytase supplementation to the NC alone or in combination with the other enzymes may have improved P availability thereby reducing the Ca:aP ratio and improving mineral absorption. Protease and xylanase alone were not able to liberate enough P from the diet to improve tibia ash. Previous reports suggest xylanase, amylase, and protease supplementation improved P and Ca retention in corn and soybean meal diets fed to broilers (Olukosi et al., 2007a), and xylanase supplementation improved P digestibility of wheat diets fed to pigs (Nortey et al., 2008). The increase in mineral availability may be an indication of the ability of carbohydrases to release cations that are bound to fiber molecules (Olukosi et al., 2008). Pinheiro et al. (2004) reported no additional improvement in broiler BW or FI when birds were fed corn/soybean meal diets supplemented with amylase and protease and broiler BWG and FC were reduced when fed corn/soy diets formulated to contain marginal levels of ME, Ca, and aP, and amylase, protease, and xylanase supplementation were not able to improve performance to that of the PC diets (Olukosi et al., 2007b). While the NC diet in this experiment contained analyzed Ca levels substantially higher than formulated, broiler daily gain and FI were comparable to Cobb 500 standards[7].

Coccivac B™ utilizes live oocysts from three of the most common species of *Eimeria* known to infect poultry. *Eimeria acervulina*, *E. maxima*, and *E. tenella* invade enterocytes in the duodenum, jejunum, and ceca, respectively, in a site specific manner, induce an inflammatory immune response, and confer protective immunity to a second exposure of the same species. In this experiment, vaccination negatively affected FI and BWG, which is in agreement with other published reports (Yi et al., 2005; Parker et al., 2007; Lehman et al., 2009). The reductions in broiler performance may be attributed to small intestinal epithelial damage associated with the initial coccidia infection. Vaccination increased cell turnover in the small intestine, as indicated

[7] Cobb 500 Broiler Growth and Nutrition Supplement

by larger crypts, especially in the duodenum and jejunum. Proliferating cells in the mucosal crypts differentiate into enterocytes and mature as they migrate up the villus (Uni et al., 2000). As the enterocyte matures, the activity of the digestive enzymes sucrase and maltase increase (Uni et al., 1998). While villus height was not affected by vaccination in this experiment, an increase in crypt depth may suggest cell turnover was increased to facilitate cellular repair and maintain intestinal integrity. Rapid movement and differentiation of crypt cells into enterocytes may reduce nutrient transporter expression and enzymatic activity along the brush border. While not evaluated in this experiment, reduced expression of nutrient transporters and activity of brush border enzymes would reduce nutrient absorption and digestion, which may be associated with reductions in broiler BWG. Interestingly, jejunum crypt depth was not affected by vaccination at d 7, but did show an impact at d 18. The differences associated with vaccination and day on intestinal morphology may be attributed to the species of *Eimeria* present in the vaccine and the onset of peak oocyst shedding. While other species of *Eimeria* are shed approximately 4 to 5 days post infection, *E. maxima* shedding occurs from 6 to 9 days post infection (Allen and Fetterer, 2002). Therefore, in this experiment damage to the jejunum from *E. maxima* would be more prevalent after the d 7 sampling. Also in this experiment, villi in the ileum were longer in vaccinated than non-vaccinated birds. The *Eimeria spp*. provided in the vaccine would not naturally have infected the ileum, and previous reports suggest the ileum may compensate for proximal intestinal damage by increasing the mucosal surface area (Idris et al., 1997).

Previous research has evaluated the use of dietary enzymes to alleviate the negative impact of coccidiosis on broiler performance and nutrient digestibility (Watson et al., 2005; Parker et al., 2007; Lehman et al., 2009). In this experiment, performance was not affected by interactions between diet and vaccination. However, differences in morphological parameters

were observed between dietary treatments, day, and vaccination status. Diet by day interactions would suggest supplementation of the NC diet with phytase, reduced cellular turnover in the duodenum at d 7, as indicated by crypt depth and VCR, to levels comparable to the PC. Since the level of phytate in the PC and NC diets are presumed to be similar, the only difference in the diets would be the Ca:aP ratio. The large amount of Ca entering the small intestine of chicks fed the NC diet, may have acted as an antagonist toward the absorption of other minerals such as Zn. Mice fed diets marginally deficient in Zn had greater crypt depths in the ileum compared to mice fed diets adequate in Zn (Peterson et al., 2008). Moderate deficiencies in essential nutrients may up-regulate expression of ion channels or nutrient transporters along the brush border to facilitate nutrient absorption. Phytase supplementation may have balanced the Ca:aP ratio, reduced the antagonistic effects of Ca on Zn, and altered the small intestinal morphology in the duodenum equivalent to that of the PC, but this was only noticed at d 7. Younger chicks may obtain more benefit from enzyme supplementation targeted to reduce anti-nutrients in the diet than older chicks (Olukosi et al., 2007a). However, phytase or xylanase may have been more effective in the jejunum of older chicks due to increased substrate availability and transit time within the intestinal tract, which resulted in larger VCR in the jejunum of vaccinated broilers at d 18. Phytase supplementation also resulted in a reduction in the number of goblet cells in the jejunum of vaccinated birds at d 18 which may be indicative of a reduction in dietary phytate. Phytate may increase endogenous losses by interacting with endogenous enzymes or gastrointestinal mucin (Cowieson et al., 2004) and in the current experiment, phytase supplementation in the diets of vaccinated birds improved the digestibility of glutamine, proline, and leucine; amino acids that make up a large component of endogenous secretions (Miner-Williams et al., 2009). In older chicks, glutamine, proline, and leucine digestibility tended to be improved with phytase

and xylanase supplementation. As mentioned previously, vaccination resulted in larger crypts in the jejunum of 18-day-old broilers. An increase in cell proliferation will reduce the age and maturity of goblet cells, the quality of mucin they produce, and increase the energy requirement of the intestinal tract (Choct, 2009). Phytase and xylanase may have had a sparing effect on energy and amino acids utilized by the jejunum during exposure to coccidia. Phytase most likely reduced endogenous amino acid losses associated with dietary phytate and xylanase may have improved starch and energy digestibility for use by the enterocytes. Activation of an inflammatory immune response causes significant metabolic changes in energy expenditure, muscle catabolism, and amino acid oxidation, and results in a reduction of the amino acid requirement of the chick (Klasing and Barnes, 1988). Therefore, chicks under an immune stress may not benefit from improved nutrient digestibility in terms of performance and muscle growth, but rather via improvements in nutrient utilization within the small intestine for tissue repair and maintenance. Improvements in the non-digestible fraction of amino acids were more prevalent in vaccinated birds compared to non-vaccinated birds (Figures 2.19 and 2.20). For example, approximately 25% or 11% of the non-digestible lysine was captured by phytase or xylanase supplementation, respectively, in vaccinated broilers at d 7. However, phytase or xylanase supplementation resulted in no additional improvement in lysine digestibility in non-vaccinated broilers at d 7. On d 18, phytase and xylanase supplementation to the diets of vaccinated birds was able to capture approximately 0.8% to 12.3% of the non-digestible fraction of all amino acids analyzed, and these results were better than supplementing individual enzymes. Multiple published experiments report improvements in apparent IAAD in diets supplemented with phytase, xylanase, and the combination (Ravindran et al., 1999; Selle et al., 2003).

When evaluating dietary supplementations to alleviate symptoms associated with intestinal pathogens, it is imperative to account for other factors that may affect bird health. Vaccination most likely resulted in an inflammatory immune response. Cytokines, such as interleukin-1 (IL-1), released from macrophages during a coccidial infection (Lillehoj and Trout, 1996), cause a reduction in FI and feed efficiency (Klasing et al., 1987). In the current study, reductions in performance associated with the vaccine may have resulted from an inhibition of feed intake due to IL-1 production. Data from this experiment would also suggest the addition of protease to enzyme cocktails may reduce the effect of the other enzymes, especially at day 7. Protease addition to the NC diet supplemented with phytase increased crypt depth and reduced VCR in the duodenum and reduced the digestibility coefficients of the analyzed amino acids compared to the NC diet supplemented with phytase alone. Protease addition to an *in vitro* enzyme preparation reduced the effect of enzyme combinations on crude protein digestibility (Saleh et al., 2004), and the combination of protease and α-galactosidase reduced the performance response associated with the individual enzymes in broilers (Ghazi et al., 2003), suggesting protease may digest other enzymes in the matrix, rendering them ineffective. However, other researchers have reported improvements (Odetallah et al., 2003, 2005; Cowieson et al., 2006c, d) or no additional benefit (Simbaya et al., 1996; Marsman et al., 1997; Ravindran et al., 1999) on performance and/or amino acid digestibility with protease supplementation alone or in combination with other enzymes.

This experiment demonstrates the variability in broiler intestinal morphology and IAAD due to enzyme supplementation and live oocyst vaccination at various days measured and various parameters evaluated. Variability in the response of enzyme supplementation has been a concern of enzyme producers, and methods to determine the optimum dose, enzyme

characteristics and combination, and target substrates are important to evaluate enzyme efficiency (Bedford, 2000) and understand the mechanistic actions of enzymes, anti-nutrients, and intestinal diseases such as coccidiosis. Further understanding of the mechanisms involved in enzyme supplementation and interactions with diet and diet quality and immune function will enhance the usage of enzymes in poultry production and further our understanding of the interactions between nutrition and bird health. Governmental regulations and consumer concerns will make it necessary to delve deeper into the nutrient requirements of immuno-compromised animals. Phytase or xylanase supplementation may improve broiler performance during coccidia vaccination by reducing the non-digestible fraction of amino acids and the shunting of nutrients to the lower intestinal tract, improving energy availability for use by the intestine, and protecting the mucin layer from degradation by phytate.

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Table 2.1 Composition and nutrient content of basal diets[1]

Ingredients	PC (%)	NC (%)
Corn	53.582	54.748
Soybean Meal	33.133	32.891
DDGs	5.000	5.000
Poultry Fat	3.933	3.524
Di-calcium Phosphate	1.654	1.111
Limestone	1.139	1.162
Salt	0.481	0.482
Titanium Di-oxide	0.300	0.300
Methionine	0.253	0.252
L-Lysine	0.192	0.197
Trace Mineral Premix[2]	0.100	0.100
Vitamin Premix[3]	0.100	0.100
Choline Cl	0.070	0.070
Sand	0.062	0.062
Calculated Composition		
Dry Matter	88.12	88.00
Crude Protein	21.50	21.50
Lysine	1.30	1.30
TSAA	0.98	0.98
Threonine	0.82	0.82
Ca	0.90	0.80
Total P	0.70	0.60
Available P	0.45	0.35
Analyzed Composition		
Dry Matter	88.84	90.83
Crude Protein	20.47	19.91
Lysine	1.13	1.03
Threonine	0.61	0.55
Ca	0.88	1.40
Total P	0.68	0.60
Nutrient Composition		
Energy (ME; kcal/kg)	3025	3025

[1] Diets were fed in mash form from days 0 to 18. Negative control diets were supplemented with an *E. coli* phytase, *Bacillus subtilsin* protease, and *Trichoderma longibrachiatum* endo-1, 4-beta-xylanase.

[2] Supplied per kilogram mix: iron (Ferrous Sulfate), 40 g; manganese (Manganese Sulfate and Manganous Oxide), 120 g; zinc (Zinc Oxide), 210 g; cobalt (Cobalt Carbonate), 2.2 g; iodine (Calcium Iodate), 132 g.

[3] Supplied per kilogram mix: vitamin A, 8,818,400 IU; vitamin D_3 2,645,520 ICU; vitamin E, 22,046 IU; vitamin B_{12}, 26 mg; riboflavin, 8,818 mg; niacin, 88,184 mg; d-pantothenic acid, 22,046 mg; vitamin K, 2,646 mg; folic acid, 2,205 mg; vitamin B_6, 4,339 mg; thiamine, 3,732 mg; d-biotin, 220 mg.

Table 2.2 Recovery of enzyme activity in experimental diets

Diet	Determined Phytase Activity[1]	Determined Protease Activity[2]	Determined Xylanase Activity[3]
Positive Control	60	< 100	430
Negative Control (NC)	< 50	-	-
NC + Phytase (1000 U)	1222	-	-
NC + Protease (8000 U)	-	6759	-
NC + Xylanase (1200 U)	-	-	1787
NC + Phytase + Protease	1272	6679	-
NC+ Phytase + Xylanase	955	-	1079
NC + Phytase + Protease + Xylanase	1156	5424	2178

[1] One unit of phytase activity (FTU) is defined as the quantity of enzyme that liberates 1 µmole of inorganic P per minute from sodium phytate at pH 5.5 and 37°C.
[2] One unit is the amount of enzyme which liberates 1 µmole of phenolic compound (tyrosine equivalents) from a casein substrate per minute at pH 7.5 and 40°C.
[3] One unit is the amount of enzyme which liberates 0.5 µmole of reducing sugar (expressed as xylose equivalents) from a cross-linked oat spelt xylan substrate at pH 5.3 and 50°C in one minute.

Table 2.3 Effects of dietary enzyme supplementation and Coccivac B^{TM} on 0 to 18 day broiler performance

Dietary Treatments	Feed Intake (g)	BWG (g)	Feed:Gain (g:g)
PC	838.9	633.8	1.324^{ab}
NC	830.0	620.7	1.338^{b}
NC + PHY	839.7	639.9	1.313^{ab}
NC + PRO	821.4	615.6	1.335^{ab}
NC + XYL	827.0	629.1	1.316^{ab}
NC + PHY + PRO	819.0	625.8	1.311^{ab}
NC + PHY + XYL	828.6	634.6	1.306^{a}
NC + PHY + PRO + XYL	831.1	630.3	1.319^{ab}
Pooled SEM	9.806	8.030	0.0078
Vaccination[1]			
Non-Vaccinated	836.4^{a}	635.6^{a}	1.317
Vaccinated	822.5^{b}	621.8^{b}	1.324
Pooled SEM	4.905	4.016	0.0039
P-Value			
Diet	0.7765	0.4719	0.0442
Vaccination	0.0478	0.0169	0.1977
Diet*Vaccination	0.3288	0.1569	0.3916

[1] Vaccinated birds were exposed to Coccivac B^{TM} at day of hatch.
[a-b] Means within a row lacking a common superscript differ significantly ($P \leq 0.05$).

Table 2.4 Effects of dietary enzyme supplementation on broiler tibia ash

Dietary Treatments	Day 7 (%)	Day 18 (%)
PC	47.72[a]	51.36
NC	46.17[b]	50.88
NC + PHY	48.03[a]	51.51
NC + PRO	46.26[b]	50.85
NC + XYL	46.17[b]	50.79
NC + PHY + PRO	48.02[a]	51.25
NC + PHY + XYL	47.92[a]	51.69
NC + PHY + PRO + XYL	47.71[a]	51.03
Pooled SEM	0.2017	0.2017
P-Value		
Diet	0.0001	
Diet*Day	0.0003	

[a-b] Means within a column lacking a common superscript differ significantly ($P \leq 0.05$).

Table 2.5 Effect of Coccivac[1] B^{TM} and day on duodenum morphology in broilers

	Crypt Depth (mm)		VCR (Ln)	
Vaccination	Day 7	Day 18	Day 7	Day 18
Non-Vaccinated	0.092	0.105[b]	2.772	2.989[a]
Vaccinated	0.093	0.113[a]	2.767	2.905[b]
Pooled SEM	0.0014	0.0014	0.0144	0.0148
P-Value				
Vaccination		0.0015		0.0040
Day		0.0001		0.0001
Vaccination*Day		0.0077		0.0052

[1] Vaccinated birds were exposed to Coccivac B^{TM} at day of hatch.
[a-b] Means within a column lacking a common superscript differ significantly ($P < 0.05$).

Table 2.6 Effect of Coccivac[1] B^{TM} and day on ileal villus height in broilers

	Day 7 (mm)	Day 18 (mm)
Vaccination		
Non-Vaccinated	0.472[b]	0.715
Vaccinated	0.523[a]	0.712
Pooled SEM	0.0087	0.0086
P-Value		
Vaccination	0.0065	
Day	0.0001	
Vaccination*Day	0.0018	

[1] Vaccinated birds were exposed to Coccivac B^{TM} at day of hatch.
[a-b] Means within a column lacking a common superscript differ significantly ($P < 0.05$).

Table 2.7 Improvement in non-digestible essential amino acid fraction in non-vaccinated and vaccinated (Coccivac B™) broilers fed corn and soybean meal diets supplemented with phytase and xylanase individually and in combination

	7-day-old broilers			18-day-old broilers		
	PHY (%)	XYL (%)	PHY+XYL (%)	PHY (%)	XYL (%)	PHY+XYL (%)
Non-Vaccinated						
His	-11.0	-10.7	-12.3	- 1.2	-8.7	0.8
Arg	-16.3	-1.3	-7.0	-4.0	-2.4	3.2
Thr	-8.4	-8.6	-7.9	-3.7	-6.5	1.8
Val	-11.0	-7.0	-7.3	-0.3	-2.4	8.1
Ile	-11.6	-7.8	-6.6	0.4	-2.9	9.4
Leu	-11.5	-3.6	-3.6	3.9	0.6	11.3
Phe	-12.4	-8.4	-4.9	2.9	-2.0	10.7
Lys	-11.1	-5.1	-4.5	-0.4	-3.6	10.1
Vaccinated						
His	12.2	1.4	-8.9	-0.3	6.2	8.5
Arg	22.9	18.1	5.4	-5.6	3.6	11.4
Thr	15.9	7.5	3.9	2.3	4.7	6.4
Val	14.3	3.0	-2.6	-1.0	5.3	9.0
Ile	17.4	3.5	0.4	0.1	4.4	9.8
Leu	18.7	3.5	-0.8	-2.5	2.8	9.7
Phe	19.8	5.1	1.4	-0.3	4.4	11.5
Lys	24.6	10.6	2.2	-0.2	4.8	10.6

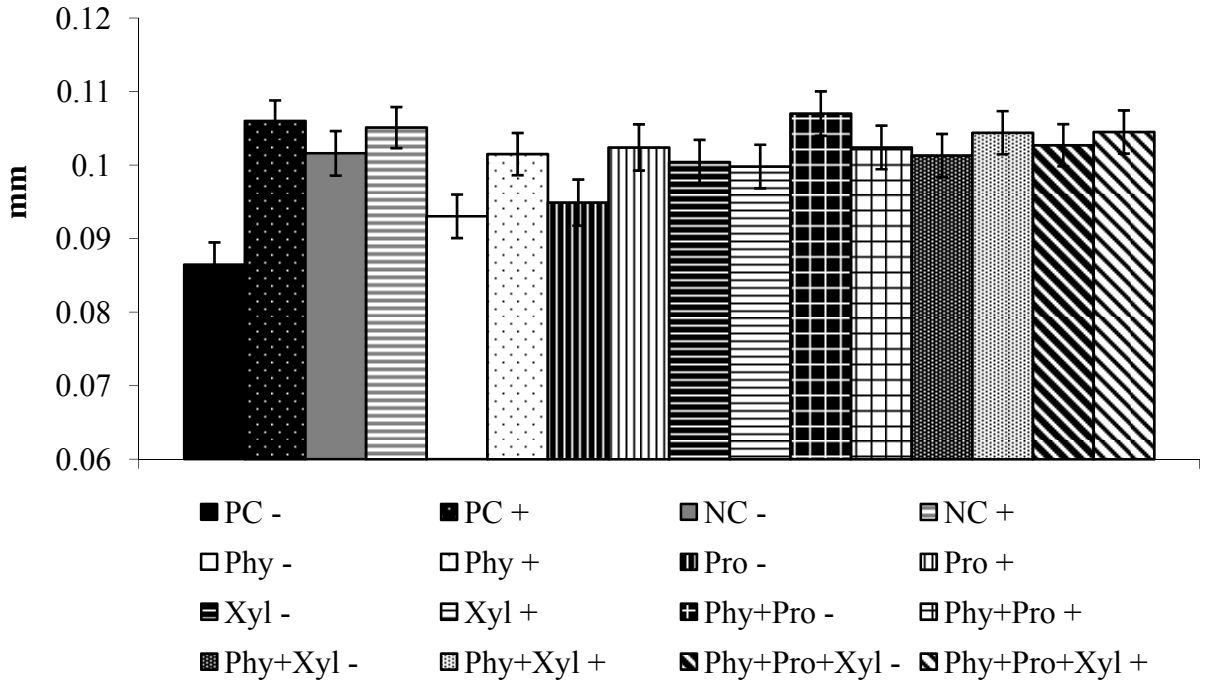
Calculated values were derived using the NC and NC supplemented with phytase, xylanase, and phytase plus xylanase treatments only. The non-digestible fraction was derived from total dietary AA – apparent ileal digestible AA, and the percent improvement was calculated.

Table 2.8 Improvement in non-digestible non-essential amino acid fraction in non-vaccinated and vaccinated (Coccivac B™) broilers fed corn and soybean meal diets supplemented with phytase and xylanase individually and in combination

Diet	7-day-old broilers			18-day-old broilers		
	PHY (%)	XYL (%)	PHY+XYL (%)	PHY (%)	XYL (%)	PHY+XYL (%)
Non-Vaccinated						
Asp	-11.1	-11.9	2.0	5.0	-9.4	7.6
Glu	-11.2	-6.7	-0.9	5.3	-2.0	11.1
Ser	-7.2	-5.5	-1.9	0.0	-5.2	4.4
Gly	-8.6	-7.1	-5.5	-1.6	-7.8	4.3
Gly + Ser	-7.9	-6.3	-3.7	-0.8	-6.6	4.3
Ala	-9.8	-2.8	-3.6	1.7	-0.9	9.1
Pro	-8.7	-6.0	-4.2	2.6	-3.7	7.5
Tyr	-7.3	-2.6	1.4	2.1	-4.1	8.5
Vaccinated						
Asp	11.8	5.2	-4.7	-3.9	9.0	8.7
Glu	15.3	6.0	-2.3	-4.1	4.7	10.8
Ser	16.0	7.5	2.2	3.0	5.0	9.0
Gly	12.6	6.0	-2.2	-0.1	5.8	9.8
Gly + Ser	14.4	6.8	0.1	1.5	5.4	9.4
Ala	15.1	3.1	-3.8	-3.0	3.0	8.2
Pro	15.3	4.8	-3.6	2.3	4.0	12.3
Tyr	27.0	17.6	3.2	3.0	5.4	10.8

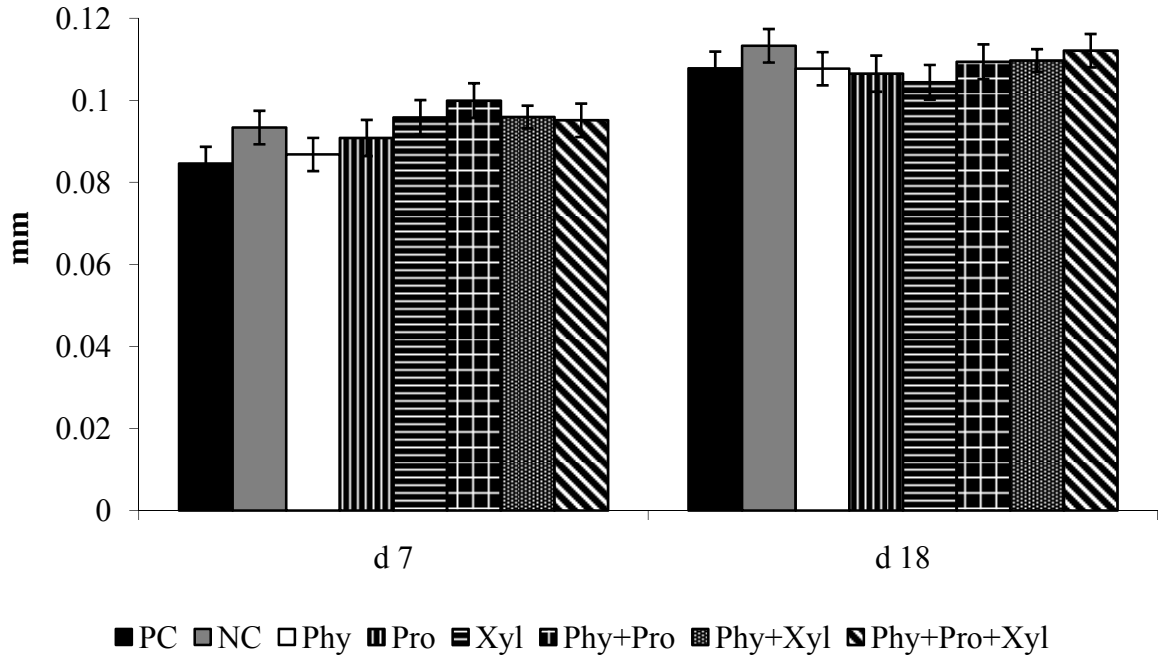
Calculated values were derived using the NC and NC supplemented with phytase, xylanase, and phytase plus xylanase treatments only. The non-digestible fraction was derived from total dietary AA – apparent ileal digestible AA, and the percent improvement was calculated.

Figure 2.1 Interaction of dietary treatment and Coccivac B^{TM} on duodenum crypt depth in broilers



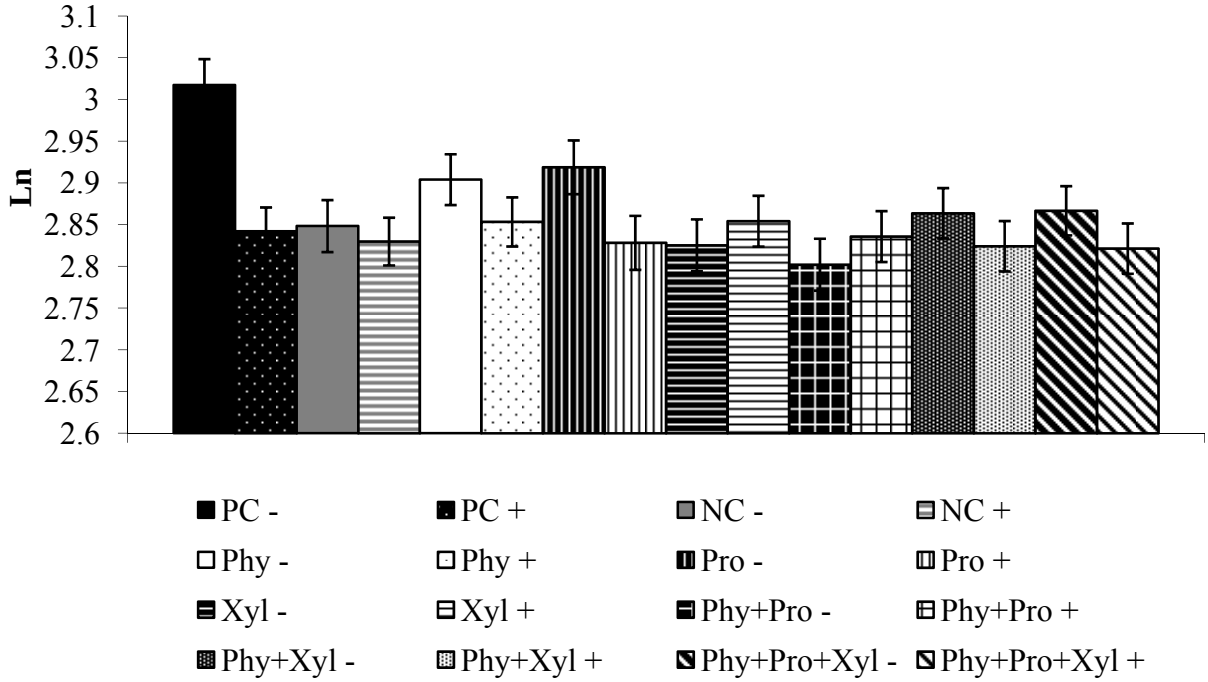
The results are the mean of 9 replicate pens/diet/vaccination (P = 0.0057). Measurements were taken from 4 villi from 3 tissue sections (n = 12). Vaccinated birds (designated by "+") were exposed to Coccivac B™ on day of hatch.

Figure 2.2 Interaction of dietary treatment and day on duodenum crypt depth in broilers



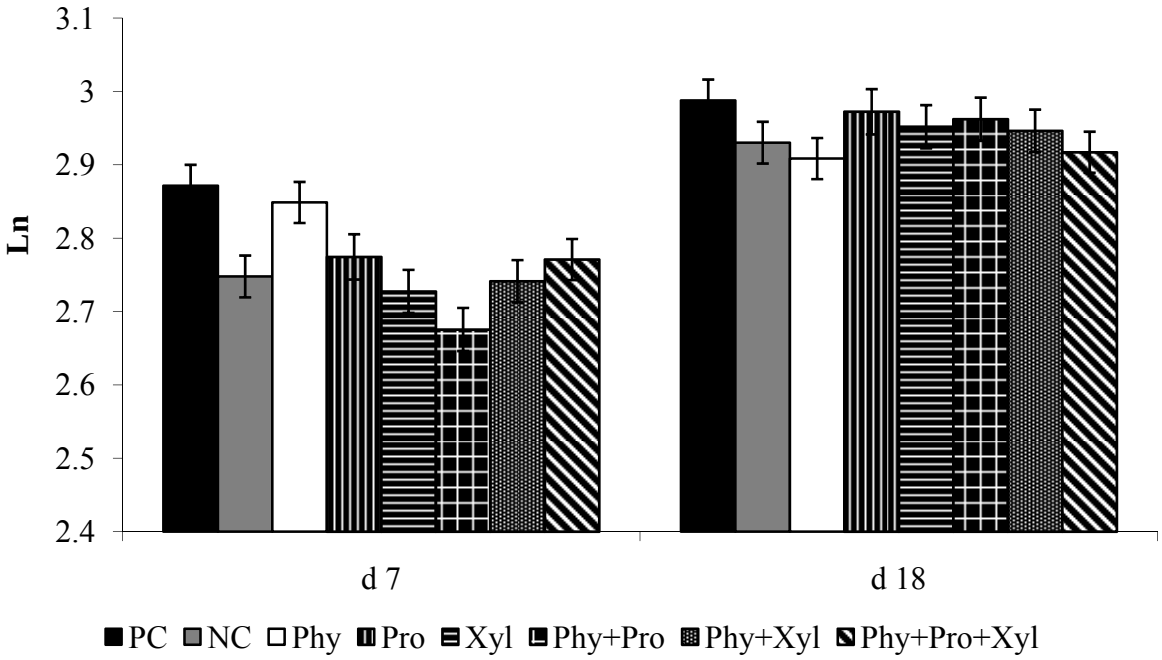
The results are the mean of 18 replicate pens/diet/day (P = 0.0528). Measurements were taken from 4 villi from 3 tissue sections (n = 12).

Figure 2.3 Interaction of dietary treatment and Coccivac B^{TM} on duodenum villus height to crypt depth ratio (VCR) in broilers



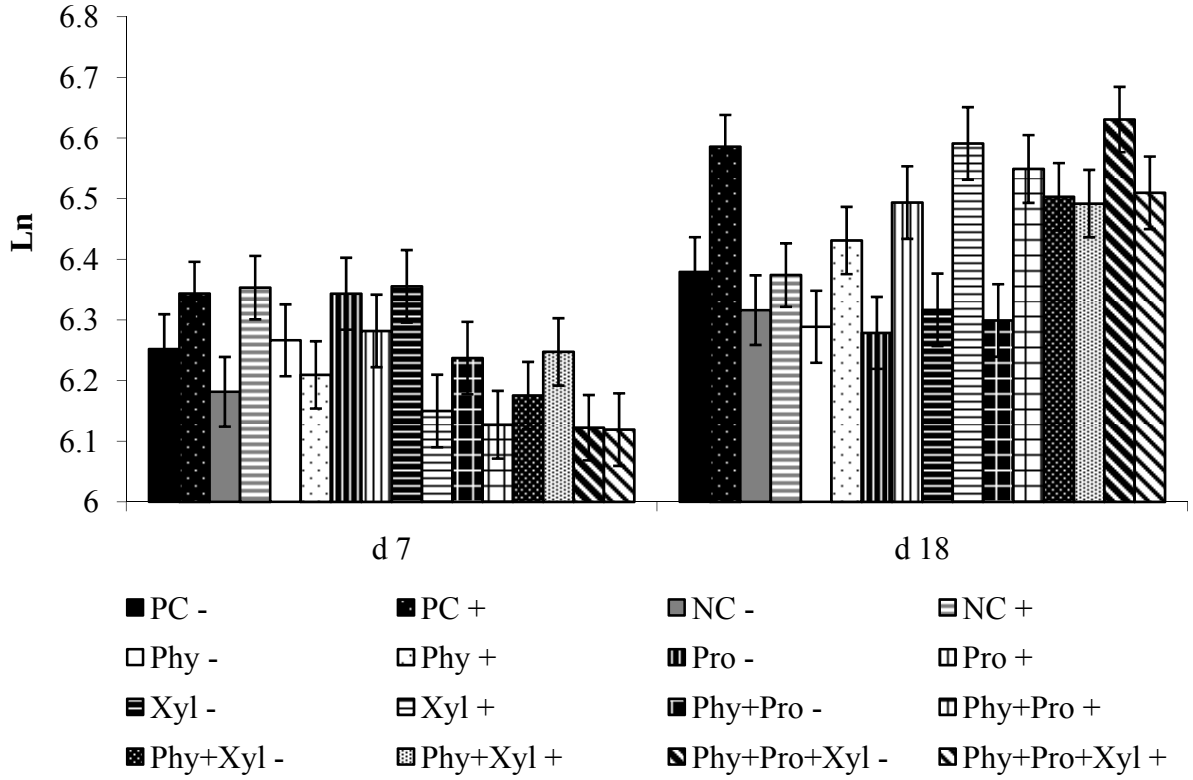
The results are the mean of 9 replicate pens/diet/vaccination (P = 0.0228). Measurements were taken from 4 villi from 3 tissue sections (n = 12). Villus height to crypt depth ratio was calculated and normalized using the natural log. Vaccinated birds (designated by "+") were exposed to Coccivac B™ on day of hatch.

Figure 2.4 Interaction of dietary treatment and day on duodenum villus height to crypt depth ratio (VCR) in broilers



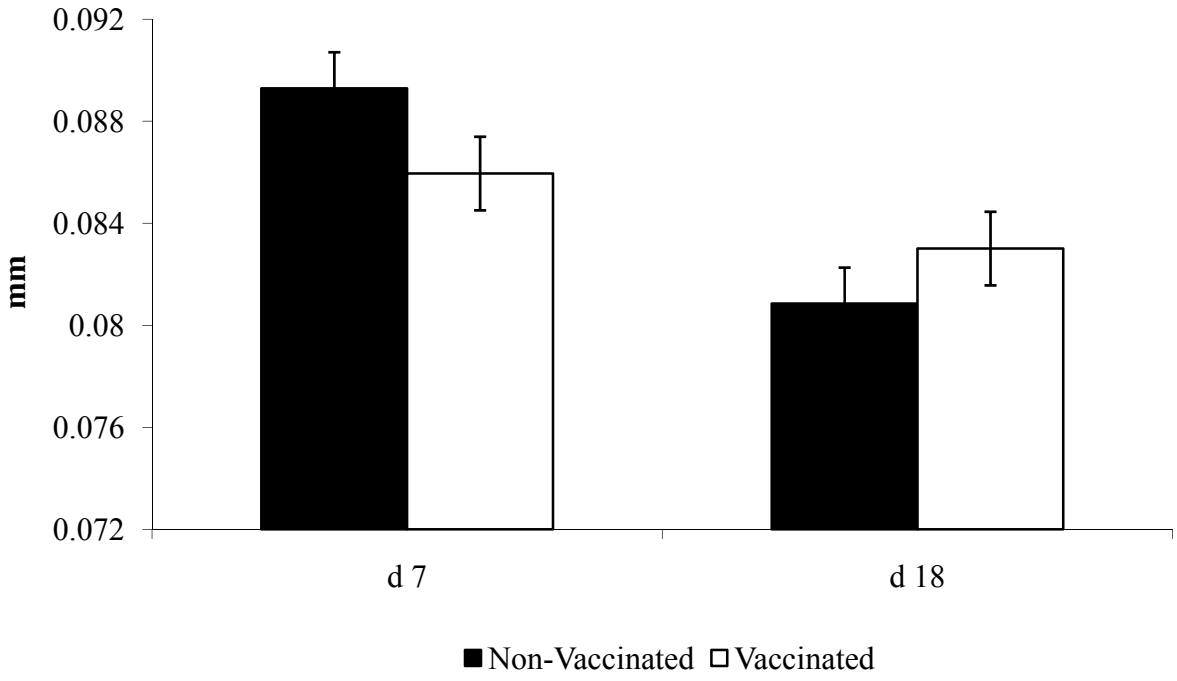
The results are the mean of 18 replicate pens/diet/day (P = 0.0040). Measurements were taken from 4 villi from 3 tissue sections (n = 12). Villus height to crypt depth ratio was calculated and normalized using the natural log.

Figure 2.5 Interaction of dietary treatment, Coccivac B^{TM}, and day on duodenum goblet cell numbers in broilers



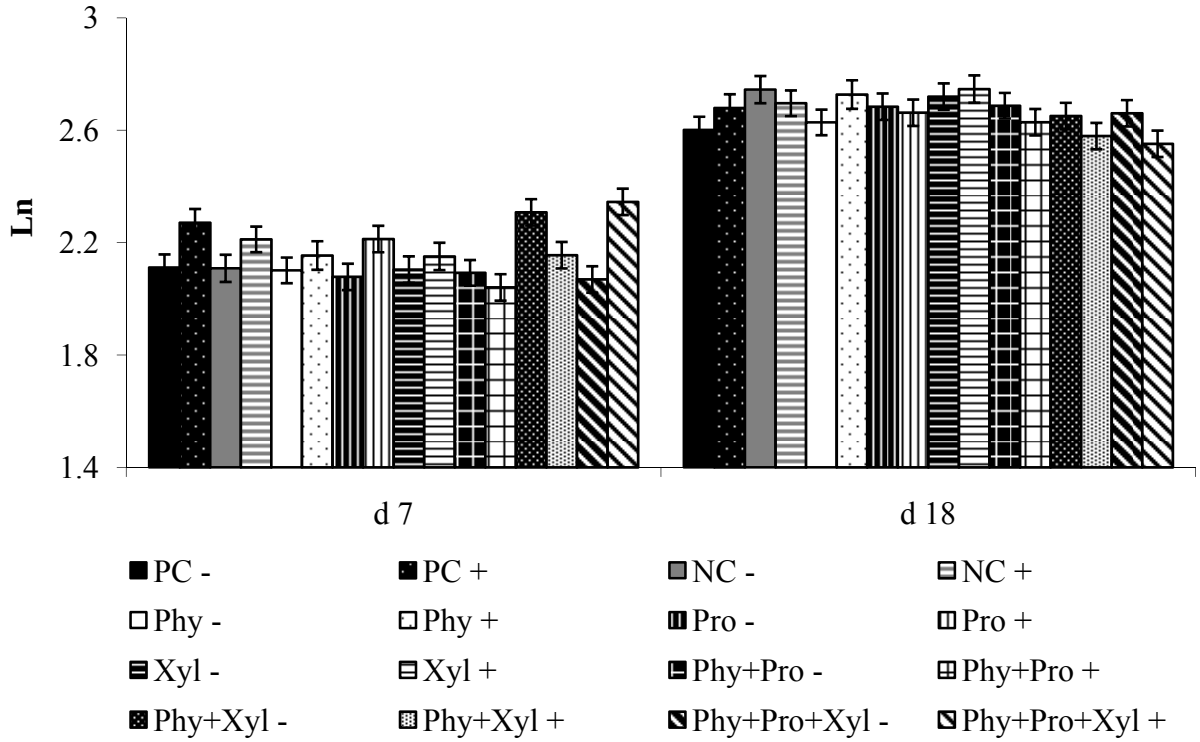
The results are the mean of 9 replicate pens/diet/vaccination/day (P = 0.0007). Cell counts and villus area were taken from 4 villi and 3 tissue sections (n = 12). Average cell number/average villus area was calculated and normalized using the natural log. Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

Figure 2.6 Interaction of Coccivac B^{TM} and day on jejunum crypt depth in broilers



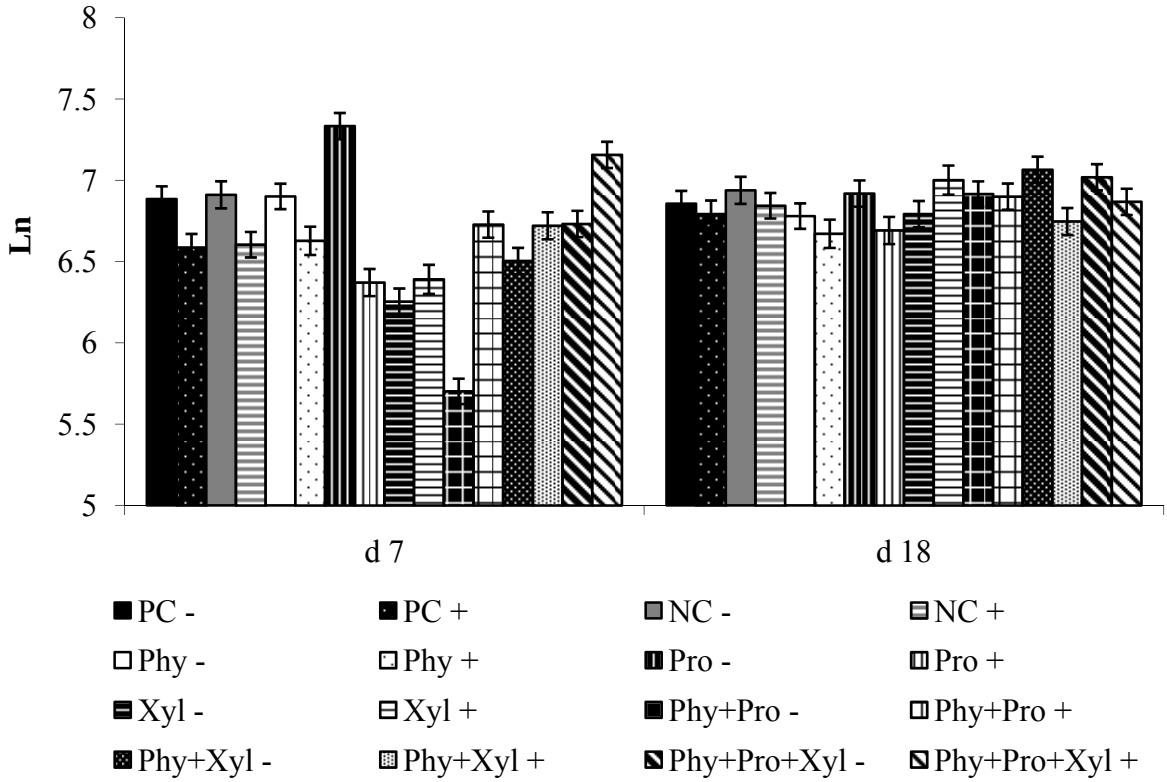
The results are the mean of 72 replicate pens/vaccination/day (P = 0.0464). Measurements were taken from 4 villi from 3 tissue sections (n = 12). Vaccinated birds were exposed to Coccivac B™ at day of hatch.

Figure 2.7 Interaction of dietary treatment, Coccivac B^{TM}, and day on jejunum villus height to crypt depth ratio (VCR) in broilers



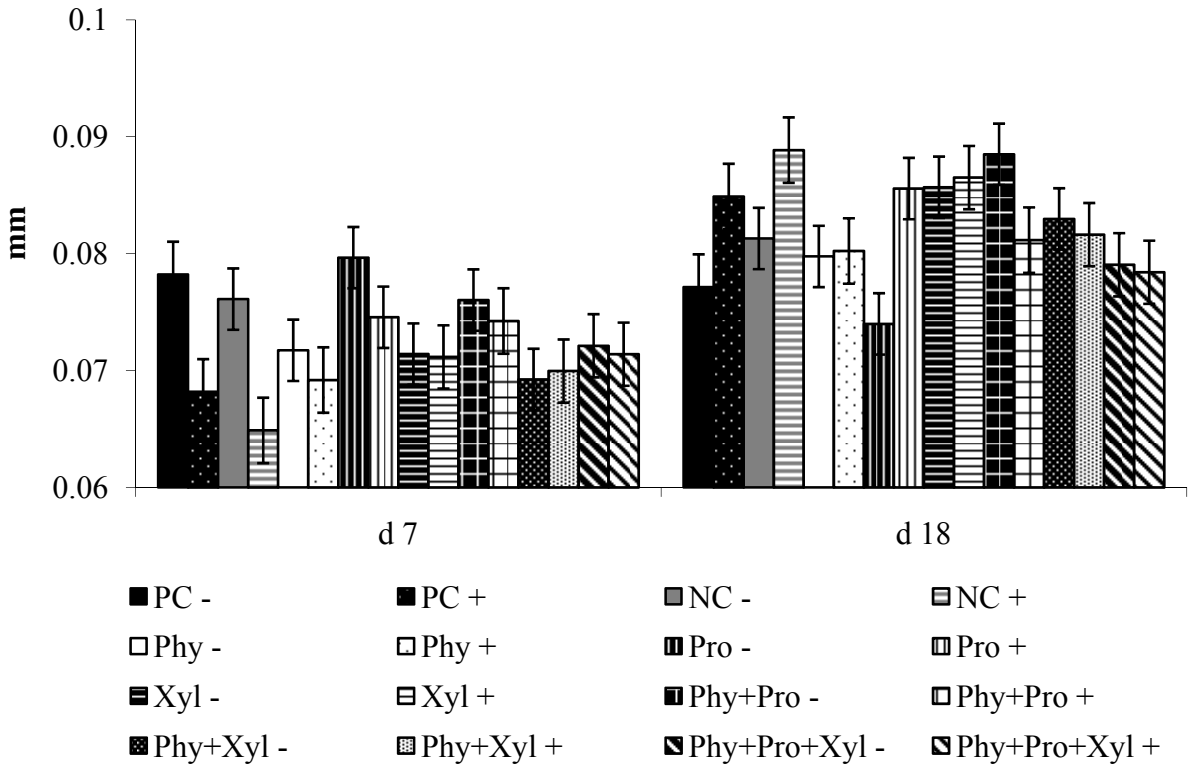
The results are the mean of 9 replicate pens/diet/vaccination/day (P = 0.0172). Measurements were taken from 4 villi from 3 tissue sections (n = 12). Villus height to crypt depth ratio was calculated and normalized using the natural log. Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

Figure 2.8 Interaction of dietary treatment, Coccivac B^{TM}, and day on jejunum goblet cell numbers in broilers



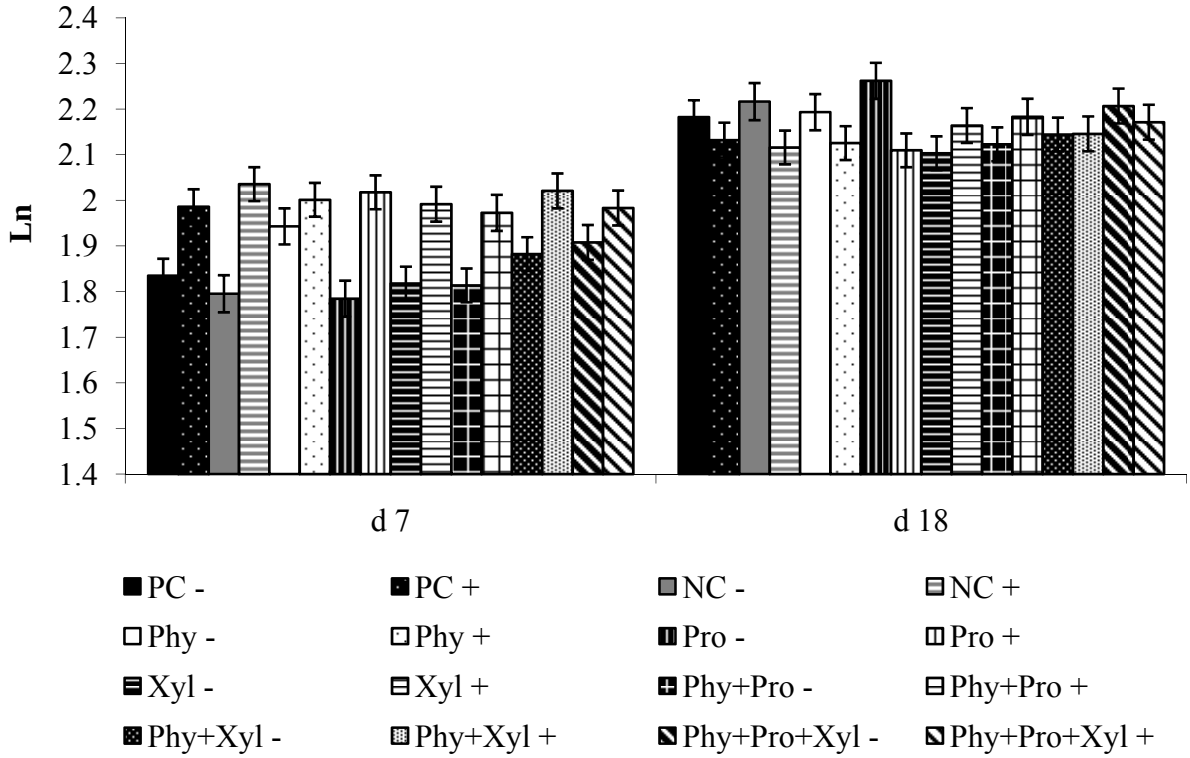
The results are the mean of 9 replicate pens/diet/vaccination/day ($P \leq 0.0001$). Cell counts and villus area were taken from 4 villi and 3 tissue sections (n = 12). Average cell number/average villus area was calculated and normalized using the natural log. Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

Figure 2.9 Interaction of dietary treatment, Coccivac B^{TM}, and day on ileum crypt depth in broilers



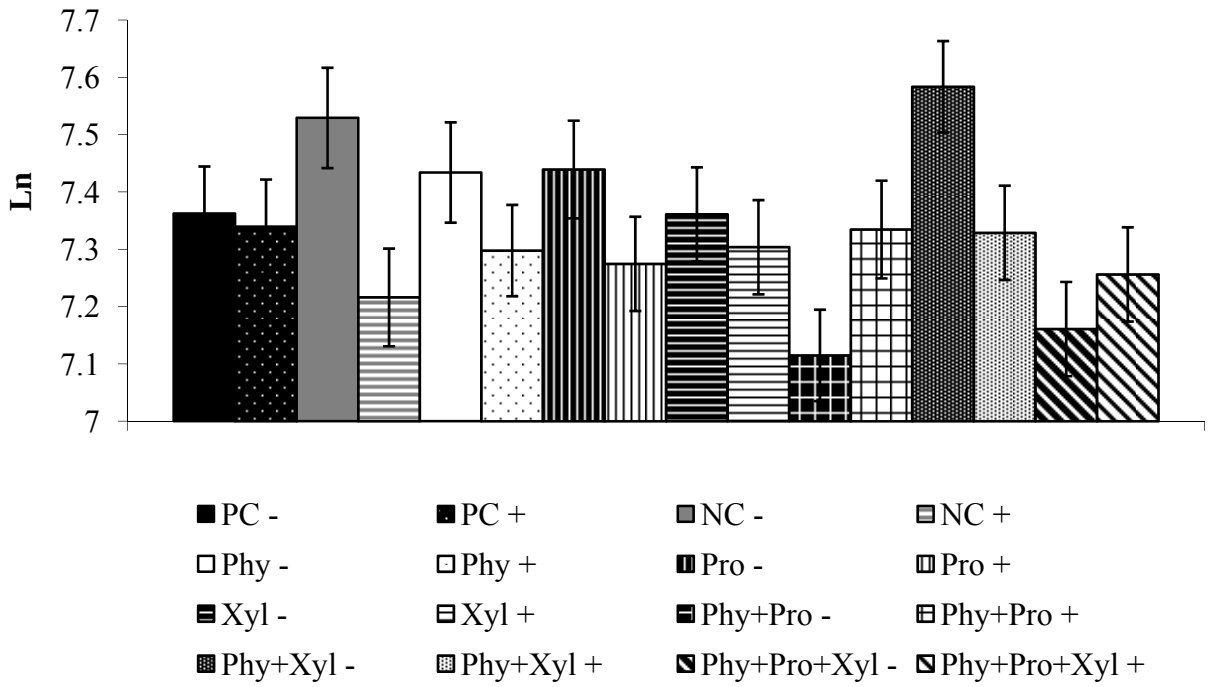
The results are the mean of 9 replicate pens/diet/vaccination/day (P = 0.0021). Measurements were taken from 4 villi from 3 tissue sections (n = 12). Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

Figure 2.10 Interaction of dietary treatment, Coccivac B^{TM}, and day on ileum villus height to crypt depth ratio (VCR) in broilers



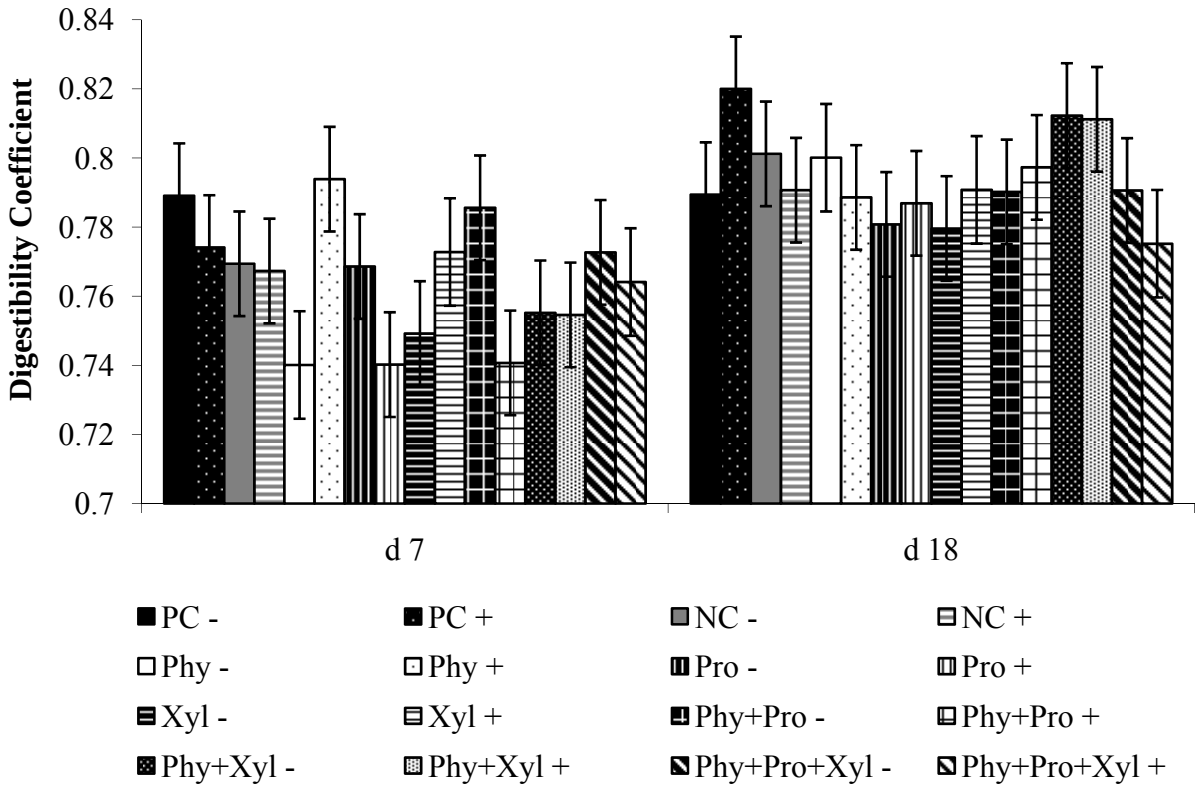
The results are the mean of 9 replicate pens/diet/vaccination/day (P = 0.0410). Measurements were taken from 4 villi from 3 tissue sections (n = 12). Villus height to crypt depth ratio was calculated and normalized using the natural log. Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

Figure 2.11 Interaction of dietary treatment and Coccivac B^{TM} on ileal goblet cell numbers in broilers



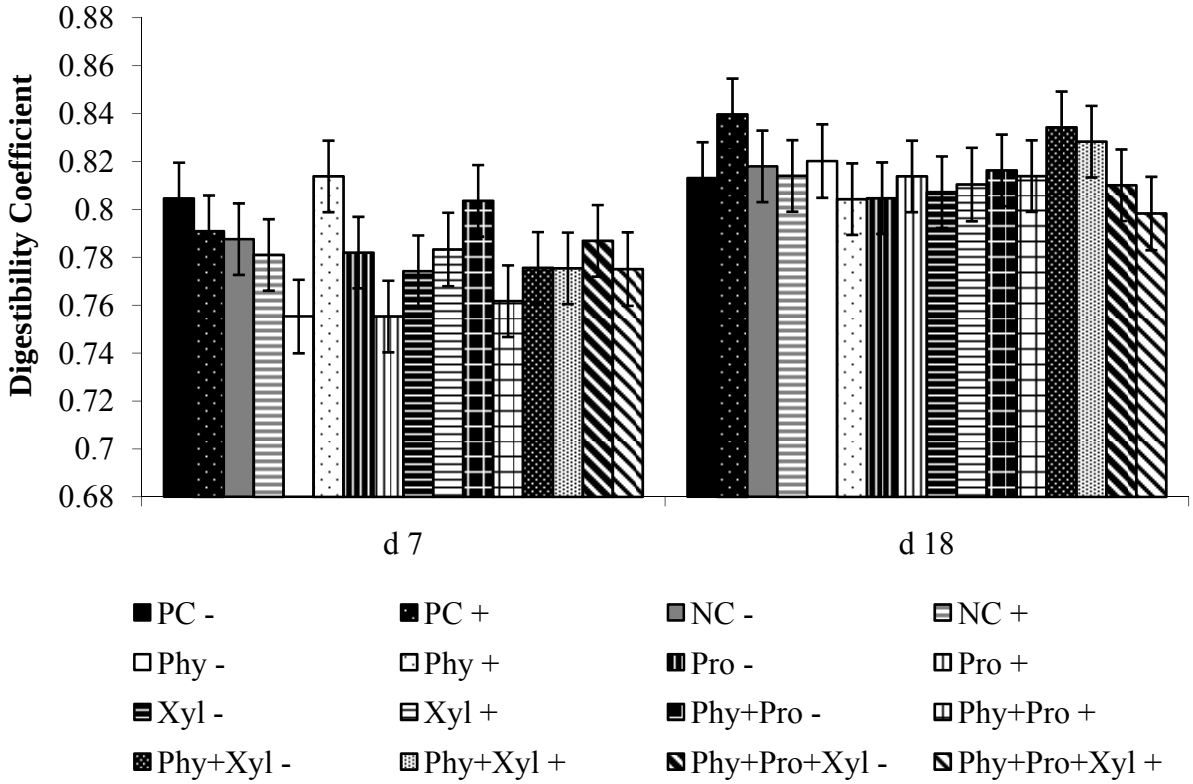
The results are the mean of 9 replicate pens/diet/vaccination (P = 0.0301). Cell counts and villus area were taken from 4 villi and 3 tissue sections (n = 12). Average cell number/average villus area was calculated and normalized using the natural log. Vaccinated birds (designated by "+") were exposed to Coccivac B™ on day of hatch.

Figure 2.12 Interaction of dietary treatment, Coccivac B^{TM}, and day on apparent ileal proline digestibility in broilers



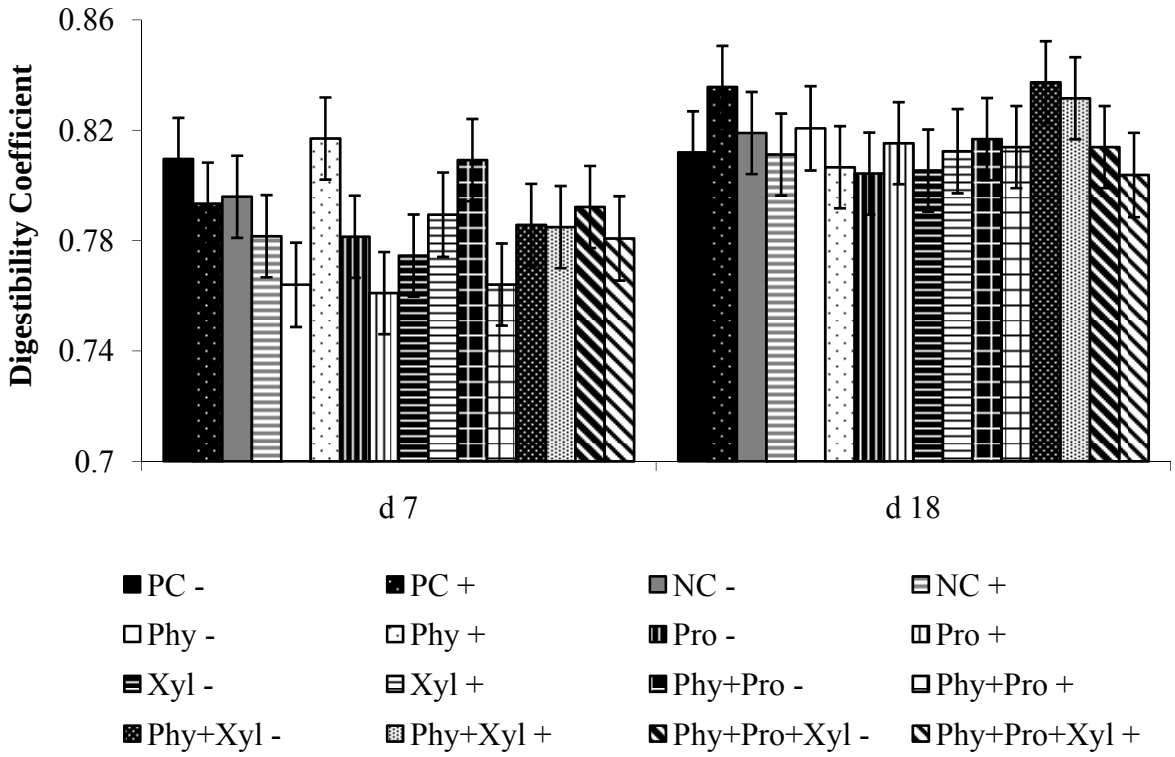
The results are the mean of 9 replicate pens/diet/vaccination/day (P = 0.0355). Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

Figure 2.13 Interaction of dietary treatment, Coccivac B^{TM}, and day on apparent ileal leucine digestibility in broilers



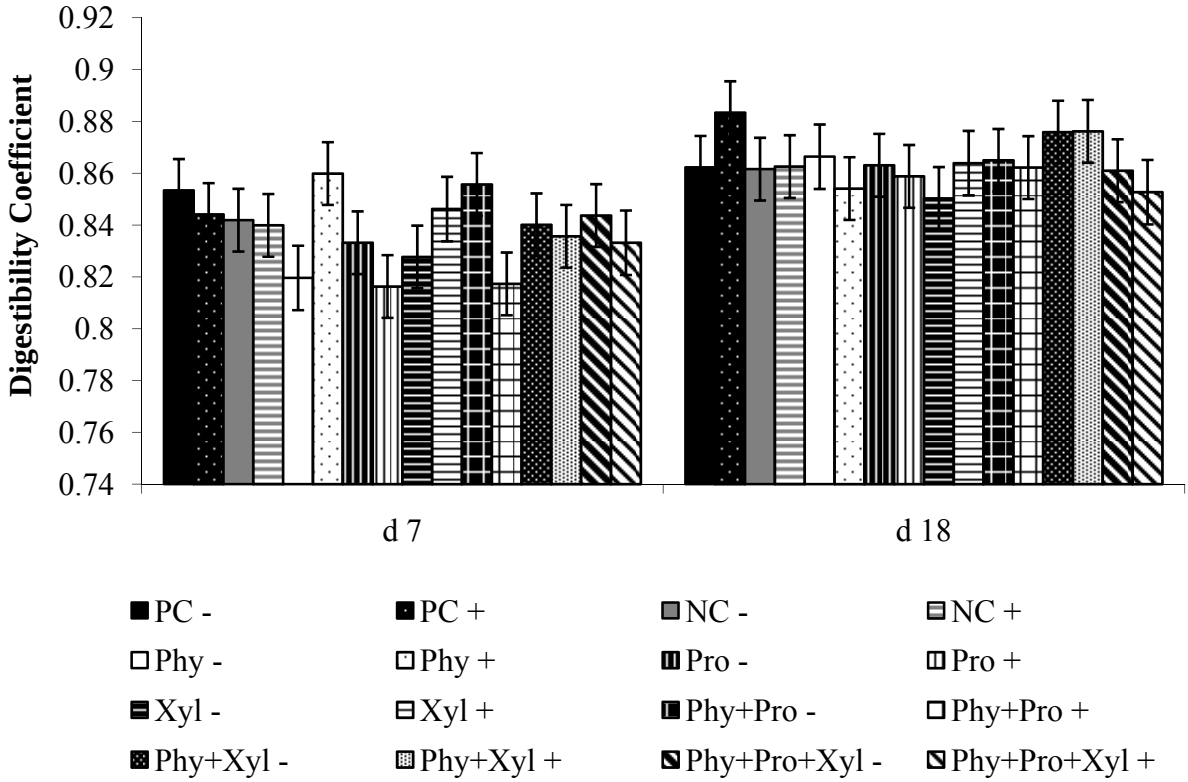
The results are the mean of 9 replicate pens/diet/vaccination/day (P = 0.0293). Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

Figure 2.14 Interaction of dietary treatment, Coccivac B™, and day on apparent ileal phenylalanine digestibility in broilers



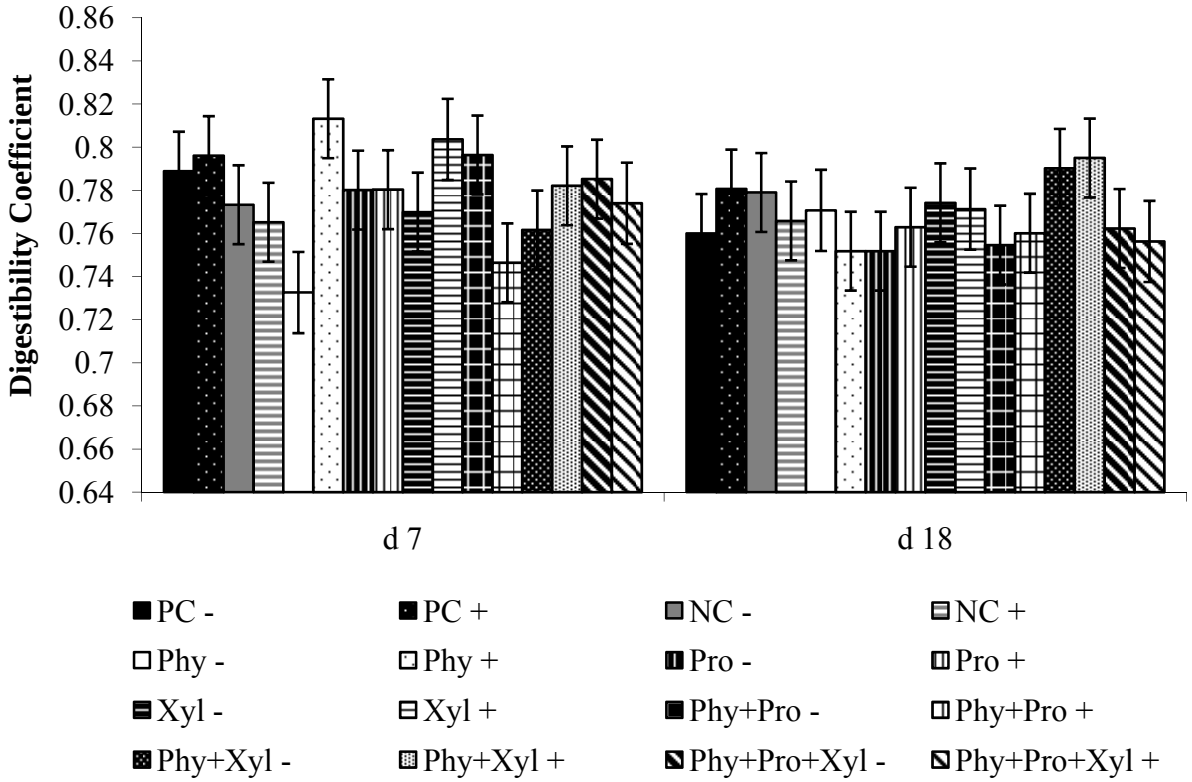
The results are the mean of 9 replicate pens/diet/vaccination/day (P = 0.0537). Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

Figure 2.15 Effects of dietary treatment, Coccivac B^{TM}, and day on apparent ileal glutamine digestibility in broilers



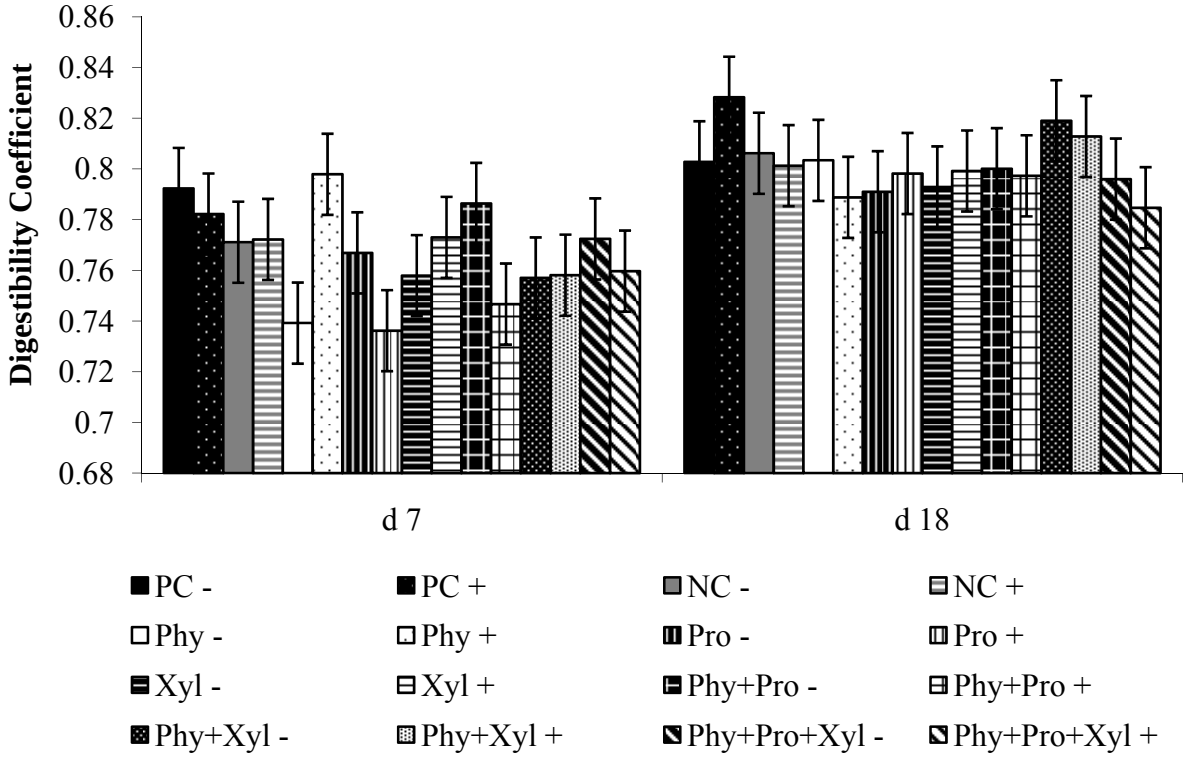
The results are the mean of 9 replicate pens/diet/vaccination/day (P = 0.1088). Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

Figure 2.16 Effects of dietary treatment, Coccivac B^{TM}, and day on apparent ileal arginine digestibility in broilers



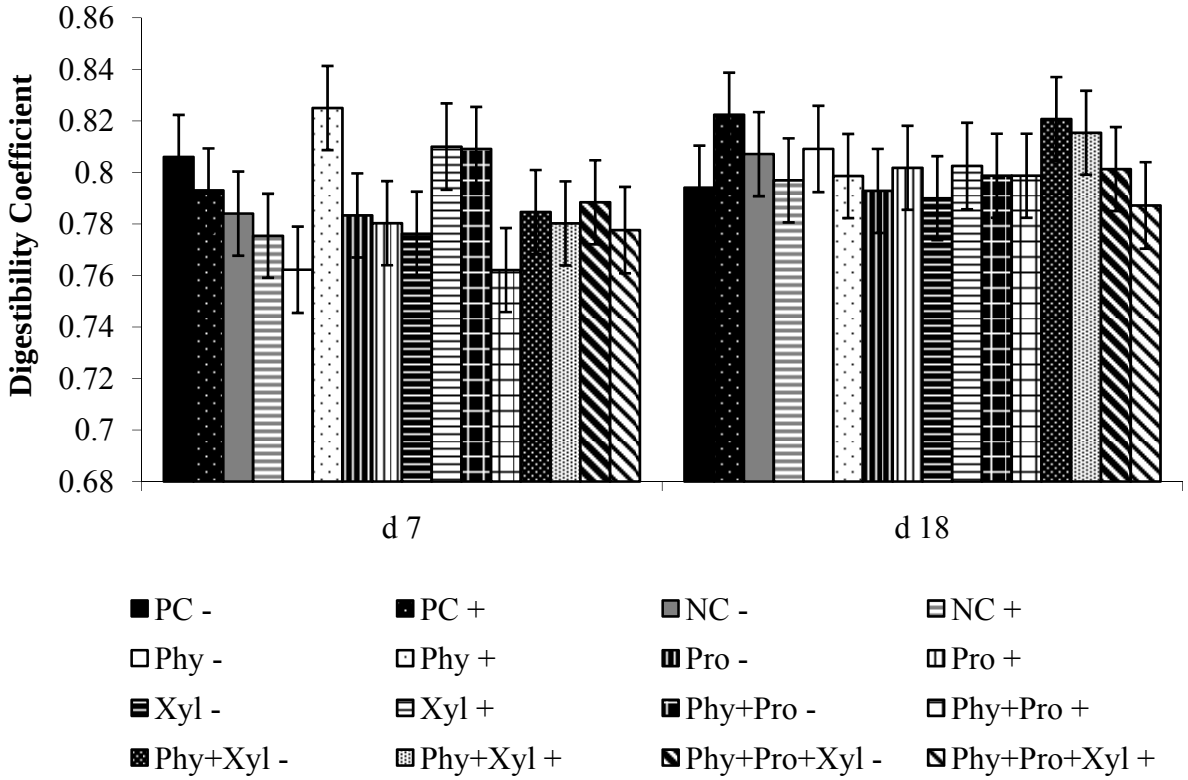
The results are the mean of 9 replicate pens/diet/vaccination/day (P = 0.0897). Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

Figure 2.17 Effect of dietary treatment, Coccivac B^{TM}, and day on apparent ileal alanine digestibility in broilers



The results are the mean of 9 replicate pens/diet/vaccination/day (P = 0.0905). Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

Figure 2.18 Effects of dietary treatment, Coccivac B^{TM}, and day on apparent ileal tyrosine digestibility in broilers



The results are the mean of 9 replicate pens/diet/vaccination/day (P = 0.1015). Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

CHAPTER III

Effect of diet and phytase on performance of broilers vaccinated with a live coccidia oocyst vaccine

ABSTRACT

An experiment was conducted to evaluate the effects of diet and phytase on performance and bone ash of broilers administered a live coccidia oocyst vaccine. One-day-old, Cobb 500, male chicks were obtained from a commercial hatchery, and half were vaccinated with Coccivac B™. All chicks were weighed and placed in battery brooders according to seven dietary treatments (8 replicate pens of 9 chicks). Dietary treatments were positive control (PC; 3050 ME, 22.0% CP, 0.95% Ca, and 0.45% aP), negative control 1 (NC1; 3005 ME, 21.6% CP, 0.84% Ca, and 0.32% aP), NC1 + phytase A (NC1+PHYA), negative control 2 (NC2; 3050 ME, 22.0% CP 0.84% Ca and 0.32% aP), NC2 + phytase B (NC2+PHYB), negative control 3 (NC3; 3010 ME, 21.7% CP, 0.84% Ca, and 0.33% aP), and NC3 + phytase C (NC3+PHYC). Phytase supplementation to the NC diets increased ($P \leq 0.0001$) feed intake (FI) from d 7 to 14 and cumulatively (d 0 to 21), and improved ($P = 0.0018$) body weight gain (BWG) from d 0 to 7, d 7 to 14 and cumulatively, and this resulted in a diet by day interaction ($P \leq 0.05$) for FI and BWG. Diet and vaccination affected feed conversion (FC) differently depending on the day, and this resulted in a three-way interaction ($P = 0.0236$). Coccidia vaccination increased ($P = 0.0143$) FI between d 14 and 21, but had no effect on FI prior to that, which resulted in vaccination by day interactions. Vaccination increased ($P = 0.0137$) FI and improved ($P = 0.0025$) BWG cumulatively (d 0 to 21). Diet and vaccination interactions ($P = 0.0516$) were noted in tibia ash at d 21, but generally phytase improved tibia ash in both non-vaccinated and vaccinated broilers. Mortality was higher ($P = 0.0103$) in non-vaccinated broilers compared to vaccinated broilers from d 0 to 21. The

results indicate that phytase supplementation improved FI and BWG in broilers fed diets with reduced ME, crude protein (CP), Ca, and available P (aP). Additionally, there was no negative impact of coccidia vaccination on broiler performance when diets were supplemented with phytase.

Key words: phytase, coccidia vaccination, broiler, tibia ash

INTRODUCTION

Coccidiosis is a common and costly disease in the poultry industry caused by a protozoan parasite of the *Eimeria spp*. Estimates suggest chemotherapeutic treatment and production losses associated with coccidiosis costs poultry producers in the United States approximately $700 million dollars annually (Lillehoj et al., 2007). Broilers become infected with coccidia upon ingestion of oocysts in contaminated litter or feed. Once in the intestinal tract, the parasite excysts and undergoes a complex, multistage lifecycle within the intestinal lumen and enterocytes. Exit of the parasite from the enterocytes results in lesions and a cell mediated inflammatory immune response (Lillehoj and Trout, 1996). Pathologies of coccidiosis result in reduced feed intake (FI) and body weight gain (BWG), poor feed conversion (FC), reduced nutrient digestibility and absorption, morbidity, and in severe cases mortality (Williams, 2005).

Historically, dietary anti-coccidials have been effectively utilized to prevent coccidiosis in commercial poultry flocks. Currently, many researchers are focusing on non-antibiotic feed ingredients to treat pathologies associated with coccidiosis (Oviedo-Rondon et al., 2006; Persia et al., 2006; Parker et al., 2007). Watson et al. (2005) found phytase was effective at increasing growth in non-infected chicks and chicks orally gavaged with 400,000 *E. acervulina* oocysts. However, the magnitude of the response was greater in healthy chicks than infected chicks. In that experiment, birds were infected with known doses of a single *Eimeria spp*. Various factors

may exacerbate the impact of coccidiosis on the intestinal tract such as: immune status, dietary ingredients and the presence of anti-nutrients, number of oocysts, type of *Eimeria spp.* ingested, and the housing environment. Another alternative for control of coccidiosis is the use of live oocyst vaccines. Commercially available vaccines have been developed to induce a primary infection by the vaccine *Eimeria spp*. isolates. The primary infection induces an immune response and promotes resistance to secondary exposure from field isolates of the same *Eimeria spp*. Mild reductions in BWG and FC may be associated with the live vaccine (Danforth, 1998) due to the coccidia lifecycle, the associated immune response, and damage within the intestinal tract such as reductions in villi height and absorptive surface area. As a result, nutrient digestibility and absorption within the intestinal epithelium may be compromised. Coccidiosis vaccination and subsequent challenge resulted in reduced amino acid digestibility in 25-day-old broilers (Parker et al., 2007), and infection with sporulated *E. acervulina* oocysts resulted in significant reductions in metabolizable energy (ME) and amino acid digestibility for chicks fed a corn and soybean meal diet (Persia et al., 2006). Therefore, broilers exposed to *Eimeria spp*. may have an increased requirement for energy and amino acids, not only as a result of damage to intestinal integrity and poor nutrient digestibility and absorption, but also as a result of the enhanced immune response. Lysine use by the immune system during an inflammatory immune response may increase by almost 6-fold (Klasing, 2007), and FI is reduced due to activation of cytokines such as interleukin-1 (IL-1; Klasing et al., 1987). Therefore, meeting the energy and amino acid requirements of the bird, and ensuring nutrient availability for utilization by the immune system are of utmost importance during an intestinal disease, such as coccidiosis.

Dietary enzymes are commonly used to improve nutrient digestion and availability, which leads to improvements in nutrient absorption. Phytase, an enzyme commonly used in

commercial poultry feeding programs, was originally developed to degrade phytate in grains and improve P utilization in monogastrics (Simons et al., 1990). Phytate is an anti-nutrient known to bind cations and possibly proteins and starches (Selle and Ravindran, 2007) and increase endogenous losses of amino acids and minerals (Cowieson et al., 2004). Recent research suggests phytase may improve amino acid digestibility and apparent ME (AME; Cowieson et al., 2006) by releasing amino acids and starches bound to the phytate molecule or reducing endogenous losses and possibly the mucoprotein (Cowieson and Ravindran, 2007) or resistant starches entering the terminal ileum, thereby, reducing nutrient sources for pathogenic bacteria. Interactions between the anti-nutrient effects of phytate and the damaging effects of coccidiosis may exacerbate the reductions in performance associated with poor nutrient digestibility and subsequent absorption within the intestinal epithelium. The objective of this trial was to determine if various *E. coli* phytases, supplemented according to manufacturer recommendations, would improve broiler performance during an intestinal infection from a live coccidia oocyst vaccination.

MATERIALS AND METHODS

Animals and Husbandry

One-day old, male, Cobb 500 broilers (n=1008) were obtained from a commercial hatchery and transported to the poultry research farm at Virginia Tech. Upon arrival, half of the chicks were sprayed with a commercially available live coccidia oocyst vaccine[8] according to manufacturer's recommendations. Chicks were then randomly selected, weighed, and placed in Petersime battery brooders with respect to non-vaccinated and vaccinated groups. Non-vaccinated birds were placed in batteries separate from vaccinated birds in the same environmentally controlled room. Birds were maintained on constant light for the 21 d trial.

[8] Coccivac B™, Schering Plough, Kenilworth, NJ

Diet and Enzyme Treatments

All diets were fed in mash form and formulated on a corn/soy basis according to Cobb nutrient recommendations with the exception of energy (mcal/kg), crude protein (CP), Ca, and available P (aP; Table 3.1). Dietary treatments consisted of a positive control (PC; 3050 mcal/kg ME, 22.0% CP, 0.95% Ca, and 0.45% aP), negative control 1 (NC1; 3005 mcal/kg ME, 21.6% CP, 0.84% Ca, and 0.32% aP), NC1 + phytase A (NC1+PHYA), negative control 2 (NC2; 3050 mcal/kg ME, 22.0% CP, 0.84% Ca, and 0.32% aP), NC2 + phytase B (NC2+PHYB), negative control 3 (NC3; 3010 mcal/kg ME, 21.7% CP, 84% Ca, and 0.33% aP), and NC3 + phytase C (NC3+PHY C). Negative control diets were formulated to account for the expected nutrient improvements associated with the *E. coli* phytases supplemented (Table 3.2). Phytases were supplemented in the NC diets in place of corn at 500 U/kg according to manufacturer recommendations. Phytase activity in the experimental diets was analyzed by Syngenta Animal Nutrition[9]. Non-vaccinated and vaccinated birds were allowed *ad libitum* access to one of the seven dietary treatments from d 0 to 21. This resulted in a total of 14 dietary treatments with eight replicate pens and nine chicks/pen. All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Sample Collection

Birds were weighed by pen prior to placement (d 0), d 7, d 14, and d 21 to measure mean body weight (BW) and calculate mean BWG for each period and cumulatively (d 0 to 21). Feed intake and FC were measured for each period and cumulatively. Mortality and room temperature were recorded daily. Any birds removed for sampling or mortality were weighed, and FI and FC were adjusted according to the number of bird days. Left tibias were collected from 3 birds/pen (n=24 birds/diet/vaccination) for determination of bone ash on d 21. Birds sampled were

[9] Syngenta Animal Nutrition, Research Triangle Park, NC

stunned by exposure to CO_2 gas and euthanized by cervical dislocation. Tibias were stripped of adhering tissues, wrapped in cheese cloth, and dried overnight at 100°C. Fat was extracted from tibias using a Soxhlet apparatus and 100% ethyl ether according to modified methods from Watson et al. (2006). Fat-extracted tibias were then dried for 24 hours at 100°C and ashed in a muffle furnace for 24 hours at 600°C to determine bone ash.

Statistical Analysis

Performance data were subjected to ANOVA using the MIXED models for completely randomized design procedure of SAS[10]. Percent mortality data were arc sine transformed prior to analysis. Pen served as the experimental unit for BWG, FI, FC, percent mortality, and tibia ash. The statistical model included diet, vaccination, the repeated factor day, and all two and three way interactions. The statistical model for cumulative performance included diet, vaccination, and diet by vaccination interactions. Mean differences were determined using Tukey's test. Statistical significance was accepted at $P \leq 0.05$.

RESULTS

Growth Performance

Analyzed dietary phytase activity was higher than formulated but within an acceptable range (Table 3.3). Dietary or vaccination treatments affected performance differently depending on the day, resulting in significant interactions. Differences in FI in response to diet were observed from d 7 to 14 and d 0 to 21 (Table 3.4). Phytase supplementation had no affect on FI from d 0 to 7 or d 14 to 21, but during d 7 to 14 phytase A and C supplementation improved ($P = 0.0001$) FI above NC1 and NC3, respectively, resulting in a significant ($P \leq 0.0001$) diet by day interaction. Vaccination had no affect on FI from d 0 to 7 or d 7 to 14, but during d 14 to 21 the vaccinated birds consumed more feed compared to the non-vaccinated birds, resulting in a

[10] SAS Institute, Cary, NC

significant (P = 0.0183) vaccination by day interaction (Table 3.4). Cumulatively, main effects showed FI was reduced (P = 0.0002) in the NC diets compared to the PC diet. Phytase supplementation improved FI to a level comparable to the PC diets, but no difference was noted relative to the NC diets (Table 3.4). Cumulatively, vaccinated birds consumed more (P = 0.0137) feed than non-vaccinated birds (1,202.8 vs. 1,182.5 g; Table 3.4). There were no other diet, vaccination, or day interactions in response to FI.

Different responses in BWG associated with dietary treatment and day resulted in a significant ($P \leq 0.0001$) two-way interaction (Table 3.5). Body weight gain was decreased in birds fed the NC diets without phytase supplementation from d 0 to 7 and d 7 to 14, and phytase supplementation to each NC diet increased (P = 0.0018) BWG comparable to the PC, during these periods. Supplementation of NC1 with phytase A increased BWG above NC1 from d 7 to 14. Phytase supplementation had no affect on BWG from d 14 to 21. Cumulatively, main dietary effects resulted in reduced (P = 0.0025) BWG in the NC diets without phytase supplementation compared to the PC (Table 3.5). Phytase supplementation to each NC diet improved BWG to a level comparable to the PC, but no difference was noted relative to the NC diets. Vaccination increased BWG from d 0 to 21 (Table 3.5). There were no other diet, vaccination, or day effects on BWG.

A three-way interaction (P = 0.0236) between diet, vaccination, and day (Figure 3.1) affected FC. Vaccinated birds fed the PC diet were less efficient than non-vaccinated birds fed the PC diet from d 0 to 7 and more efficient than the non-vaccinated birds from d 7 to 14 and d 14 to 21. From d 0 to 7, phytase supplementation to all NC diets resulted in improved FC of both non-vaccinated and vaccinated birds. Similar results were seen with NC1 from d 7 to 14 with both vaccinated and non-vaccinated birds fed NC1 supplemented with phytase A having

improved FC compared to NC1 without phytase. However, from d 14 to 21, non-vaccinated birds fed NC1 without phytase had better FC than non-vaccinated birds with phytase supplementation or vaccination with or without phytase supplementation. From d 7 to 14, there was little difference in FC between any treatments with birds fed NC2, but from d 14 to 21, both non-vaccinated and vaccinated birds fed NC2 supplemented with phytase B had more efficient FC than non-vaccinated birds without phytase. Vaccinated birds fed NC3 with phytase had improved FC compared to other NC3 treatments from d 7 to 14. In contrast, from d 14 to 21, the FC of both non-vaccinated and vaccinated birds fed NC3 with phytase was less efficient than non-vaccinated birds without dietary phytase. Vaccinated birds without phytase supplementation to NC3 had intermediate FC to these other groups. There were no diet or vaccination main effects on feed conversion. Cumulatively (d 0 to 21), non-vaccinated broilers had a higher ($P = 0.0103$) percent mortality compared to vaccinated broilers (Figure 3.2).

Bone Ash

Interaction ($P = 0.0516$) between diet and vaccination was observed for 21-day tibia ash (Table 3.6). Tibia ash was not different between non-vaccinated and vaccinated birds fed NC2 and NC3. However, in birds fed NC1, tibia ash was increased in vaccinated birds compared to non-vaccinated birds. Additionally, phytase supplementation resulted in tibia ash comparable to the PC in both non-vaccinated and vaccinated birds, except for non-vaccinated birds fed NC2 with phytase B supplementation, which still had decreased tibia ash. The main effects of diet suggest tibia ash was reduced ($P < 0.0001$) in the NC diets without phytase supplementation (Table 3.6). Phytase supplementation to each NC diet increased ($P = 0.0001$) tibia ash above the NC diets. Phytase A supplementation to NC1 improved tibia ash comparable to the PC. The main effect of vaccination did not significantly affect 21-day tibia ash (Table 3.6).

DISCUSSION

The present results are suggestive of complex responses to diet and vaccination that vary depending on the stage of coccidia infection (day), dietary nutrient inclusion, and phytase source. The three phytases used in this experiment were added to NC diets with adjustments in the nutrient matrices according to manufacturer recommendations for the expected benefit of the enzyme. However, the results would suggest further evaluation of the nutrient matrices for some of the enzymes may be necessary to establish recommended nutrient inclusion levels during intestinal disease. Intestinal pathology may alter nutrient requirements of broilers due to associated reductions in absorptive surface area, brush border enzyme activity, and nutrient absorption. Damage to the intestinal epithelium from coccidiosis may also alter nutrient requirements by inducing an inflammatory immune response (Yun et al., 2000; Dalloul and Lillehoj, 2005). Activation of the immune system results in metabolic changes such as a redistribution of divalent cations, increased energy expenditure, increased muscle protein catabolism, increased amino acid oxidation, increased gluconeogenesis from amino acids, and decreased fatty acid uptake (Klasing and Barnes, 1988). The poultry industry commonly utilizes dietary enzymes such as phytase to improve digestibility and absorption of cations, such as Ca, P, Zn, and certain amino acids, and improve energy availability (Wyatt et al., 2008).

In this experiment, phytase supplementation of NC diets, formulated to account for the enzyme associated improvements in nutrient availability, improved broiler performance from d 0 to 21 and bone ash as compared to diets without phytase, suggesting the various phytases were effective at liberating starches, amino acids, and cations from the phytate molecule and allowing them to be more available for utilization by the bird. Previous research has shown that inclusion of an *E. coli* phytase improved FI, BWG, and tibia ash in corn and soy diets formulated to

contain marginal levels of Ca, aP, and/or energy (Onyango et al., 2005; Olukosi et al., 2007). Interestingly, in this experiment, the effects of phytase supplementation on bird performance and bone ash were variable and differed depending on the day evaluated, resulting in significant three- and two-way interactions. For example, phytase supplementation initially (d 0 to 7 or d 7 to 14) resulted in improved FI and BWG, but later resulted in no difference in BWG or FI (d 14 to 21). Body weight gain was numerically lower in birds fed NC3 supplemented with phytase C compared to birds fed NC3 without phytase C supplementation from d 14 to 21. A nutrient such as energy or CP may have been limiting in NC3, which resulted in no measured improvement in BWG during that time. Analyzed dietary CP values in NC3 were approximately 1.1% (Table 3.7) lower than formulated, which may have resulted in numerically reduced BWG associated with phytase C supplementation from d 14 to 21. Fungal phytase supplementation was unable to alleviate the negative effects on performance associated with low protein diets in pigs (Sands et al., 2009). However, essential amino acid and phytase supplementation to low protein diets resulted in laying hen performance similar to that of birds fed control diets (Keshavarz and Austic, 2004). Phytase C is reportedly able to liberate approximately 0.28% protein from the phytate molecule (Table 3.2). However, based on the analyzed dietary protein levels, a required release of approximately 1.36% protein was needed to equal that of the PC. Phytase C may have been effective at liberating proteins and amino acids associated with the phytate molecule; however, the low CP levels may have attributed to an imbalance in essential amino acids or in the ME to CP ratio. Nutrient imbalances, regardless of intestinal disease status may result in reductions in BWG, decreases in mineral absorption, and increases in broiler mortality. Vaccinated birds had lower percent mortality than non-vaccinated birds. However, the total

percent mortality from days 0 to 21 was relatively low and there were no dietary treatment differences.

In young vaccinated broilers (d 0 to 7 and d 7 to 14), phytase A supplementation of NC1 diets resulted in birds with three and five points, respectively, better FC compared to vaccinated broilers fed NC1 without phytase supplementation. Phytase C supplementation to NC3 diets of vaccinated broilers resulted in birds with two (d 0 to 7) and three points (d 7 to 14) better FC compared to vaccinated birds fed NC3 without phytase supplementation. Phytase A and C were effective at liberating enough ME, CP, Ca, and aP for the chick to utilize, even during an intestinal response to coccidia, which resulted in improvements in FC in vaccinated birds and improvements in BWG and tibia ash comparable to the PC. However, these improvements in FC with phytase supplementation to NC1 or NC3 diets were not apparent from d 14 to 21. Interestingly, vaccinated birds fed NC2 diets supplemented with phytase B had 3 points better FC at d 7 to 14 and d 14 to 21, compared to vaccinated birds fed NC2 diets without phytase supplementation. The different responses in FC associated with diet, day, and enzyme supplementation may be due to the effect of coccidia vaccination in regards to the nutrient inclusions in the diet. Research evaluating dietary protein levels and coccidiosis interactions suggest diets containing lower CP may reduce oocyst sporulation by reducing trypsin secretion from the pancreas (Britton et al., 1964). While purely speculative, in this experiment, the low CP content of NC3 may have reduced the sporulation of the oocysts within the intestinal lumen and reduced the initial infection, allowing improvements in FC in young broilers (d 0 to 7 and 7 to 14). Reductions of nutrients in NC1 and NC3 without phytase supplementation resulted in the poorest FC in vaccinated broilers during the initial stages of the infection (d 0 to 7 and d 7 to 14), suggesting the combination of marginal nutrient content and reductions in nutrient utilization

from coccidia damage to the intestine may have reduced bird efficiency. Feed conversion was improved by phytase A supplementation in the NC1 diet in vaccinated broilers from d 0 to 7, phytase B supplementation in vaccinated birds fed NC2 from d 7 to 14 and 14 to 21, and phytase C supplementation in the NC3 diet in vaccinated broilers from d 7 to 14. Therefore, in general phytase was effective at liberating nutrients for utilization in vaccinated broilers to allow for improvements in FC.

The differences in diet and vaccination responses can be correlated to the results of tibia ash and performance. Tibia ash is the most sensitive indicator of P absorption in broilers. Calcium and aP reductions in the NC diets resulted in reductions in performance and bone ash. However, phytase supplementation of the NC diets in both the vaccinated and non-vaccinated broilers improved tibia ash. In a previous report, phytase supplementation increased tibia ash but only in chicks not infected with *E. acervulina* (Watson et al., 2005). The improvements in BWG and FI associated with the vaccination are different from an experiment conducted by Parker et al. (2007) and may be partially explained by the lifecycle of the *Eimeria* parasite and the battery cages utilized in this experiment. After ingestion, the parasite enters the host by penetration of intestinal epithelial cells causing serious damage to gut integrity (Yun et al., 2000). Immunity is stimulated, boosted, and maintained by multiple re-infections initiated by oocyst cycling in the litter (Williams, 2002). The normal Coccivac B™ reaction pattern in broilers will produce a peak in oocyst shedding between d 18 and 23 with a smaller peak earlier associated with the primary infection between days 5 and 9 (Schering-Plough, 2007). In this trial, the primary infection may have resulted in slight intestinal damage and an inflammatory immune response. However, these birds were housed in battery cages on raised wire floors. Lack of access to excreta would have prevented further oocyst cycling and promoted intestinal healing after the

initial infection. Vaccinated broilers may have benefited from compensatory FI following infection and initial recovery, especially during the last 7 days, allowing these birds to have heavier body weights at the conclusion of the trial. The birds ate more feed and gained more weight after the initial infection but had similar FC to non-vaccinated birds. Improvements in FC in both the vaccinated and non-vaccinated birds fed phytase compared to the NC diets suggest phytase may have beneficial effects in young broilers during an initial coccidia infection from live oocyst vaccines.

While early performance differences were seen, there were no measured improvements in performance due to dietary phytase supplementation from d 14 to 21. The digestive tract of young broilers is considered quite immature and must undergo extensive metabolic changes when moving from yolk absorption to an exogenous diet (Noy and Sklan, 1999). Pancreatic and mucosal enzymes and nutrient transporters must be available in sufficient quantities for digestion and absorption (Noy and Sklan, 1999). Pancreatic enzyme activity rapidly increased during the first 14 to 21 days after hatch in turkey poults (Krogdahl and Sell, 1989) and 4 to 21 days in chicks (Uni et al., 1995). Phytase supplementation in young broilers is more efficacious than older broilers (Bedford, 2000), and as the bird ages, digestive enzyme secretion increases. The activity of amylase, sucrase, and maltase was increased in broilers fed corn-soy diets supplemented with phytase (Liu et al., 2008). Therefore, the lack of beneficial effects of supplementing phytase from days 14 to 21 may be a result of improved endogenous digestive enzymes and nutrient digestibility within the bird.

In conclusion, diets formulated to be marginal in ME, CP, Ca, and aP may exacerbate the negative effects of a mild coccidia infection by reducing FC. Early in the infection, the negative effects of vaccination on FC were more prevalent in NC1, which contained the lowest level of

ME, and supplementation of phytase A was effective at improving FC in vaccinated broilers. Energy is necessary for all aspects of survival including activation of the immune system. Even mild coccidia infections result in an inflammatory immune response, which may cause reductions in FI, changes in nutrient allocation, increased energy requirement, and muscle protein break down. Coccidia invade the intestinal epithelium causing damage to gut integrity and lesions, which reduce nutrient digestibility and absorption. In most instances, coccidiosis results in reductions in bird performance and FC until immunity to secondary exposure is established. In this experiment, birds were housed on raised wire floors and after the initial infection, coccidia were cleared from the intestinal tract, and vaccinated broilers consumed more feed and gained more than non-vaccinated broilers. Phytase was effective at improving broiler performance in diets formulated to contain marginal nutrient levels, but these effects were more beneficial in younger broilers. Supplementation of broiler diets with an *E. coli* phytase during a live coccidia oocyst vaccination did not negatively affect broiler performance and may provide protection against intestinal damage and improve nutrient absorption. Further studies evaluating phytase and coccidia interactions on intestinal morphometric parameters and nutrient digestibility will aid in further characterizing the mechanisms surrounding phytase supplementation during a coccidia infection.

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Table 3.1 Composition and nutrient content of basal diets[1]

Ingredients	PC (%)	NC 1 (%)	NC 2 (%)	NC 3 (%)
Corn	60.79	61.87	59.88	61.60
Soybean Meal	31.74	30.11	31.24	30.36
Poultry Meal	3.00	3.00	3.00	3.00
Wheat Midds	0.00	2.00	2.00	2.00
Poultry Fat	1.43	0.50	1.49	0.54
Di-calcium Phosphate	1.20	0.50	0.50	0.55
Limestone	0.78	0.96	0.93	0.90
Salt	0.42	0.42	0.42	0.42
DL-Methionine	0.27	0.26	0.27	0.26
Lysine	0.15	0.18	0.16	0.17
Trace Mineral Premix[2]	0.10	0.10	0.10	0.10
Vitamin Premix[3]	0.10	0.10	0.10	0.10
Threonine	0.01	0.00	0.01	0.01
Calculated Composition				
Dry Matter	87.4	86.9	86.7	87.2
Protein	22.0	21.6	22.0	21.7
Fat	4.15	3.33	4.24	3.36
Calcium	0.95	0.84	0.84	0.84
Available Phosphorus	0.45	0.32	0.32	0.33
TSAA	0.97	0.95	0.97	0.95
Threonine	0.85	0.82	0.85	0.83
Lysine	1.30	1.29	1.30	1.29
Sodium	0.20	0.20	0.20	0.20
Nutrient Composition				
Energy (ME; kcal/kg)	3050	3005	3050	3010

[1] Diets were fed in mash form from days 0 to 21. Negative control diets were supplemented with three different *E. coli* phytases according to manufacturer recommendations.

[2] Supplied per kilogram mix: iron (Ferrous Sulfate), 40 g; manganese (Manganese Sulfate and Manganous Oxide), 120 g; zinc (Zinc Oxide), 210 g; cobalt (Cobalt Carbonate), 2.2 g; iodine (Calcium Iodate), 132 g.

[3] Supplied per kilogram mix: vitamin A, 8,818,400 IU; vitamin D_3 2,645,520 ICU; vitamin E, 22,046 IU; vitamin B_{12}, 26 mg; riboflavin, 8,818 mg; niacin, 88,184 mg; d-pantothenic acid, 22,046 mg; vitamin K, 2,646 mg; folic acid, 2,205 mg; vitamin B_6, 4,339 mg; thiamine, 3,732 mg; d-biotin, 220 mg.

Table 3.2 Phytase matrices utilized to formulate negative control diets

Nutrient	Phytase A	Phytase B	Phytase C
Protein, %	0.37	--	0.28
ME, kcal/kg	45	--	40
Calcium, %	0.11	0.11	0.11
Available Phosphorus, %	0.13	0.13	0.12
Cystine, %	0.02	--	0.02
Met + Cys (TSAA), %	0.02	--	0.02
Lysine, %	0.01	--	0.01
Threonine, %	0.03	--	0.02

Table 3.3 Recovery of phytase activity in experimental diets

Diet	Formulated Phytase Values	Determined Phytase Activity[1]
Positive Control	0	< 50
Negative Control 1 (NC1)	0	67
NC1 + Phytase A	500	661
Negative Control 2 (NC2)	0	57
NC2 + Phytase B	500	659
Negative Control 3 (NC3)	0	64
NC3 + Phytase C	500	770

[1]One unit of phytase activity (FTU) is defined as the quantity of enzyme that liberates 1 μmole of inorganic P per minute from sodium phytate at pH 5.5 and 37°C.

Table 3.4 Effect of diet, Coccivac[1] B^{TM}, and day on period and cumulative broiler feed intake

Dietary Treatment	d 0 - 7 (g)	d 7- 14 (g)	d 14 - 21 (g)	d 0 - 21 (g)
PC	162.4	405.2[a]	668.0	1234.1[a]
NC 1	156.6	374.7[b]	635.1	1168.8[b]
NC 1 + PHY A	160.0	394.8[a]	650.5	1205.4[ab]
NC 2	157.4	375.3[b]	641.5	1174.2[b]
NC 2 + PHY B	158.5	389.9[ab]	654.5	1202.9[ab]
NC 3	157.1	377.4[b]	635.5	1170.0[b]
NC 3 + PHY C	157.5	395.0[a]	640.8	1193.3[ab]
Pooled SEM	1.5047	2.8666	7.3566	10.673
Vaccination				
Non-vaccinated	158.3	385.3	638.9[b]	1182.5[b]
Vaccinated	158.7	389.6	654.2[a]	1202.8[a]
Pooled SEM	0.8043	1.7512	3.9325	5.7053

P-Value		
Diet	0.0001	0.0002
Vaccination	0.0143	0.0137
Diet*Vaccination	0.2816	0.2627
Day	0.0001	
Diet*Day	0.0001	
Vaccination*Day	0.0183	
Diet*Vaccination*Day	0.2403	

[a-b] Means within a column lacking a common superscript differ significantly ($P \leq 0.05$).
[1] Vaccinated birds were exposed to Coccivac B^{TM} at day of hatch.

Table 3.5 Effect of diet, Coccivac[1] B[TM], and day on period and cumulative broiler body weight gain

Dietary Treatment	d 0 - 7 (g)	d 7- 14 (g)	d 14 - 21 (g)	d 0 - 21 (g)
PC	140.2[a]	304.0[a]	424.7	861.1[a]
NC 1	129.3[b]	274.1[c]	407.1	805.7[b]
NC 1 + PHY A	135.5[ab]	299.5[a]	409.0	837.8[ab]
NC 2	132.3[b]	282.3[bc]	398.1	805.2[b]
NC 2 + PHY B	133.8[ab]	294.4[ab]	416.3	835.9[ab]
NC 3	130.6[b]	279.0[bc]	407.7	810.2[b]
NC 3 + PHY C	134.5[ab]	293.7[ab]	398.5	818.3[ab]
Pooled SEM	1.5052	3.1237	8.3527	10.757
Vaccination				
Non-vaccinated	133.5	287.5	404.4	816.6[b]
Vaccinated	133.9	291.6	413.2	833.1[a]
Pooled SEM	0.8045	1.6698	4.4650	5.7503
P-Value				
Diet			0.0018	0.0025
Vaccination			0.1075	0.0452
Diet*Vaccination			0.8543	0.8578
Day			0.0001	
Diet*Day			0.0001	
Vaccination*Day			0.1710	
Diet*Vaccination*Day			0.7920	

[a-c] Means within a column lacking a common superscript differ significantly ($P \leq 0.05$).
[1] Vaccinated birds were exposed to Coccivac B[TM] at day of hatch.

Table 3.6 Effect of diet and Coccivac[1] B^{TM} on 21-day-old broiler tibia ash

Diet*Vaccination	Non-Vaccinated (%)	Vaccinated (%)	
PC	52.30[a]	52.31[a]	
NC 1	48.69[f]	50.45[bcde]	
NC 1 + PHY A	51.88[ab]	51.43[ab]	
NC 2	49.41[ef]	49.57[def]	
NC 2 + PHY B	50.56[bcde]	51.05[abcd]	
NC 3	49.43[ef]	49.72[cdef]	
NC 3 + PHY C	51.33[ab]	51.23[abc]	
Pooled SEM			0.3341
Diet			
PC	52.31[a]		
NC 1	49.57[c]		
NC 1 + PHY A	51.66[ab]		
NC 2	49.49[c]		
NC 2 + PHY B	50.81[b]		
NC 3	49.57[c]		
NC 3 + PHY C	51.28[b]		
Pooled SEM	0.2362		
Vaccination			
Non-vaccinated	50.52		
Vaccinated	50.82		
Pooled SEM	0.1263		
P-Value			
Diet			0.0001
Vaccination			0.0887
Diet*Vaccination			0.0516

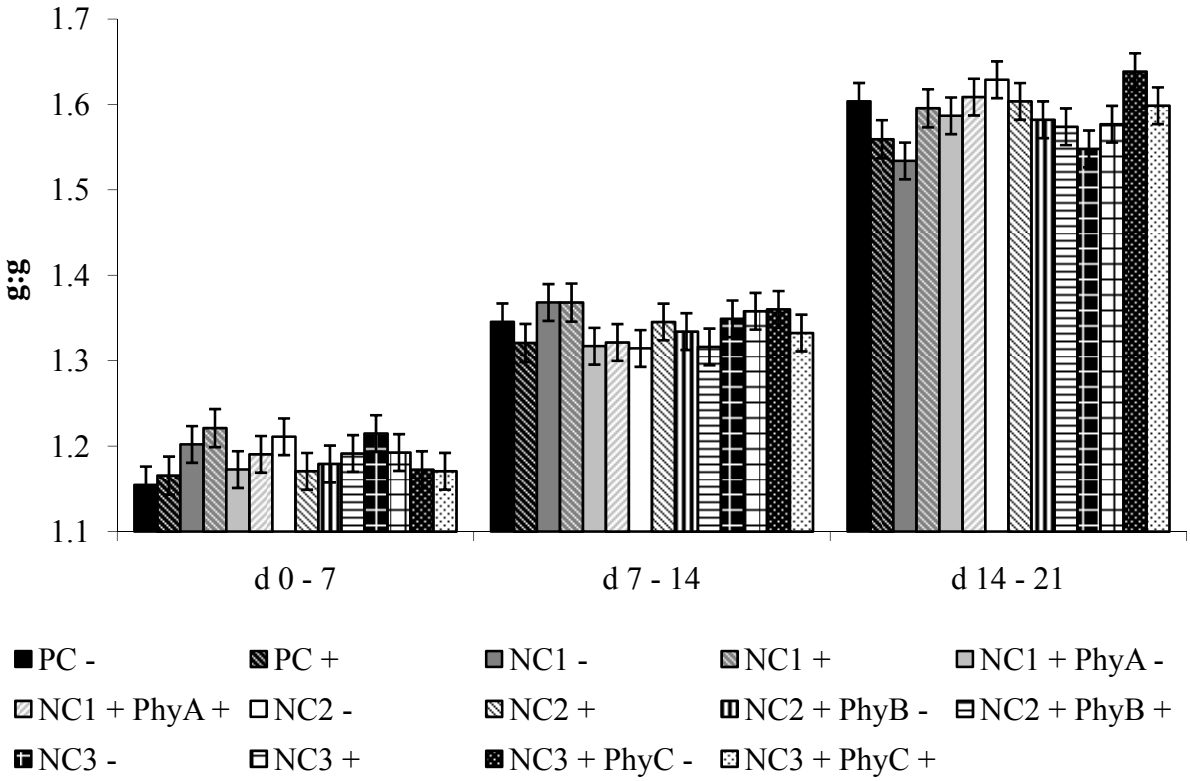
[a-f] Means within a row and column lacking a common superscript differ significantly ($P \leq 0.05$).
[1] Vaccinated birds were exposed to Coccivac B^{TM} at day of hatch.

Table 3.7 Analyzed composition of basal diets[1]

Ingredients	PC (%)	NC 1 (%)	NC 2 (%)	NC 3 (%)
Dry Matter	91.48	88.18	89.93	89.28
Protein	21.61	21.73	21.88	20.64
Fat	2.29	2.34	2.57	2.37
Calcium	0.91	0.86	0.80	0.87
Phosphorus	0.61	0.55	0.50	0.57

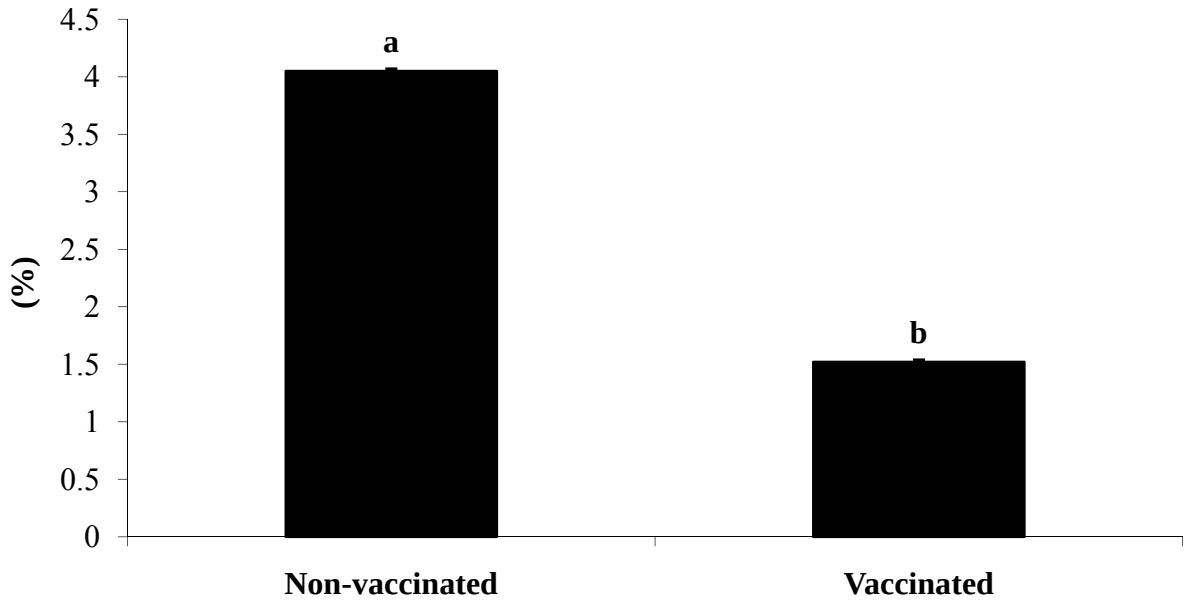
[1] Nutrient analyses were performed by A&L Eastern Laboratories, Inc., Richmond, VA.

Figure 3.1 Interaction of dietary treatment, Coccivac B^{TM}, and day on broiler feed conversion



The results are the mean of 8 replicate pens/ diet/ vaccination/ day (P = 0.0236). Vaccinated birds (designated by "+") were exposed to Coccivac B™ at day of hatch.

Figure 3.2 Effect of Coccivac B^{TM} on broiler mortality from days 0 to 21



[a-b] Data lacking a common superscript differ significantly (P = 0.0103). The results are the mean of 56 replicate pens/ vaccination. Vaccinated birds were exposed to Coccivac B™ at day of hatch.

CHAPTER IV

Effects of phytase on broiler performance, bone ash, small intestinal morphology, and apparent ileal amino acid digestibility of broilers exposed to a live coccidia oocyst vaccine

ABSTRACT

An experiment was conducted to evaluate the effects of phytase supplementation on broiler performance, bone ash, and gut morphology when administered a live coccidia oocyst vaccine. One-day-old, Cobb 500 male broilers were obtained from a commercial hatchery, and half were sprayed with Coccivac B^{TM}. Chicks were weighed and placed in floor pens on clean pine shavings according to five dietary treatments (7 replicate pens/diet/treatment). Dietary treatments were positive control (PC; formulated to meet or exceed Cobb nutrient requirements), PC + 1000 FTU phytase (PC+1000), negative control (NC; reduced Ca and aP), NC + 1000 FTU phytase (NC+1000), and NC + 5000 FTU phytase (NC+5000). Negative control diets were formulated to meet or exceed Cobb nutrient requirements with the exception of Ca and available P (aP), which were reduced 0.13% in the starter, grower, and finisher phase. Mortality was low throughout the starter and grower phases and not related to treatment. Mortality associated with a natural necrotic enteritis (NE) outbreak was increased ($P \leq 0.0001$) in the PC compared to all other dietary treatments, and increased ($P \leq 0.0001$) in the non-vaccinated birds compared to the vaccinated birds from d 31 to 41. Phytase supplementation generally improved ($P \leq 0.05$) feed intake (FI), body weight gain (BWG), feed conversion (FC), and tibia ash from d 0 to 30, and apparent ileal amino acid digestibility (IAAD) at d 18. Coccidia vaccination reduced ($P < 0.05$) FI, BWG, and tibia ash, negatively ($P < 0.05$) affected FC from d 0 to 30, and reduced ($P \leq 0.05$) apparent IAAD at d 18. Birds fed the PC diet had reduced ($P \leq 0.05$) performance compared to all other dietary treatments from d 31 to 41 and cumulatively (d 0 to 41). Tibia ash was

improved (P = 0.0288) in NC + 5000 compared to the NC diet at d 41. There were no differences in apparent IAAD associated with diet at d 41. Vaccination improved ($P \leq 0.05$) apparent IAAD but did not affect tibia ash at d 41. Crypt depth was affected ($P \leq 0.05$) by diet and vaccination interactions in the duodenum and ileum. Vaccination increased ($P < 0.05$) crypt depth and reduced villus height to crypt depth ratio (VCR) in all intestinal sections measured. These data indicate vaccination using live coccidia oocysts reduced apparent IAAD and altered gut integrity in broilers raised in floor pens, which may lead to associated reductions in broiler performance. Phytase supplementation improved performance, bone ash, and apparent IAAD. Necrotic enteritis substantially changed performance data, and phytase supplementation reduced NE associated mortality in the PC diets.

Keywords: phytase, broiler, coccidia, vaccination, necrotic enteritis

INTRODUCTION

Previous experiments in our laboratory demonstrated beneficial effects of supplemental phytase on apparent ileal amino acid digestibility (IAAD) in young broilers exposed to a live coccidia oocyst vaccination. Although the data were not significant at the $P \leq 0.05$ level, phytase was able to reduce the percentage of non-digestible amino acids by approximately 12 to 27% in 7-day-old broilers vaccinated with a commercial coccidia vaccine. No improvements in the non-digestible amino acid fractions were apparent with phytase supplementation in non-vaccinated broilers. In the same experiment, phytase supplementation in vaccinated broilers significantly improved apparent proline, leucine, and phenylalanine digestibility compared to the non-vaccinated broilers. Proline and leucine are large components of endogenous secretions in the ileum (Miner-Williams et al., 2009). Phytase may have a sparing effect on mucin production in the distal small intestine by reducing endogenous losses of amino acids associated with phytate

(Cowieson and Ravindran, 2007; Cowieson et al., 2008) or by facilitating an increase in proline, phenylalanine, and leucine digestion and absorption for use by the innate immune system. The ability of phytase supplementation to reduce the non-digestible fraction of amino acids shunted toward the distal intestine in vaccinated birds may also reduce the incidence of necrotic enteritis (NE).

Coccidia invade the intestinal epithelium, damage intestinal integrity, and reduce nutrient digestion and absorption (Williams, 2005). Invasion of the parasite also activates an inflammatory immune response, which elicits specific metabolic changes such as reductions in feed intake (FI) and fatty acid oxidation and an increase in energy expenditure and protein catabolism (Klasing and Barnes, 1988). Coccidia may facilitate the occurrence of NE by increasing mucin production and reducing nutrient digestion and absorption allowing an excess of protein and amino acids to flow into the distal intestine. Phytase supplementation may reduce the anti-nutrient effects of phytate and reduce the incidence of NE in vaccinated broilers by improving amino acid digestibility, reducing the non-digestible amino acid fraction, and promoting a healthy mucin layer in vaccinated broilers. Therefore, the objective of this experiment was to evaluate the effect of supplemental phytase on performance, bone ash, apparent IAAD, and small intestinal morphology during a live coccidia oocyst vaccination in broilers housed in floor pens.

MATERIALS AND METHODS

Animals and Husbandry

A total of 2,940, one-day-old, Cobb 500, male broilers were obtained from a commercial hatchery and transported to the poultry research farm at Virginia Tech. Upon arrival, half of the

chicks (n=1,470) were sprayed with a commercially available live oocyst vaccine[11] according to manufacturer's recommendations. Chicks were then randomly selected, weighed, and placed in floor pens on clean pine shavings with respect to the vaccinated and non-vaccinated groups at a stocking density of 12.6 chicks/m^2. Birds were maintained on a lighting program of 23L:1D d 0 to 7, 18L:6D d 8 to 14, 14L:10D d 15 to 26, 18L:6D d 27 to 35, and 22L:2D d 36 to 41, and they were allowed *ad libitum* access to feed and water for the 41 d trial.

Diets and Enzyme Treatments

All diets were fed in crumbled (starter) or pelleted (grower and finisher) form and formulated on a corn/ soybean meal basis according to Cobb nutrient recommendations with the exception of Ca and aP in the negative control (NC; Table 4.1). Dietary treatments consisted of a positive control (PC), PC + 1000 FTU phytase/kg diet (PC+1000), NC (0.13% reduction in Ca and aP for all feeding phases), NC + 1000 FTU phytase/kg diet (NC+1000), and NC + 5000 FTU phytase/kg diet (NC+5000). Non-vaccinated and vaccinated birds were allowed *ad libitum* access to one of the five dietary treatments from d 0 to 41. This resulted in a total of 10 treatments with seven replicate pens of 42 birds per pen.

The phytase used in the experiment was an evolved *E. coli* 6-phytase (AB Vista[12]) expressed in *Pichia pastoris*. Corn was supplemented in place of phytase in the PC and NC diets without phytase. Diets were formulated to contain 0.1% titanium oxide as an indigestible marker. Phytase activities in the experimental diets were analyzed by AB Vista[3]. All experimental procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

[11] Coccivac B™, Schering Plough, Kenilworth, NJ
[12] AB Vista Feed Ingredients, Marlborough, UK

Sample Collection

Birds were weighed by pen prior to placement (d 0), d 17, d 30, and d 41 to measure mean BW and calculate mean BW gain (BWG) for each period and cumulatively (d 0 to 41). Feed intake and feed conversion (FC) were also measured for each period and cumulatively. Building temperature and mortality were recorded daily and any birds removed for sampling or mortality were weighed, and FI and FC were adjusted according to the number of bird days. Birds sampled were stunned by exposure to CO_2 gas and euthanized by cervical dislocation for collection of digesta for digestibility assays, and tissues for histology, and bone ash.

On d 18, one bird/pen (n=7 birds/diet/treatment) was euthanized to obtain tissues for morphometric and histopathological measurements. Tissues were collected from the duodenum, jejunum, and ileum for determination of villus height, crypt depth, villus height to crypt depth ratio (VCR), and goblet cell number. Intestinal segments (approximately 2 to 3 cm) were obtained and gently flushed with cold phosphate buffered saline to remove luminal contents. Collected tissues were placed in 10% neutral buffered formalin until further processing. Each fixed intestinal segment was cut into five-5 mm sections and embedded in paraffin. Embedded samples were cut at 5 μm and mounted onto slides. Slides were stained using Periodic Acid-Schiff's reagent and Alcian Blue and examined by light microscopy[13]. Measurements of villus height, crypt depth, and goblet cell number/villus were obtained using Sigma Scan Pro 5[14] and digitized using Image Pro Plus[15]. Villus height (n=12) and crypt depth (n=12) were measured from 3 tissues/slide following procedures according to Sun et al. (2005). Briefly, villus height was measured from the tip of a villus to the crypt opening, and crypt depth was measured from the crypt opening to the crypt bottom. Villus height to crypt depth ratio was calculated and

[13] Olympus Polaroid DMC-IE camera, Polaroid Corporation, MA
[14] SPSS, Chicago, IL
[15] Media Cybernetics, Silver Springs, MD

normalized using the natural log. The number of goblet cells/villus were counted, and the villus area was obtained from 4 villi/3 tissues/slide (n=12). The goblet cell number/villus area was calculated and normalized using the natural log.

On d 18 and d 41, digesta was obtained from the ileum, consisting of the Meckel's diverticulum to the ileo-cecal junction, and pooled from four birds/pen (n=28 birds/treatment). Samples were snap frozen on dry ice and stored at -80°C until amino acid analysis. Digesta samples were lyophilized, and diet and digesta samples were ground to pass through a 1 mm screen. Titanium dioxide concentrations in the diet and digesta were determined according to methods of Short et al. (1996). Amino acid concentrations of the diet and ileal digesta were determined using HPLC following acid hydrolysis according to modified methods of Albin et al. (2000). Briefly, 100 mg of ileal digesta were purged for 30 seconds with N_2 and hydrolyzed for 24 hours at 100°C in approximately 6 ml 6 M HCl. Hydrolyzed samples were filtered using 0.45 µm luer lock syringe filters. Filtered, hydrolyzed samples (100 µL) were added to 100 µL of the internal standard Norleucine, centrifuged, dried twice, and analyzed using a Pico-Tag column and HPLC. Amino acids were identified and integrated using Pierce hydrolyzed standards (Fisher P120088), and grams amino acid/100 g of sample was calculated. Amino acid values were then used to calculate apparent IAAD using the following equation:

$$\text{Digestibility Coefficient} = ((\text{amino acid/TiO})_d - (\text{amino acid/TiO})_i) / (\text{amino acid/TiO})_d * 100$$

Left tibias were obtained from four birds/pen (n=28 birds/treatment) and pooled for determination of bone ash on d 18 and d 41. Tibias were stripped of adhering tissues, wrapped in cheese cloth, and dried overnight at 100°C. Fat was extracted from tibias using a Soxhlet

apparatus and 100% ethyl ether according to modified methods adapted from Watson et al. (2006). Fat-extracted tibias were then dried for 24 hours at 100°C and ashed in a muffle furnace for 24 hours at 600°C to determine bone ash.

Statistical Analysis

Performance data were subjected to ANOVA using the MIXED models for completely randomized design procedure of SAS[16]. Data were analyzed by feeding phase and cumulatively prior to NE outbreak (d 0 to 30) and after NE outbreak (d 0 to 41) to evaluate the effects of diet, vaccination, and NE. Percent mortality data were arc sine transformed prior to analysis. Pen served as the experimental unit for BWG, FI, FC, percent mortality, apparent IAAD, and tibia ash. Bird served as the experimental unit for histological measurements. The statistical model included diet, vaccination, and the interaction of diet and vaccination. Mean differences were determined using Tukey's test. Statistical significance was accepted at $P \leq 0.05$.

RESULTS

Growth Performance

Dietary analysis revealed Ca levels in the PC during the starter period were higher than formulated (Table 4.1). All other analyzed dietary nutrients were within acceptable ranges for the feeding phases. Recovery of enzymatic activities in the experimental diets were in approximate agreement with formulated activities for all phases measured (Table 4.2). Mortality was relatively low during the starter and grower period, and there were no period or cumulative (d 0 to 30) treatment differences (Table 4.3). However, on approximately d 31 all treatments broke with necrotic enteritis (NE), and treatment associated differences in mortality were significant ($P \leq 0.0001$) between d 31 and 41 and cumulatively (d 0 to 41). Necropsied birds

[16] SAS Institute, Cary, NC

presented with fragile, enlarged, gas filled intestines, the intestinal epithelium was eroded and the mucosa was covered by a membrane of necrotic tissue. The liver was enlarged and dark. Birds fed the PC diet had the highest ($P \leq 0.0001$) percent mortality (37%) associated with NE compared to birds fed all other diets. Mortality associated with NE was lowest ($P \leq 0.0001$) in birds fed the NC diet (13%), and this was not different than the NC diets supplemented with phytase. Phytase supplementation to the PC reduced ($P \leq 0.0001$) NE associated mortality compared to the PC diet, and equivalent to the NC diets supplemented with phytase. Vaccination with live coccidia oocysts resulted in reduced ($P \leq 0.0001$) NE associated mortality compared to non-vaccinated birds from d 31 to 41 and cumulatively (d 0 to 41; Table 4.3).

Diet and vaccination influenced FI from d 0 to 17, which resulted in a significant ($P = 0.0543$) interaction (Table 4.4). Vaccination did not affect FI within dietary treatment, except when birds were fed the NC diet supplemented with 5000 FTU/kg, which resulted in lower FI in vaccinated broilers than non-vaccinated broilers fed this diet. Feed intake was increased ($P \leq 0.0001$) in birds fed the PC and the NC diets supplemented with 1000 FTU/kg from d 0 to 17 compared to birds fed the diets without phytase. Phytase supplementation to the NC diet at 5000 FTU/kg increased ($P \leq 0.0001$) FI compared to the PC but no differences were seen when compared to the NC diet without phytase supplementation or PC+1000. Feed intake was not affected by diet during the grower phase. However, from d 0 to 30 phytase supplementation at 1000 or 5000 FTU/kg to the NC increased ($P = 0.0066$) FI compared to the PC. During the finisher phase and NE outbreak, FI was lower ($P = 0.0021$) in birds fed the PC diet compared to birds fed all other dietary treatments. Cumulatively (d 0 to d 41) FI was lower ($P \leq 0.0001$) in birds fed the PC diet compared to birds fed all other dietary treatments.

Diet similarly influenced broiler BWG regardless of vaccination treatment, which resulted in no significant diet by vaccination interactions for BWG. Phytase supplementation to the PC or NC diets improved ($P \leq 0.0001$) broiler BWG from d 0 to 17 compared to birds fed the PC or NC diets without phytase supplementation (Table 4.5). Birds fed the PC diet had the lowest BWG from d 0 to 17, compared to broilers fed all other diets. From d 17 to 30, only phytase supplementation at 5000 FTU/kg diet improved ($P = 0.0045$) broiler BWG above the PC or NC diets without phytase supplementation. From d 0 to 30 phytase supplementation to the PC or NC diet improved ($P \leq 0.0001$) broiler BWG compared to broilers fed the NC or PC diet without phytase supplementation. From d 30 to 41, BWG was heavier ($P = 0.0009$) in broilers fed the NC diet or NC diet supplemented with 5000 FTU/kg compared to the PC, but this was not different than PC+1000 or NC+1000. Cumulatively (d 0 to 41), broilers fed the PC had the least ($P \leq 0.0001$) BWG compared to broilers fed all other diets.

As expected from the differences in FI and BWG, FC was affected by diet (Table 4.6). Phytase supplementation to the PC improved ($P \leq 0.0001$) FC compared to the PC without phytase supplementation from d 0 to 17 and cumulatively (d 0 to 41). Feed conversion was improved ($P \leq 0.0001$) in broilers fed the NC diet supplemented with 5000 FTU/kg compared to the PC diet, NC diet, or NC+1000 from d 0 to 17. Only phytase supplementation to the NC diet at 5000 FTU/kg improved FC above the PC or NC diets without phytase from d 17 to 30 and d 0 to 30. Broilers fed the PC diet were less ($P = 0.0018$) efficient than broilers fed the NC or NC+5000 diets from d 30 to 41.

Broiler FI (Table 4.4), BWG (Table 4.5), and FC (Table 4.6) were reduced ($P \leq 0.05$) in vaccinated birds compared to non-vaccinated birds from d 0 to 17, d 17 to 30, and d 0 to 30. Interestingly, from d 30 to 41 FI, BWG, and FC, were improved ($P \leq 0.05$) in vaccinated birds

compared to non-vaccinated birds, which resulted in no cumulative (d 0 to 41) differences in performance between non-vaccinated and vaccinated broilers.

Bone Ash

The percent tibia ash of non-vaccinated and vaccinated birds was similarly influenced by the dietary treatments, which resulted in no significant diet by vaccination interactions. Birds fed the PC diet had a higher ($P \leq 0.0001$) percent tibia ash than birds fed the NC diet at d 18 (Table 4.7). Phytase supplementation to the PC or NC diets improved ($P \leq 0.0001$) the percent tibia ash at d 18. Phytase supplementation at 1000 or 5000 FTU/kg to the NC diet resulted in tibia ash comparable to the PC and PC+1000. The only difference from diet at d 41 was from phytase supplementation to the NC diet at 5000 FTU/kg which improved ($P = 0.0288$) tibia ash above the NC diet. Tibia ash was reduced ($P \leq 0.0001$) in birds vaccinated with live coccidia oocysts compared to non-vaccinated birds at d 18, but tibia ash was not affected by vaccination at d 41.

Small Intestinal Morphology

Due to the large mortality and pathogenesis associated with the NE outbreak, small intestinal samples obtained at d 41 were not included in the analysis. Duodenum, jejunum, or ileum villus height was not affected by diet, vaccination, or the interaction of diet and vaccination (data not shown). However, differences were observed in crypt depth and VCR in the duodenum, jejunum, and ileum.

Vaccination with live coccidia oocysts resulted in larger duodenum crypts compared to the non-vaccinated birds, regardless of dietary treatment (Table 4.8). Non-vaccinated birds fed the NC diet supplemented with 5000 FTU/kg had the shallowest crypt depths, while vaccinated birds on this diet had the deepest crypts, which resulted in a larger difference due to vaccination between non-vaccinated and vaccinated birds in this diet compared to the others and a diet by

vaccination interaction (P = 0.0036; Table 4.8). Duodenum VCR was not affected by diet or the interaction between diet and vaccination. However, vaccination resulted in reduced (P ≤ 0.0001) duodenum VCR compared to the non-vaccinated broilers (Table 4.8). Duodenum goblet cells were similarly affected by diet and vaccination, which resulted in no significant diet by vaccination interactions (Table 4.9). There were more goblet cells in the duodenum (P = 0.0400) of birds fed the PC diets supplemented with phytase compared to birds fed the NC diets supplemented with 5000 FTU phytase/kg, while all other treatments were similar in number to both of these. Vaccination reduced (P = 0.0001) the number of goblet cells in the duodenum compared to non-vaccinated birds.

Vaccination with live coccidia oocysts resulted in deeper (P ≤ 0.0001) jejunum crypts and smaller (P ≤ 0.0001) VCRs compared to non-vaccinated broilers (Table 4.8). There were no effects of diet or interaction of diet and vaccination on jejunum crypt depths, jejunum VCR, or jejunum goblet cells. Vaccination reduced (P = 0.0001) the number of goblet cells in the jejunum compared to non-vaccinated birds (Table 4.9).

In the ileum, in birds fed the NC diets with 1000 or 5000 FTU phytase/kg, vaccination resulted in deeper crypts compared to non-vaccinated birds. These differences were not seen with any other dietary treatments, which resulted in a diet and vaccination interaction (P = 0.0122; Table 4.8). The main effect of diet and vaccination was also noted with ileal crypt depth. Phytase supplementation to the NC diet at 5000 FTU/kg increased (P = 0.0129) ileal crypt depth compared to phytase supplementation of the PC diet at 1000 FTU/kg, while results from other diets were similar to both of these (Table 4.8). Vaccination increased (P = 0.0001) ileal crypt depth compared to the non-vaccinated broilers. Ileal VCR was not affected by diet or the interaction of diet and vaccination. However, vaccination with live coccidia oocysts reduced

($P \leq 0.0001$) ileal VCR compared to the non-vaccinated birds (Table 4.8). An interaction ($P = 0.0268$) of diet and vaccination was observed for goblet cell number, with lower numbers of goblet cells in vaccinated birds compared to non-vaccinated birds on the PC+1000 or NC diets (Table 4.9). In all other diets, goblet cell numbers were similar between non-vaccinated and vaccinated broilers. Birds fed the NC diet had the least ($P \leq 0.0001$) number of ileal goblet cells compared to all other dietary treatments. Supplementation of the PC diet with phytase at 1000 FTU/kg had no effect on the number of goblet cells compared to the PC diet without phytase. However, NC+1000 and NC+5000 fed broilers had more goblet cells in the ileum than did birds fed the NC diet without phytase supplementation. Additionally, feeding NC+1000 or NC+5000 resulted in goblet cell numbers similar to the PC. Vaccination reduced ($P \leq 0.0001$) ileal goblet cell numbers compared to non-vaccinated broilers (Table 4.9).

Apparent Ileal Amino Acid Digestibility

Apparent IAAD was similarly affected by diet and vaccination which resulted in no significant diet by vaccination interactions at d 18 or d 41. Diet affected ($P \leq 0.0001$) apparent IAAD for all amino acids analyzed at d 18 (Tables 4.10 and 4.11). In general, phytase supplementation to the PC or NC diets improved ($P \leq 0.0001$) apparent IAAD above the NC or PC diets without phytase supplementation. There was no difference in the apparent digestibility of histidine between the PC and NC + 5000, and arginine digestibility was only improved ($P = 0.0002$) in the NC diet supplemented with 1000 FTU/kg compared to the NC diet without phytase supplementation. Vaccination reduced ($P \leq 0.0001$) apparent IAAD in all amino acids analyzed compared to the apparent IAAD in non-vaccinated broilers at d 18, with the exception of arginine which was not affected by vaccination (Tables 4.10 and 4.11). Apparent ileal amino acid digestibility was not affected by diet or the interaction of diet and vaccination at d 41 (Table

4.12). In contrast to the results at d 18, vaccination improved ($P \leq 0.05$) apparent IAAD in all amino acids analyzed at d 41 compared to apparent IAAD of non-vaccinated birds (Table 4.12).

DISCUSSION

The objective of this experiment was to evaluate the effect of supplementing an *E. coli* 6-phytase on performance, bone ash, small intestinal morphology, and apparent IAAD in broilers exposed to a live coccidia oocyst vaccine. Broiler performance and tibia ash were generally improved with phytase supplementation to the PC or NC diets. Previous authors have reported improvements in performance and tibia ash associated with phytase supplementation to both nutritionally adequate and deficient diets (Watson et al., 2006). Vaccination reduced broiler FI, BWG, and FC from d 0 to 30, and this has been previously reported (Yi et al., 2005; Parker et al., 2007; Lehman et al., 2009). Tibia ash, the most sensitive indicator of bone mineralization, was also reduced due to live oocyst vaccination. Reductions in tibia ash associated with the vaccination may suggest that mineral absorption was reduced in vaccinated birds. Feed intake was affected by an interaction between diet and vaccination from d 0 to 17, with vaccinated birds fed the NC diet supplemented with 5000 FTU/kg eating less than non-vaccinated birds fed the NC supplemented with 5000 FTU/kg diet, while with other diets FI was similar between non-vaccinated and vaccinated birds. In all dietary treatments, vaccination numerically reduced FI, except in birds fed the NC diet supplemented with 1000 FTU/kg. Phytase has been implicated in improving FI in low Ca and aP diets (Pirgozliev et al., 2008) and the NC+5000 fed non-vaccinated birds had the highest FI, which may have resulted in the larger difference between non-vaccinated and vaccinated birds fed this diet. It is interesting to note that diet and vaccination interactions affected FI during the peak oocyst shedding cycle (Schering-Plough, 2007). Vaccinated birds initially shed oocysts in the feces approximately 5 to 7 days post

vaccination, and oocyst shedding peaks at approximately 18 to 23 days post vaccination. Exit of the oocyst from the enterocyte results in cell damage and an inflammatory immune response, which has been known to reduce FI due to IL-1 secretion (Klasing et al., 1987).

Throughout the experiment, the PC appeared to yield results similar to the NC, which were generally less beneficial than the diets supplemented with phytase. The PC diet was formulated to meet or exceed Cobb nutrient requirements, and dietary nutrient analysis revealed no major differences between the PC and NC basal diets, with the exception of Ca and aP. Broiler FI and BW were in agreement with published Cobb 500 standards in birds fed the PC. Therefore, phytase supplementation may have liberated excess nutrients such as amino acids and starches for use by the bird, which would facilitate an increase in FI and BWG and result in differences associated with phytase supplementation, even in the PC. Watson et al. (2006) reported improvements in nutrient adequate diets with phytase supplementation compared to nutrient adequate diets without phytase supplementation. A broader performance response between the PC and NC diets in this experiment may have been seen had the Ca and aP levels in the NC diet been reduced approximately 0.2%. The fact that the PC diet performed the poorest cumulatively can be attributed to the higher percentage of NE associated mortality in the PC compared to all other dietary treatments. Interestingly, mortality associated with NE was lowest in the NC diet and the only differences between the PC and NC diets were the Ca and aP levels. The alpha-toxin produced by *C. perfringens* is a phospholipase enzyme that hydrolyzes phospholipids and promotes membrane disorganization (Titball et al., 1999). High dietary levels of Zn and Ca have been implicated as a predisposing factor of NE due to the mineral usage in the alpha-toxin pathway (Titball et al., 1999). High Ca levels entering the proventriculus and gizzard may have also acted as a buffer. Calcium carbonate reacts with and neutralizes gastric

hydrochloric acid to increase pH and subsequently inhibit pepsin activity (Maton and Burton, 1999). The large amount of limestone present in the PC diet may have acted as an antacid and increased gastric pH in the proventriculus and gizzard, thereby reducing pepsin conversion from pepsinogen and inhibiting protein digestion. Limestone supplementation in beef cattle rations increased the amount of crude protein (CP) entering the duodenum compared to de-fluorinated rock phosphate or control diets formulated without a supplemental Ca source (Christiansen and Webb, 1990). In the current experiment, excess protein flow to the distal intestine may have exacerbated *C. perfringens* growth. Phytase supplementation in the PC and NC diets resulted in a reduction in NE associated mortality compared to the PC. Phytase would have liberated Ca, P, and amino acids from the phytate molecule allowing them to be readily absorbed by the bird rather than shunted to the distal intestine. The higher levels of Ca in the PC would have increased proventricular pH and may have reduced the solubility of phytate (Selle et al., 2000), which may in turn have reduced the ability of phytase to break down the phytate molecule, thereby allowing nutrients to flow to the lower intestine for bacterial proliferation. Vaccination with live coccidia oocysts did not affect mortality from d 0 to 30. Non-vaccinated birds may have been exposed to a late coccidia infection, which resulted in an increase in NE associated mortality compared to the vaccinated birds. Vaccinated birds would have acquired a level of protective immunity due to the first cycling of oocysts, and therefore would have experienced less intestinal damage from subsequent coccidia exposure, which may have protected them from the *C. perfringens* outbreak. By d 41, there were no differences in broiler performance or tibia ash due to vaccination, which may be a result of the increased intestinal damage and mortality associated with NE in the non-vaccinated birds.

Due to the large percentage of mortality associated with NE and the differences in performance parameters after the NE outbreak, small intestinal samples collected at d 41 were not analyzed. Therefore, only samples obtained at d 18 were used in this experiment for evaluation of small intestinal morphology. Phytase supplementation or vaccination did not affect villus height in any intestinal section measured, but the interaction of phytase and vaccination did increase crypt depth in the duodenum and ileum. In general, vaccination increased cellular turnover in the duodenum as demonstrated by deeper crypts. In non-vaccinated birds, phytase supplementation at 5000 FTU/kg diet may have reduced the phytate content of the diet, thereby reducing the endogenous losses of amino acids, minerals, and mucin associated with phytate (Cowieson and Ravindran, 2007; Cowieson et al., 2008) which presumably resulted in less turnover and shallower crypts compared to the NC diet without phytase supplementation. Interestingly, the ileum was also affected by interactions between the diet and vaccination. The vaccination was composed of oocysts from *E. acervulina*, *E. maxima*, and *E. tenella* which invade the duodenum, jejunum, and ceca of broilers, respectively. However, mucin production is higher in the ileum (Uni et al., 2003) and may be more affected by diet and vaccination status. Vaccination did not affect ileal crypt depth in birds fed the PC supplemented with 1000 FTU/kg diet. In addition, the number of goblet cells in the ileum of birds fed the NC diets supplemented with phytase was not different between the vaccinated and non-vaccinated broilers, but ileal crypt depth was deeper in vaccinated birds. It is possible that the distal small intestinal morphological effects may be associated with the interaction of phytate, coccidia, dietary minerals, and morphology in the proximal small intestine. For example, supplementation of the NC diet with 5000 FTU/kg resulted in the lowest number of goblet cells in the duodenum compared to the PC supplemented with 1000 FTU/kg. However, phytase supplementation to the

NC diet at 5000 FTU/kg resulted in the highest number of goblet cells in the ileum, and this was significantly higher than the PC diet supplemented with 1000 FTU/kg. The ileum may compensate for morphological changes in the proximal small intestine by increasing mucosal surface area (Idris et al., 1997). Dietary phytase has been implicated as a cause for wet litter in a few occasions. Excess Na^+ availability associated with the destruction of phytate from phytase (Cowieson et al., 2004) may alter the ionic balance between the lumen and enterocyte tight junctions, resulting in excess water or mucus secretion and an increase in goblet cell numbers. In broilers, excreta dry matter is reduced as dietary electrolyte balance (DEB) increases, and phytase supplementation does not affect excreta dry matter even at the highest DEB level (Ravindran et al., 2008). There were no overt differences in the litter conditions in this experiment. However, evaluating litter quality was not an original objective and differences in litter dry matter content may have been more apparent upon closer evaluation. Experimental diets were formulated to contain approximately 0.20% Na, and reducing the Na content of the diet has been recommended with phytase supplementation in order to reduce the chance of wet litter.

Goblet cells, crypt depth, and VCR were reduced in the duodenum, jejunum, and ileum of vaccinated birds compared to non-vaccinated birds. This may suggest that while villi height was not affected by vaccination or diet, the damage to the intestinal tract was minimal and could be repaired quickly by increasing cell turnover. The intestinal tract of vaccinated birds may be functionally immature, which may affect nutrient digestion and absorption. The lower number of goblet cells, reductions in tibia ash, and reduced apparent IAAD associated with the vaccination at d 18 would also be suggestive of functionally immature enterocytes that may lack digestive enzymes and nutrient transporters. Reduced goblet cell numbers would be indicative of

a shallow protective mucin layer, which is necessary to stabilize brush border enzymes and trap food particles for absorption. While phytase supplementation was able to increase both tibia ash and apparent IAAD in all amino acids evaluated at d 18, there were no significant diet and vaccination interactions. Other authors have reported improvements in apparent IAAD with phytase supplementation (Cowieson et al., 2006; Pirgozliev et al., 2007; Ravindran et al., 2008). In the current experiment, trends would suggest phytase was able to improve (P = 0.10; Table 4.13) apparent IAAD in the vaccinated birds and this was higher than vaccinated birds fed diets without phytase supplementation. Previous experiments in our lab have demonstrated similar effects of phytase on apparent IAAD during a live coccidia oocyst vaccination. Significant diet by vaccination interactions may not be apparent due to an increase in intestinal damage, cell sloughing, and endogenous losses associated with the vaccination. While phytase supplementation may have been able to improve amino acid digestibility in vaccinated broilers, the increase in endogenous losses associated with the vaccine may have reduced differences associated with amino acid digestibility. Research to evaluate nutrient partitioning and amino acid requirements of the intestinal tract and the immune system of broilers during a coccidia infection may be beneficial to establish nutrient recommendations and reduce the incidence of NE by reducing endogenous losses and excess amino acids or minerals flowing to the distal intestine. Interestingly, arginine digestibility was not different between the non-vaccinated and vaccinated birds. Fast growing chickens have a high arginine requirement and arginine is a substrate in the nitric oxide (NO) pathway. Nitric oxide is a free radical known to have antimicrobial properties and is increased in coccidia infections (Allen, 1999). In this experiment the lack of differences in arginine digestibility between the vaccinated and non-vaccinated birds was unexpected. Phytase supplementation above 1000 FTU/kg diet did not appear to provide

addition benefit to apparent IAAD. By d 41, diet did not affect apparent IAAD, and the vaccinated birds had better apparent IAAD than the non-vaccinated broilers. These data may have been influences by the high percentage of mortality associated with NE and the differences in performance parameters associated with the vaccination and NE interactions.

This experiment demonstrates the need to further understand nutrient partitioning and dietary requirements of birds exposed to a live coccidia oocyst vaccine. Vaccination reduced performance prior to the NE outbreak, but may have protected birds from mortality associated with NE. While amino acid digestibility was not improved in vaccinated broilers due to phytase supplementation, trends would suggest there are benefits to supplementing phytase during a live coccidia oocyst vaccination or a subsequent NE outbreak. Phytase supplementation reduced broiler mortality and improved FC in broilers experiencing NE. Further characterization of the alpha-toxin and the nutrients used by *C. perfringens* may also be beneficial to establish dietary recommendations to reduce the incidence of NE. In this experiment amino acid digestibility was not improved with phytase supplementation above 1000 FTU/kg diet. However, bird performance and FC were improved during a NE outbreak with phytase supplementation at 5000 FTU/kg. Phytase has many beneficial effects on broiler performance and nutrient digestion, and inclusion rate in the diet may depend on environment, economic value, production and vaccination status, and the presence or absence of antibiotics in the diet.

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Table 4.1 Composition and nutrient content of basal diets[1]

	Starter		Grower		Finisher	
Ingredients	PC (%)	NC (%)	PC (%)	NC (%)	PC (%)	NC (%)
Corn	59.55	60.55	61.79	62.80	63.60	64.63
Soybean Meal	28.60	28.43	22.68	22.51	18.63	18.45
DDGs	4.00	4.00	5.00	5.00	6.00	6.00
Poultry-by-Product	2.50	2.50	4.00	4.00	5.00	5.00
Poultry Fat	1.65	1.29	3.21	2.85	3.82	3.45
Limestone	0.86	0.93	0.89	0.96	0.79	0.86
Di-calcium Phosphate	1.48	0.78	1.06	0.35	0.85	0.15
Salt	0.39	0.39	0.36	0.36	0.32	0.32
L-Lysine	0.31	0.31	0.29	0.30	0.30	0.30
DL-Methionine	0.31	0.31	0.27	0.27	0.23	0.23
Titanium Di-oxide	0.00	0.00	0.10	0.10	0.10	0.10
Quantum® Phytase XT	0.04	0.20	0.04	0.20	0.04	0.20
L-Threonine	0.10	0.10	0.10	0.10	0.12	0.12
Trace Mineral Premix[2]	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin Premix[3]	0.10	0.10	0.10	0.10	0.10	0.10
Selenium Premix[4]	0.012	0.012	0.010	0.010	0.009	0.009
Calculated Composition						
Dry Matter	87.40	87.28	87.62	87.50	88.48	88.37
Crude Protein	21.00	21.00	19.50	19.50	18.50	18.50
Lysine	1.30	1.30	1.18	1.18	1.08	1.08
TSAA	0.96	0.96	0.89	0.89	0.82	0.82
Threonine	0.88	0.88	0.82	0.82	0.78	0.78
Ca	0.92	0.79	0.89	0.76	0.84	0.71
Total P	0.66	0.53	0.59	0.46	0.55	0.43
Available P	0.45	0.32	0.40	0.27	0.38	0.25
Analyzed Composition						
Dry Matter	86.81	86.37	87.24	87.67	86.16	85.72
Crude Protein	22.87	22.68	20.72	19.36	18.64	18.76
Lysine	n/a	n/a	0.97	0.96	0.85	0.86
Threonine	n/a	n/a	0.59	0.58	0.56	0.57
Ca	1.13	0.73	0.90	0.59	0.84	0.70
Total P	0.62	0.56	0.61	0.45	0.59	0.41
Nutrient Composition						
Energy (ME; kcal/kg)	3003	3003	3124	3124	3186	3186

[1] Diets were fed in pelleted form from day 0 to 41. Quantum® Phytase XT was added in place of corn in the diets containing supplemental phytase.

[2] Supplied per kilogram mix: iron (Ferrous Sulfate), 40 g; manganese (Manganese Sulfate and Manganous Oxide), 120 g; zinc (Zinc Oxide), 210 g; cobalt (Cobalt Carbonate), 2.2 g; iodine (Calcium Iodate), 132 g.

[3] Supplied per kilogram mix: vitamin A, 8,818,400 IU; vitamin D_3 2,645,520 ICU; vitamin E, 22,046 IU; vitamin B_{12}, 26 mg; riboflavin, 8,818 mg; niacin, 88,184 mg; d-pantothenic acid,

22,046 mg; vitamin K, 2,646 mg; folic acid, 2,205 mg; vitamin B_6, 4,339 mg; thiamine, 3,732 mg; d-biotin, 220 mg.
[4] Supplied per kilogram mix: 600 ppm.

Table 4.2 Recovery of enzyme activity in experimental diets

	Determined Phytase Activity[1]		
Diet	Starter	Grower	Finisher
Positive Control (PC)	< 50	< 50	< 50
PC + 1000 FTU	892	892	873
Negative Control (NC)	~ 50	~ 50	< 50
NC + 1000 FTU	940	1017	1086
NC + 5000 FTU	4279	4485	4466

[1]One unit of phytase activity (FTU) is defined as the quantity of enzyme that liberates 1 μmole of inorganic P per minute from sodium phytate at pH 5.5 and 37°C.

Table 4.3 Effect of phytase supplementation and Coccivac[1] B^{TM} on period and cumulative broiler mortality[2]

Dietary Treatments	d 0 - 17 (%)	d 17 - 30 (%)	d 30 - 41 (%)	d 0 - 30 (%)	d 0 - 41 (%)
PC	1.099	1.313	37.03^{c}	2.381	32.97^{c}
PC + 1000	2.015	1.695	22.60^{b}	3.663	22.16^{b}
NC	1.190	0.521	13.16^{a}	1.701	12.76^{a}
NC + 1000	1.832	0.377	18.02^{ab}	2.198	17.22^{ab}
NC + 5000	1.654	0.753	17.56^{ab}	2.198	16.67^{ab}
Pooled SEM					
Vaccination					
Non-Vaccinated	1.541	0.504	28.81^{b}	2.031	25.98^{b}
Vaccinated	1.488	1.371	13.69^{a}	2.827	14.14^{a}
Pooled SEM					
P-Value					
Diet	0.8358	0.6911	0.0001	0.7375	0.0001
Vaccination	0.8090	0.1811	0.0001	0.2962	0.0001
Diet*Vaccination	0.8252	0.6771	0.7880	0.9035	0.7294

[a-c] Means lacking a common superscript differ significantly ($P < 0.05$).
[1] Vaccinated birds were exposed to Coccivac B^{TM} at day of hatch.
[2] Mortality data were transformed using Arc Sine prior to analysis.

Table 4.4 Effect of phytase supplementation and Coccivac[1] B[TM] on period and cumulative broiler feed intake

Dietary*Vaccination		d 0 - 17 (kg)	d 17 - 30 (kg)	d 30 - 41 (kg)	d 0 - 30 (kg)	d 0 - 41 (kg)
PC	-	0.762[bcd]	1.784	1.862	2.546	4.411
PC	+	0.752[d]	1.749	1.953	2.501	4.454
PC + 1000	-	0.798[a]	1.834	1.959	2.632	4.590
PC + 1000	+	0.785[abcd]	1.753	2.077	2.538	4.657
NC	-	0.769[abcd]	1.802	2.000	2.571	4.571
NC	+	0.761[cd]	1.791	2.089	2.551	4.641
NC + 1000	-	0.797[ab]	1.830	1.973	2.627	4.600
NC + 1000	+	0.797[abc]	1.806	2.056	2.603	4.659
NC + 5000	-	0.804[a]	1.817	2.008	2.621	4.629
NC + 5000	+	0.759[d]	1.825	2.062	2.584	4.621
Pooled SEM		0.0016	0.0228	0.0355	0.0258	0.0440
Dietary Treatments						
PC		0.757[c]	1.766	1.908[b]	2.523[b]	4.432[b]
PC + 1000		0.792[a]	1.794	2.018[a]	2.585[ab]	4.624[a]
NC		0.765[bc]	1.797	2.045[a]	2.561[ab]	4.606[a]
NC + 1000		0.797[a]	1.818	2.014[a]	2.615[a]	4.629[a]
NC + 5000		0.782[ab]	1.821	2.035[a]	2.603[a]	4.625[a]
Pooled SEM		0.0055	0.0161	0.0251	0.0183	0.0312
Vaccination						
Non-Vaccinated		0.786[a]	1.814[a]	1.960[b]	2.600[a]	4.560
Vaccinated		0.771[b]	1.785[b]	2.048[a]	2.555[b]	4.606
Pooled SEM		0.0035	0.0102	0.0159	0.0115	0.0197
P-Value						
Diet		0.0001	0.1290	0.0021	0.0066	0.0001
Vaccination		0.0034	0.0486	0.0003	0.0090	0.1056
Diet*Vaccination		0.0543	0.3785	0.9377	0.6327	0.8988
Orthogonal Contrasts[2]						
Linear		0.0331	0.2877	0.7884	0.1123	0.6623
Quadratic		0.0010	0.6506	0.4111	0.1524	0.7212

[a-d] Means lacking a common superscript differ significantly ($P < 0.05$).
[1] Vaccinated birds (designated by "+") were exposed to Coccivac B[TM] at day of hatch.
[2] Orthogonal contrasts were performed on the NC diets with and without phytase.

Table 4.5 Effect of phytase supplementation and Coccivac[1] B[TM] on period and cumulative broiler body weight gain

Dietary Treatments	d 0 - 17 (kg)	d 17 - 30 (kg)	d 30 - 41 (kg)	d 0 - 30 (kg)	d 0 - 41 (kg)
PC	0.571[c]	1.163[b]	0.914[b]	1.734[c]	2.649[b]
PC + 1000	0.620[a]	1.195[ab]	1.006[ab]	1.814[ab]	2.842[a]
NC	0.589[b]	1.174[b]	1.048[a]	1.763[bc]	2.811[a]
NC + 1000	0.619[a]	1.210[ab]	1.009[ab]	1.829[a]	2.838[a]
NC + 5000	0.629[a]	1.241[a]	1.073[a]	1.869[a]	2.923[a]
Pooled SEM	0.0030	0.0151	0.0260	0.0158	0.0299
Vaccination					
Non-Vaccinated	0.622[a]	1.217[a]	0.970[b]	1.839[a]	2.809
Vaccinated	0.590[b]	1.176[b]	1.051[a]	1.765[b]	2.817
Pooled SEM	0.0019	0.0095	0.0164	0.0100	0.0189
P-Value					
Diet	0.0001	0.0045	0.0009	0.0001	0.0001
Vaccination	0.0001	0.0029	0.0009	0.0001	0.7745
Diet*Vaccination	0.2518	0.2763	0.4953	0.2726	0.8918
Orthogonal Contrasts[2]					
Linear	0.0001	0.0026	0.4903	0.0001	0.0098
Quadratic	0.0074	0.8971	0.1112	0.5090	0.4394

[a-c] Means lacking a common superscript differ significantly ($P < 0.05$).
[1] Vaccinated birds (designated by "+") were exposed to Coccivac B[TM] at day of hatch.
[2] Orthogonal contrasts were performed on the NC diets with and without phytase.

Table 4.6 Effect of phytase supplementation and Coccivac[1] B[TM] on period and cumulative broiler feed conversion

Dietary Treatments	d 0 - 17 (kg:kg)	d 17 - 30 (kg:kg)	d 30 - 41 (kg:kg)	d 0 - 30 (kg:kg)	d 0 - 41 (kg:kg)
PC	1.325[c]	1.520[b]	2.120[b]	1.456[b]	1.675[c]
PC + 1000	1.278[ab]	1.505[ab]	2.014[ab]	1.427[ab]	1.628[ab]
NC	1.299[bc]	1.530[b]	1.957[a]	1.453[b]	1.639[bc]
NC + 1000	1.288[b]	1.503[ab]	2.003[ab]	1.430[ab]	1.632[bc]
NC + 5000	1.244[a]	1.470[a]	1.905[a]	1.394[a]	1.583[a]
Pooled SEM	0.0088	0.0124	0.0357	0.0091	0.0112
Vaccination					
Non-Vaccinated	1.265[a]	1.490[a]	2.040[b]	1.414[a]	1.626
Vaccinated	1.309[b]	1.521[b]	1.960[a]	1.450[b]	1.637
Pooled SEM	0.0056	0.0079	0.0226	0.0058	0.0071
P-Value					
Diet	0.0001	0.0128	0.0018	0.0001	0.0001
Vaccination	0.0001	0.0081	0.0151	0.0001	0.2532
Diet*Vaccination	0.1442	0.7064	0.1163	0.4795	0.6093
Orthogonal Contrasts[2]					
Linear	0.0001	0.0010	0.2960	0.0001	0.0007
Quadratic	0.1463	0.8469	0.1074	0.5535	0.1387

[a-c] Means lacking a common superscript differ significantly ($P < 0.05$).
[1] Vaccinated birds (designated by "+") were exposed to Coccivac B[TM] at day of hatch.
[2] Orthogonal contrasts were performed on the NC diets with and without phytase.

Table 4.7 Effect of phytase supplementation and Coccivac[1] B^{TM} on broiler tibia ash

Dietary Treatments	Day 18 (%)	Day 41 (%)
PC	51.72^{b}	52.94^{ab}
PC + 1000	52.53^{a}	53.71^{ab}
NC	50.40^{c}	52.51^{b}
NC + 1000	52.22^{ab}	53.65^{ab}
NC + 5000	52.14^{ab}	53.79^{a}
Pooled SEM	0.1579	0.3307
Vaccination		
Non-Vaccinated	52.17^{a}	53.40
Vaccinated	51.43^{b}	53.24
Pooled SEM	0.0999	0.2092
P-Value		
Diet	0.0001	0.0288
Vaccination	0.0001	0.6091
Diet*Vaccination	0.2644	0.2710
Orthogonal Contrasts[2]		
Linear	0.0001	0.0094
Quadratic	0.0001	0.2225

[a-c] Means lacking a common superscript differ significantly ($P < 0.05$).

[1] Vaccinated birds (designated by "+") were exposed to Coccivac B^{TM} at day of hatch.

[2] Orthogonal contrasts were performed on the NC diets with and without phytase.

Table 4.8 Effect of phytase supplementation and Coccivac[1] B[TM] on small intestinal morphology of 18-day-old broilers

		Duodenum		Jejunum		Ileum	
		CD[2]	VCR[3]	CD[2]	VCR[3]	CD[2]	VCR[3]
Diet*Vaccination							
PC	-	0.122[bc]	2.916	0.102	2.599	0.075[b]	2.229
PC	+	0.182[a]	2.481	0.135	2.267	0.100[ab]	2.098
PC + 1000	-	0.121[bc]	2.837	0.097	2.577	0.084[b]	2.259
PC + 1000	+	0.172[a]	2.500	0.132	2.382	0.086[b]	2.214
NC	-	0.128[b]	2.833	0.100	2.564	0.086[b]	2.229
NC	+	0.179[a]	2.456	0.149	2.164	0.096[ab]	2.082
NC + 1000	-	0.118[bc]	2.925	0.104	2.604	0.082[b]	2.287
NC + 1000	+	0.183[a]	2.496	0.123	2.364	0.115[a]	2.015
NC + 5000	-	0.098[c]	3.041	0.090	2.627	0.085[b]	2.187
NC + 5000	+	0.191[a]	2.434	0.137	2.168	0.120[a]	1.936
Pooled SEM		0.0058	0.0481	0.0058	0.0616	0.0057	0.0589
Dietary Treatments							
PC		0.152	2.699	0.118	2.433	0.088[ab]	2.164
PC + 1000		0.146	2.668	0.115	2.480	0.085[b]	2.236
NC		0.154	2.644	0.125	2.364	0.091[ab]	2.155
NC + 1000		0.151	2.711	0.113	2.484	0.099[ab]	2.151
NC + 5000		0.144	2.737	0.113	2.398	0.103[a]	2.062
Pooled SEM		0.0041	0.0340	0.0041	0.0436	0.0040	0.0416
Vaccination							
Non-Vaccinated		0.117[b]	2.910[a]	0.099[b]	2.594[a]	0.082[b]	2.238[a]
Vaccinated		0.181[a]	2.473[b]	0.135[a]	2.269[b]	0.103[a]	2.069[b]
Pooled SEM		0.0026	0.0215	0.0026	0.0276	0.0025	0.0263
P-Value							
Diet		0.4799	0.3308	0.2580	0.2391	0.0129	0.0765
Vaccination		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Diet*Vaccination		0.0036	0.0674	0.0826	0.2040	0.0122	0.2866
Orthogonal Contrasts[4]							
Linear		0.1178	0.0550	0.0551	0.5888	0.0484	0.1221
Quadratic		0.7306	0.6186	0.2718	0.0519	0.6930	0.4061

[a-c] Means lacking a common superscript differ significantly ($P < 0.05$).
[1] Vaccinated birds (designated by "+") were exposed to Coccivac B[TM] at day of hatch.
[2] CD = crypt depth (mm)
[3] VCR = villus height to crypt depth ratio, normalized using the natural log (Ln).
[4] Orthogonal contrasts were performed on the NC diets with and without phytase.

Table 4.9 Effect of phytase supplementation and Coccivac[1] B[TM] on 18-day-old broiler small intestinal goblet cell[2] numbers

Dietary Treatments		Duodenum (Ln)	Jejunum (Ln)	Ileum (Ln)
PC	-	6.359	6.760	7.151[ab]
PC	+	6.117	6.533	6.808[bc]
PC + 1000	-	6.561	6.806	7.093[ab]
PC + 1000	+	6.228	6.410	6.684[c]
NC	-	6.355	6.619	6.941[bc]
NC	+	6.175	6.342	6.265[d]
NC + 1000	-	6.504	6.712	7.053[ab]
NC + 1000	+	6.167	6.490	6.877[bc]
NC + 5000	-	6.400	6.735	7.309[a]
NC + 5000	+	6.056	6.203	7.021[abc]
Pooled SEM		0.0611	0.0755	0.0763
Dietary Treatments				
PC		6.238[ab]	6.647	6.980[ab]
PC + 1000		6.395[a]	6.608	6.888[b]
NC		6.265[ab]	6.481	6.603[c]
NC + 1000		6.336[ab]	6.601	6.965[ab]
NC + 5000		6.228[b]	6.469	7.165[a]
Pooled SEM		0.0432	0.0535	0.0540
Vaccination				
Non-Vaccinated		6.436[a]	6.727[a]	7.109[a]
Vaccinated		6.149[b]	6.396[b]	6.731[b]
Pooled SEM		0.0273	0.0338	0.0341
P-Value				
Diet		0.0400	0.0715	0.0001
Vaccination		0.0001	0.0001	0.0001
Diet*Vaccination		0.5796	0.2015	0.0268
Orthogonal Contrasts[3]				
Linear		0.5428	0.8773	0.0001
Quadratic		0.0874	0.0521	0.2214

[a-d] Means lacking a common superscript differ significantly ($P < 0.05$).
[1] Vaccinated birds (designated by "+") were exposed to Coccivac B[TM] at day of hatch.
[2] Means represent the number of goblet cells/villus area normalized using the natural log (Ln).
[3] Orthogonal contrasts were performed on the NC diets with and without phytase.

Table 4.10 Effect of phytase supplementation and Coccivac[1] B[TM] on apparent ileal essential amino acid digestibility of 18-day-old broilers

Dietary Treatments	HIS (%)	ARG (%)	THR (%)	VAL (%)	ILE (%)	LEU (%)	PHE (%)	LYS (%)
PC	0.739^{bc}	0.734^{bc}	0.678^{b}	0.675^{b}	0.702^{b}	0.736^{b}	0.739^{b}	0.801^{b}
PC + 1000	0.792^{a}	0.775^{ab}	0.771^{a}	0.761^{a}	0.780^{a}	0.811^{a}	0.812^{a}	0.856^{a}
NC	0.724^{c}	0.726^{b}	0.673^{b}	0.677^{b}	0.703^{b}	0.737^{b}	0.739^{b}	0.795^{b}
NC + 1000	0.796^{a}	0.791^{a}	0.745^{a}	0.748^{a}	0.773^{a}	0.798^{a}	0.806^{a}	0.854^{a}
NC + 5000	0.777^{ab}	0.756^{abc}	0.763^{a}	0.740^{a}	0.767^{a}	0.795^{a}	0.804^{a}	0.848^{a}
Pooled SEM	0.0111	0.0105	0.0117	0.0126	0.0117	0.0103	0.0102	0.0083
Vaccination								
Non-Vaccinated	0.794^{a}	0.760	0.767^{a}	0.759^{a}	0.781^{a}	0.806^{a}	0.809^{a}	0.854^{a}
Vaccinated	0.737^{b}	0.753	0.685^{b}	0.682^{b}	0.710^{b}	0.745^{b}	0.751^{b}	0.808^{b}
Pooled SEM	0.0070	0.0066	0.0074	0.0080	0.0074	0.0065	0.0065	0.0053
P-Value								
Diet	0.0001	0.0002	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Vaccination	0.0001	0.4333	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Diet*Vaccination	0.1815	0.5135	0.1323	0.1755	0.1580	0.1176	0.1346	0.1390
Orthogonal Contrasts[2]								
Linear	0.0013	0.0531	0.0001	0.0009	0.0003	0.0002	0.0001	0.0001
Quadratic	0.0012	0.0002	0.0713	0.0147	0.0100	0.0152	0.0091	0.0022

[a-c] Means lacking a common superscript differ significantly ($P < 0.05$).
[1] Vaccinated birds (designated by "+") were exposed to Coccivac B[TM] at day of hatch.
[2] Orthogonal contrasts were performed on the NC diets with and without phytase.

Table 4.11 Effect of phytase supplementation and Coccivac[1] B^{TM} on apparent ileal non-essential amino acid digestibility of 18-day-old broilers

Dietary Treatments	ASP (%)	GLU (%)	SER (%)	GLY (%)	ALA (%)	PRO (%)	TYR (%)	TOTAL (%)
PC	0.757[b]	0.794[b]	0.651[b]	0.662[b]	0.714[b]	0.694[b]	0.725[b]	0.725[b]
PC + 1000	0.838[a]	0.855[a]	0.747[a]	0.751[a]	0.795[a]	0.775[a]	0.790[a]	0.799[a]
NC	0.745[b]	0.789[b]	0.644[b]	0.650[b]	0.717[b]	0.691[b]	0.710[b]	0.720[b]
NC + 1000	0.811[a]	0.845[a]	0.737[a]	0.730[a]	0.777[a]	0.758[a]	0.793[a]	0.790[a]
NC + 5000	0.825[a]	0.843[a]	0.735[a]	0.718[a]	0.772[a]	0.747[a]	0.784[a]	0.784[a]
Pooled SEM	0.0130	0.0091	0.0127	0.0126	0.0112	0.0118	0.0106	0.0103
Vaccination								
Non-Vaccinated	0.823[a]	0.848[a]	0.739[a]	0.738[a]	0.790[a]	0.767[a]	0.794[a]	0.793[a]
Vaccinated	0.768[b]	0.803[b]	0.666[b]	0.666[b]	0.719[b]	0.700[b]	0.727[b]	0.735[b]
Pooled SEM	0.0082	0.0057	0.0081	0.0080	0.0071	0.0074	0.0067	0.0065
P-Value								
Diet	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Vaccination	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Diet*Vaccination	0.3722	0.1723	0.1100	0.2030	0.1709	0.1902	0.1817	0.1496
Orthogonal Contrasts[2]								
Linear	0.0001	0.0001	0.0001	0.0004	0.0010	0.0017	0.0001	0.0001
Quadratic	0.1048	0.0115	0.0037	0.0045	0.0182	0.0089	0.0009	0.0036

[a-b] Means lacking a common superscript differ significantly ($P < 0.05$).
[1] Vaccinated birds (designated by "+") were exposed to Coccivac B^{TM} at day of hatch.
[2] Orthogonal contrasts were performed on the NC diets with and without phytase.

Table 4.12 Effect of Coccivac[1] B[TM] on apparent ileal amino acid digestibility of 41-day-old broilers

Essential AA	HIS (%)	ARG (%)	THR (%)	VAL (%)	ILE (%)	LEU (%)	PHE (%)	LYS (%)
Vaccination								
Non-Vaccinated	0.786[b]	0.660[b]	0.688[b]	0.704[b]	0.733[b]	0.770[b]	0.786[b]	0.835[b]
Vaccinated	0.835[a]	0.722[a]	0.749[a]	0.764[a]	0.787[a]	0.815[a]	0.829[a]	0.869[a]
Pooled SEM	0.0107	0.0143	0.0138	0.0131	0.0120	0.0103	0.0096	0.0081
P-Value								
Diet	0.6519	0.6371	0.3355	0.4029	0.3005	0.2323	0.2253	0.2559
Vaccination	0.0020	0.0032	0.0027	0.0023	0.0023	0.0029	0.0028	0.0038
Diet*Vaccination	0.9569	0.8758	0.8837	0.8641	0.8522	0.7805	0.8717	0.9894

Non-essential AA	ASP (%)	GLU (%)	SER (%)	GLY (%)	ALA (%)	PRO (%)	TYR (%)	TOTAL (%)
Vaccination	---------- % ----------							
Non-Vaccinated	0.901[b]	0.865[b]	0.687[b]	0.695[b]	0.739[b]	0.734[b]	0.752[b]	0.762[b]
Vaccinated	0.925[a]	0.899[a]	0.746[a]	0.748[a]	0.792[a]	0.778[a]	0.805[a]	0.809[a]
Pooled SEM	0.0051	0.0070	0.0141	0.0128	0.0114	0.0113	0.0102	0.0104
P-Value								
Diet	0.4238	0.3728	0.4329	0.7225	0.3793	0.4881	0.1941	0.3757
Vaccination	0.0013	0.0011	0.0043	0.0050	0.0016	0.0081	0.0005	0.0020
Diet*Vaccination	0.9977	0.9631	0.9541	0.9555	0.8234	0.8791	0.8207	0.9220

[a-b] Means lacking a common superscript differ significantly ($P < 0.05$).

[1] Vaccinated birds (designated by "+") were exposed to Coccivac B[TM] at day of hatch.

Table 4.13 Effect of phytase supplementation and Coccivac[1] B^{TM} on apparent ileal amino acid digestibility of 18-day-old broilers

Dietary*Vaccination		THR (%)	ILE (%)	LEU (%)	PHE (%)	LYS (%)	SER (%)	TOTAL (%)
PC	-	0.746	0.764	0.792	0.792	0.842	0.718	0.778
PC	+	0.610	0.640	0.681	0.686	0.760	0.584	0.672
PC + 1000	-	0.796	0.806	0.833	0.831	0.868	0.769	0.818
PC + 1000	+	0.745	0.755	0.789	0.793	0.844	0.725	0.780
NC	-	0.711	0.731	0.759	0.761	0.818	0.676	0.745
NC	+	0.636	0.676	0.715	0.717	0.771	0.612	0.695
NC + 1000	-	0.782	0.801	0.822	0.829	0.871	0.766	0.813
NC + 1000	+	0.707	0.746	0.773	0.782	0.837	0.708	0.767
NC + 5000	-	0.801	0.801	0.822	0.831	0.869	0.766	0.809
NC + 5000	+	0.726	0.733	0.768	0.778	0.828	0.704	0.758
Pooled SEM		0.0166	0.0166	0.0145	0.0144	0.0117	0.0180	0.0145
P-Value								
Diet*Vaccination		0.1323	0.1580	0.1176	0.1346	0.1390	0.1100	0.1496

[1] Vaccinated birds (designated by "+") were exposed to Coccivac B^{TM} at day of hatch.

CHAPTER V

Epilogue

Eimeria spp. provide a multifaceted challenge to the broiler industry. The complex lifecycle of the parasite and changes in the intestinal integrity resulting from the diet, inflammatory immune response, and bacterial colonies inhabiting the intestine confound the results presented in this dissertation. Nutrient digestion and goblet cell numbers were reduced, and crypt cell proliferation was increased in the jejunum of vaccinated birds. Goblet cells and enterocytes mature as they migrate from the crypts to the villi tip and a reduction in the number of goblet cells may be the result of a functionally immature intestine. Nutrient transporter expression along the brush border membrane may also be reduced in functionally immature intestines. In addition, a thin mucin layer may reduce the binding of digestive enzymes and nutrients for absorption, allow access of harmful bacteria into the cell, or reduce the colonization of beneficial bacteria.

Results from this research project would suggest dietary enzyme supplementation had beneficial responses associated with intestinal integrity and amino acid digestibility in broilers exposed to a live coccidia oocyst vaccine. Similar to published reports, administration of a live coccidia oocyst vaccine reduced broiler performance and nutrient digestion in two of the three studies presented. Phytase, xylanase, or the combination of phytase and xylanase may improve apparent ileal amino acid digestibility (IAAD) and promote mucin integrity in the jejunum of vaccinated broilers through the reduction of anti-nutrients such as phytate and non-starch polysaccharides, and the improvement in nutrient availability and digestion. Dietary phytate has been associated with an increase in endogenous losses of amino acids, minerals, and mucin and cell walls may encapsulate starches and amino acids, rendering them inaccessible for use by the

broiler. Improvements in amino acid, mineral, and energy availability may have provided a source of nutrients for the small intestine to activate an immune response, repair the intestine, and maintain homeostasis during a coccidia vaccination. Energy utilization by the small intestine may be increased during an intestinal disease. However, anorexia is a common symptom associated with coccidiosis due to secretion of cytokines such as IL-1 and IL-6 that stimulate leptin secretion from adipocytes. Stimulation of feed intake (FI) in morbid animals may be beneficial to alleviate reductions in body weight (BW) associated with the disease and anorexia, and reduce the use of amino acids for gluconeogenesis. Administration of pain relievers such as aspirin may temporarily relieve the pain and fevers associated with the inflammatory immune response and encourage FI in broilers. However, intestinal damage may result in a reduction in nutrient digestion, which can increase nutrients entering the distal intestine and facilitate pathogenic bacterial growth. Designing experiments to evaluate only one exogenous enzyme, its substrate and products, and utilization of comparative slaughter techniques to determine the energy or amino acids requirement of the intestine and skeletal muscle during coccidia vaccination may be advantageous to determine the nutrient requirements of vaccinated broilers and provide beneficial insight to the partitioning of nutrients during an intestinal disease. In addition, evaluation of nutrient transporter expression, digestive enzyme secretion, cell proliferation, mucin maturity, and phytate degradation may be beneficial in future studies to further characterize the shift in nutrient digestion and intestinal morphology associated with coccidia vaccination and enzyme supplementation.

From the previous trials, it appears that dietary mineral concentrations effect small intestinal morphology and bacterial proliferation. For example, Zn deficiency stimulated crypt cell proliferation as demonstrated by larger crypts in the ileum of mice fed Zn deficient diets. A

large Ca:aP ratio (4:1) may have resulted in a Zn deficiency which increased crypt cell depth. Calcium may have also been the predisposing factor that influenced the NE outbreak. Calcium may have acted as a buffer to reduce gastric pH and subsequent protein digestion. Calcium is essential for the alpha-toxin to bind to the phospholipid and may have acted as a catalyst to increase alpha-toxin activity and intestinal damage. Experiments evaluating the interaction between dietary minerals such as Ca and Zn, gastric acid secretion, nutrient digestion, other bacterial species, and *Clostridium perfringens* production of NE may facilitate the formulation of diets and development of nutritional supplements to alleviate NE. Ironically, phytate may have beneficial effects in regards to reducing the Ca available for use by the alpha-toxin in the distal intestine. Phytate is a strong chelator of cations and high dietary phytate may reduce the availability of Ca for use by the alpha-toxin. However, it would be assumed that most bacterial species within the lower intestinal tract would produce intrinsic phytase and therefore be able to release and utilize the nutrients bound to phytate. Unfortunately, experiments evaluating the effects of phytate supplementation on alpha-toxin activity may only be valid *in vitro* due to the anti-nutrient qualities of phytate *in vivo*.

Characterization of the composition of the receptors used by *Eimeria spp*. to bind and invade the intestinal epithelium, and the characterization of bacterial species prevalent during a coccidia infection, may provide pathways to develop dietary strategies to reduce parasite infestation. It is believed *Eimeria* and certain bacterial species bind to carbohydrate groups lining the intestine. Dietary ingredients such as enzymes, prebiotics, or probiotics are known to alter the intestinal mucin carbohydrate composition or promote beneficial bacterial growth or competitively exclude harmful bacteria in the distal small intestine and ceca. Therefore, supplementation of specific dietary ingredients may prevent coccidia binding by altering the

composition of carbohydrate moieties or reducing the number of receptors available for attachment. In addition, the supplementation of prebiotics such as mannans or glucans may act as an adjuvant for coccidia vaccinations. While the live oocyst vaccine stimulates an immune response and provides protection from secondary exposure, the large number of oocysts necessary to induce a protective response results in a reduction in broiler performance, feed efficiency, and alters intestinal integrity. Therefore, stimulation of an immune response by mannans or glucans may facilitate the use of a lower number of live oocysts included in the vaccine while promoting protection from secondary exposure of coccidia.

Unfortunately, interactions between coccidia and bacteria encompass a multi-factorial interaction between animal health and production status, immune function, dietary ingredients, and genetics. Therefore, one dietary recommendation may be beneficial to stimulate an immune response in coccidia vaccinated broilers but harmful in broilers not exposed to coccidia due to stimulation of the immune response. A dietary ingredient known to increase FI in sick or anorexic broilers may exacerbate bacterial proliferation by increasing digesta transit time and allowing excess nutrients to flow to the distal intestine. Altering mucin composition to reduce coccidia binding may promote the binding of other harmful bacteria. Therefore, understanding the physiology of the immune response, which cells are active during the primary and secondary infections, and how the nutrient requirements change regarding the innate verses humoral immune response and dietary supplementation of enzymes, probiotics, or minerals, characterization of intestinal bacteria, and their interactions should be critical components evaluated when designing dietary regimes or ingredients to protect against intestinal parasites.