The Effects of Dietary Calcium and Enzyme Supplementation on the Occurrence of Necrotic Enteritis

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Abstract

Diet composition and nutrient balance can have a critical impact on intestinal integrity during exposure to enteric pathogens. Researchers have extensively reported benefits on nutrient availability and broiler performance as a consequence of the impact of phytase supplementation. However, the poultry industry has little information on the effects of phytase supplementation in disease settings. The objective of these studies was to evaluate phytase supplementation impact on bird performance, intestinal morphology and pH, nutrient digestibility and bone mineralization during necrotic enteritis (NE). In each experiment, Cobb 500 broilers were obtained from a commercial hatchery and housed in floor pens at the Virginia Tech Turkey Research Center. Birds were placed on used litter from a previous flock that had presented clinical signs of NE. Broilers were fed non-medicated diets formulated to meet NRC (1994) nutrient requirements, except for calcium and phosphorus. In the first experiment, birds began exhibiting clinical signs of NE on d 9, and elevated NE-associated mortality persisted until d 26. Mortality was influenced by the main effects of dietary Ca or phytase. Dietary Ca supplemented at 0.9% or 1000 FTU/kg of phytase increased mortality compared to 0.6% Ca or 0 FTU/kg phytase, respectively, from d 0 to 19. Feed intake (FI) and feed conversion (FC) were affected by Ca x P interaction. From d 0 to 19, birds fed 0.9% Ca and 0.3% available P (avP) had decreased FI and improved FC compared to birds fed 0.9% Ca and 0.45% avP, while FI and FC were similar in birds fed diets with 0.6% Ca, regardless of avP level. Calcium x P x phytase interaction influenced

BW or BWG from d 0-12. In general, birds fed 0.9% Ca and 0.45% avP with phytase were heavier compared to birds fed 0.6% Ca, 0.45% avP, and phytase. Calcium at 0.9% increased gizzard (d 19) and jejunum (d 12) pH. Dietary Ca supplemented at 0.9%, avP supplemented at 0.45%, and 1,000 FTU/kg phytase significantly increased tibia ash weight compared to 0.6% Ca, 0.3% avP, and 0 FTU/kg phytase, respectively, on d 12. A 3-way interaction was observed on d 35 for tibia ash percentage; birds fed 0.9% Ca and 0.45% avP had a significant increase in tibia ash percentage, regardless of phytase supplementation. A 3-way interaction was also observed for Ca and P digestibility on d 35. Phytase supplementation significantly increased Ca digestibility regardless of Ca and P levels of the diets. In addition, diets containing 0.6% Ca and 1,000 FTU/Kg of phytase resulted in a significant increase in P digestibility, regardless of P levels. In the second experiment, birds also began exhibiting clinical signs of NE on d 9, and elevated NE-associated mortality persisted until the end of the trial (d 21). Mortality was significantly affected by an interaction between Ca source and Ca levels. Significantly higher mortality was observed when animals were fed 0.9% Ca diets formulated with calcified seaweed from d 0-21 compared to 0.6% Ca diets (regardless of Ca source). From d 0-7, birds fed 0.6% Ca in diets supplemented with phytase had heavier BW than the other treatments regardless of Ca source. From d 0-14 and 0-21, animals fed diets with calcified seaweed had significantly higher FC than animals fed diets with limestone. On d 21, the gizzard of birds fed 0.9% Ca was significantly less acidic than the gizzard of birds fed 0.6% Ca. In conclusion, reducing dietary levels of Ca associated with phytase supplementation improved bird performance and nutrient digestibility. In addition, these experiments indicate that Ca is an important dietary factor in the pathogenesis of NE.

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Chapter I: Introduction

In sight of new market demands concerning the decreased use of growth promoters and anticoccidial drugs in feed formulations, the poultry industry has been trying to reduce or eliminate the inclusion of sub-therapeutic doses of antimicrobials into feed. Formulating diets for its effects on gastrointestinal health is becoming a reality for poultry nutritionists. In addition, nutritionists realized that maintenance and enhancement of gut health is essential for animal performance when antimicrobials are not allowed in feed.

Necrotic enteritis has reemerged as an important disease of poultry in recent years. The reduction in the use of sub-therapeutical doses of antimicrobials in poultry feeds has been attributed as one of the mains causes of the increasing incidence of necrotic enteritis in commercial poultry. Mortality due to necrotic enteritis is extremely high (1% daily mortality), which results in great economical losses. Economic losses due to necrotic enteritis are not only associated with high mortality, but also associated with decreases in bird performance and feed efficiency. Animals that survive necrotic enteritis outbreaks usually have a reduced ability to digest and absorb nutrients due to extensive damage to the mucosal lining.

The gastrointestinal system is not only the major site for nutrient digestion and absorption, but also works as the largest immunological organ in the animal, protecting the host against pathogens. The gastrointestinal tract ecosystem is very susceptible to dietary composition. Feed additives, such as enzymes, probiotics and prebiotics, can alter intestinal microbiota and ultimately impact bird performance and animals' susceptibility to diseases.

Exogenous enzymes are added to poultry feed with the objective to improve nutrient availability and digestibility, which ultimately results in improvements in bird performance.

The mechanisms in which these enzymes improve bird performance have been extensively researched in healthy birds. However, limited literature is available in regards to how animals respond to disease challenges when they are being supplemented with exogenous phytases. Therefore, it is essential to research the impact of enzyme supplementation and nutrient balance in disease settings.

Chapter II: Literature Review

Necrotic Enteritis

Necrotic enteritis (NE) is an enterotoxaemia of poultry with an important economic impact on poultry production. It has been estimated that NE costs the poultry industry US $2 billion globally, as a result of reduction in bird performance and disease treatment (Van der Sluis, 2000; McReynolds et al., 2004). The reduction in bird performance is not only associated with impaired growth rate and feed conversion (FC) during production, but also with increased condemnation rates in broilers due to hepatitis at the processing plant (Immerseel et al., 2004; Opengart, 2008).

In the past, the use of antimicrobial growth promoters (AGP) in poultry feed helped controll NE in poultry flocks (Williams, 2005; Lee et al., 2012). However, NE has reemerged as a significant problem in poultry production, likely as a result of national and international policies that ban or limit the use of AGP in poultry feeds (Williams, 2005; Opengart, 2008; Cooper and Songer, 2009). In addition, consumers' preferences have had a large impact on animal production, and the push for poultry production with less medication in poultry diets have also had a significant impact on the increase in the incidence of NE in the past few years (Cooper and Songer, 2009).

Etiology and Epidemiology

Clostridium perfringens (types A and C) is the etiological agent associated with NE (Opengart, 2008). *C. perfringens* is a Gram-positive, rod-shaped, anaerobic encapsulated

bacterium that causes a broad spectrum of human and veterinary diseases (Smith, 1992; McClane, 2001; Wrigley, 2001). *C. perfringens* differs from many other clostridia in being nonmotile, reducing nitrate, and carrying out a stormy fermentation of lactose in milk (Setlow and Johnson, 2001; Wrigley, 2001). *C. perfringens* also ferments glucose, fructose, galactose, inositol, maltose, mannose, starch and sucrose. The fermentation products include acetic and butyric acids with or without butanol (Smith, 1992). *C. perfringens* will grow over a wide pH range varying from 5.5 to 8.5, while optimum growth of this bacterium occurs at pH 6 to 7 (McClane, 2001; Setlow and Johnson, 2001). The virulence of *C. perfringens* largely results from its prolific toxin-producing ability. The classification scheme for *C. perfringens* assigns isolates to one of the five types (A-E) depending upon their ability to express one or all four "typing" toxins (alpha, beta, epsilon, and iota) (Smith, 1992; McClane, 2001; Wrigley, 2001).

C. perfringens has a wide distribution being isolated from the intestine of warm blooded animals, and a variety of environments such as soil, water and feed (Smith, 1992; Immerseel et al., 2004). Colonization in the intestines of poultry may happen as early as day-of-hatch in the hatcheries. In fact, Craven et al. (2001) were able to isolate *C. perfringens* from eggshell fragments, chicken fluff, and paper pads in commercial broiler hatcheries. Given that *C. perfringens* is a naturally occurring bacterium in the intestinal environment of poultry, disease development is dependant on other predisposing factors that will be discussed later in this review.

Although chickens from 2 weeks to 6 months are susceptible to NE, incidence varies greatly according to type of bird and raising conditions. Most NE outbreaks in broiler flocks raised on litter are reported between the 2^{nd} and 5^{th} week of age (Williams, 2005; Opengart,

2008; Timbermont et al., 2011). On the other hand, NE outbreaks in commercial layers raised in floor pen settings have been reported between 3 to 6 months of age (Opengart, 2008). Mortality rates associated to NE in broilers are often between 2 and 10%, however rates as high as 50% have been reported (McDevitt et al., 2006; Lee et al., 2011).

Pre-disposing Factors

C. perfringens is naturally occurring bacterium in the intestines of warm blooded animals, and its presence itself is not a determining factor for disease development. Therefore, pre-disposing factors that could lead to an outgrowth of *C. perfringens* are crucial to NE onset and development. Several different factors have been identified as pre-disposing factors for NE: diets, immune status and stress, intestinal physiopathology, and coccidiosis.

Dietary factors. Several different dietary components may favor *C. perfringens* growth, and consequently NE onset and development. The type of cereal used in poultry diets was identified as one of these components. Research has shown that the use of diets formulated using cereals (barley, rye, oats and wheat) containing high levels of indigestible, water-soluble, non-starch polysaccharides (NSP) predispose birds to NE (Branton et al., 1987; Riddell and Kong, 1992). While the mechanism in which NSP predisposes birds to NE is not clear, the impact that NSP have on bird performance, nutrient digestibility and digesta viscosity has been extensively reported. Complex carbohydrates are known to have a negative impact on bird performance (Antoniou et al., 1981; Hesselman and Aman, 1986). The reduction in performance can be associated with a decrease in nutrient digestibility of several nutrients such as amino acids, fat and cholesterol, and decreased dry matter retention

(Hesselman and Aman, 1986; Ward and Marquardt, 1987; Fengler and Marquardt, 1988). These nutrients then become available to microbial attack in the lower small intestine, and could potentially change the type and balance of intestinal microbiota (Fengler and Marquardt, 1988; Langhout et al., 1999).

Jia et al. (2009) reported that digesta viscosity was greater in birds consuming diets containing high levels of NSP. The increase in digesta viscosity due to NSP has been associated with an increase in mucous secretion (Langhout et al., 1999) and an increase in water binding capacity by complex carbohydrates (Antoniou et al., 1981; Fengler and Marquardt, 1988). High digesta viscosity impairs nutrient digestion by interfering with the interaction between digestive enzymes and their substrates, and impeding nutrient uptake in the gastrointestinal tract (Antoniou et al., 1981; Hesselman and Aman, 1986; Fengler and Marquardt, 1988). As a result, an influx of undigested nutrients enters the distal segments of the small intestine increasing microbial growth. High digesta viscosity is also related to an increase in mucous secretion (Langhout et al., 1999). This nutrient rich environment favors *C. perfringens* over other bacterial species, as this bacterium is known to have mucolytic activity and a short generation time (Deplancke et al., 2002; Collier et al., 2003, 2008).

Dietary protein levels and protein source are also dietary factors that can pre-dispose birds to the development of NE. Protein rich diets result in a high concentration of protein in the gastrointestinal tract that will serve as substrate for microbial growth (Timbermont et al., 2011). In addition, in the small intestine, proteins are degraded to nitrogenous compounds (ammonia and amines) that not only increase intestinal pH (high pKa of nitrogenous compounds), but also favor the proliferation of pathogenic bacteria, including *C. perfringens* (Smith, 1965; McDevitt et al., 2006). Evidence suggests that protein source is more

important than the protein levels in the diet itself. High inclusion levels of animal protein sources (fishmeal, meat and bone meal) are usually associated with an increase in the incidence of NE in broilers (Drew et al., 2004). Timbermont et al. (2011) hypothesized that animal protein sources would have higher levels of indigestible protein that would reach the ceca and serve as substrate for *C. perfringens*. Another possibility is that the amino acid balance (specially methionine and glycine) in these feed ingredients would ultimately favor *C. perfringens* growth in the ceca, leading to NE (Drew et al., 2004; Williams, 2005). However, it is still unclear why high inclusion levels of animal protein in the diet of broilers results in an increase in the prevalence of NE.

Depending on diet composition (especially cereal type), feed form (mash vs. pellet) may also influence the incidence of NE (Branton et al., 1987; McDevitt et al., 2006). Branton et al. (1987) reported that birds fed diets in the mash form were associated with higher mortality due to NE. This might be due to the fact that the feed particles in mash diets are smaller. Smaller particles are not only more easily digested by birds' digestive enzymes, but also by microbial populations in the ceca (Immerseel et al., 2004; Cooper and Songer, 2009). Additionally, when birds are fed mash diets, and increase in digesta viscosity is usually observed. The effects of digesta viscosity in the incidence of NE will be discussed further in this chapter.

Immune Status and Stress. Immunosupression predisposes animals to NE because the factors that usually lead to immunosupression likely alter the intestinal environment and the intestinal microbial population (McDevitt et al., 2006; Timbermont et al., 2011). Infections with pathogens that lead to immunosupression (*Eimeria* spp., infectious bursal

disease virus (IBDV), chick anemia virus, and Marek's disease virus) have been reported to predispose chickens to NE (McReynolds et al., 2004; Lee et al., 2011). In a NE disease model study, McReynolds et al. (2004) reported that birds infected with IBDV often have secondary infections with *C. perfringens*. Stress has probably the same immunosuppressive effects of the aforementioned pathogens. Environmental (heat or cold stress) and managerial (feed changes, litter conditions, stocking density, vaccination programs) stressors have been shown to cause immunosupression, predisposing animals to disease (McReynolds et al., 2004; McDevitt et al., 2006; Tsiouris et al., 2009).

Intestinal Physiopathology. When intestinal conditions are not favorable, even highly virulent *C. perfringens* strains fail to produce disease. However, there are some intestinal physiopathological circumstances that favor the development of NE, such as intestinal stasis, gastrointestinal pH, and damage to the intestinal mucosa (Williams, 2005; Cooper and Songer, 2009; Lee et al., 2011). When intestinal motility is reduced (i.e. increased digesta viscosity) feed passage is delayed. This increases nutrient availability to the microbial population in the gastrointestinal tract, allowing *C. perfringens* to outgrow other species (Williams, 2005). Associated with an increase in nutrient availability, an increase in transit time also means a reduction in microbial *flushing* from the gastrointestinal tract, which increases the opportunity of pathogens to proliferate, colonize and cause disease.

Intestinal acidity can also have an impact on NE onset. Higher intestinal pH can predispose birds to develop NE, since *C. perfringens* growth is inhibited in more acidic intestines (Kmet et al., 1993). Damage of the intestinal mucosa is another condition that predisposes broilers to develop NE. This predisposition is likely a combination of effects

such as changes in intestinal pH, excess nutrient in the gastrointestinal tract (birds are usually unable to digest and absorb nutrients), leakage of plasma proteins and growth factors to the intestinal environment, and loss of intestinal integrity. These factors will be discussed in more detail further in the coccidiosis section of this chapter.

Coccidiosis. Although coccidiosis appears to be the most studied pre-disposing factor of NE, it is still the one we understand the least. Coccidiosis in chickens is usually caused by the association of two or more species of *Eimeria*: *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella* (McDougald, 2008). Coccidia oocysts are ubiquitous in areas where poultry are raised allowing this parasite to cycle and remain in the environment (Williams, 2005). The *Ei*meria sp. life cycle is complex and involves stages inside and outside of the host. Once inside the host, the cycle consists of extracellular and intracellular phases, and sexual and asexual reproduction. Coccidiosis infection occurs via the fecal-oral route. Excreted oocysts are dependent on moisture and oxygen to sporulate and become infective. Animals ingest sporulated oocysts that excystates and release individual sporozoites into the intestinal lumen. Sporozoites invade the enterocytes and form trophozoites. Trophozoites undergo nuclear divisions forming immature meronts. Inside the meronts, schizonts reproduce asexualy (multiple fission) forming merozoites. Mature meronts rupture the host enterocyte releasing the merozoites in the intestinal lumen, which undergo differentiation stages of merogony. Merezoites invade enterocytes and develop into microgamonts or macrogamonts. Microgamonts undergo multiple divisions until they become microgametes (flagellated), and macrogamonts develop into a macrogamete. Microgametes rupture the enterocyte in which they were developing and invade the

enterocyte that contains a macrogamete. Microgametes fertilize the macrogamete, and the oocyst wall is formed. Following oocyst maturation, the enterocyte ruptures releasing oocysts in the intestinal lumen, which will be excreted in the environment.

The consequence of the *Eimeria* cycle described above is extensive damage to the intestinal mucosa. The intestinal lumen then becomes rich with plasma proteins, which are themselves rich in amino acids, growth factors and vitamins that serve as substrate for clostridial growth (Immerseel et al., 2004; Williams, 2005; Timbermont et al., 2011). In addition, digestibility of nutrients is reduced due to extensive gastrointestinal damage, which may substantially increase nutrient availability for *C. perfringens* (Lillehoj and Trout, 1996; Williams, 2005). In addition, coccidiosis induces a local T cell-mediated inflammatory response that increases mucin (and mucous) production (Lillehoj and Trout, 1996; Collier et al., 2008). The increase in mucous production ultimately favors *C. perfringens* growth due to its mucolytic ability (Collier et al., 2003, 2008).

Clostridium perfringens **Toxins**

C. perfringens are extra-cellular pathogens, thus its virulence is mostly associated with its ability to produce toxins. *C. perfringens* can produce up to seventeen toxic or potentially toxic exoproteins (Songer, 1996). The species is divided into types (A, B, C, D, and E) according to the production of four major toxins: alpha, beta, epsilon, and iota. Type A comprises the strains that produce alpha-toxin, type B as strains that produce alpha, beta, and epsilon toxins, type C as strains that produce alpha and beta toxins, type D as strains that produce alpha and epsilon toxins, and type E as strains that produce alpha and iota toxins

(Songer, 1996; Petit et al., 2009). Since only *C. perfringens* types A and C are associated with NE, only alpha and netB toxins will be discussed in this review.

Alpha-toxin. Alpha-toxin is a zinc-dependent phospholipase sphingomyelinase C, and is encoded by the *Plc* gene (Guillouard et al., 1997; Titball et al., 1999; Cooper and Songer, 2009). Alpha-toxin is organized in two distinct domains: the N-terminal region (247 residues), that carries the active site required for phospholipid hydrolysis; and the C-terminal region (123 residues) that carries the lipid-binding site (Guillouard et al., 1997; Petit et al., 1999). Alpha-toxin requires zinc for substrate hydrolysis (Guillouard et al., 1997; Titball et al., 1999; McDevitt et al., 2006). In addition to zinc, alpha-toxin also depends on Ca for full activity. Calcium ions are essential for the binding of alpha-toxin to lipid films (Moreau et al., 1988; Petit et al., 1999; Titball et al., 1999). High Ca concentrations are required for optimal enzymatic activity with physiological substrates (Guillouard et al., 1997; Sakurai et al., 2004). Alpha-toxin has been shown to be cytotoxic leading to cell lysis of erythrocytes, phagocytes, fibroblasts, platelets, leukocytes, endothelial cells, and myocytes (Songer, 1996; Guillouard et al., 1997).

Alpha-toxin hydrolyses phospholipids and promotes cellular membrane disorganization. *In vivo*, the cellular substrates for alpha-toxin are phosphatidylcholine and sphingonyelin, which are both components of the cellular membrane of epithelial cells in the gastrointestinal system (Titball et al., 1999; McDevitt et al., 2006). The mechanism of membrane recognition is a complex event. This mechanism involves a Ca mediated phospholipid recognition, where Ca ions are partially coordinated by acidic amino acid side chains of alpha-toxin, and partially by phosphate groups or membrane phsopholipids (Titball

et al., 1999). Hydrolysis of cell membrane phospholipds results in the formation of diacylglycerol, following an activation of protein kinase C, and consequent activation of arachidonic acid cascade (Petit et al., 1999; Sakurai et al., 2004; McDevitt et al., 2006). The outcome of the activation of the arachidonic acid cascade is the synthesis of inflammatory mediators (leukotrienes, tromboxane, platelet-agglutinating factor, and prostacyclin), which cause blood vessel contraction, platelet aggregation, myocardial dysfunction, leading to acute death (Petit et al., 1999; Immerseel et al., 2004; Sakurai et al., 2004).

Alpha-toxin has been indicated as the main virulence mediator for NE in poultry. Al-Sheikhly and Truscott (1976) were able to successfully reproduce NE in birds infused intraduodenally with bacteria-free crude toxin. Another indication of alpha-toxin participation in NE pathogenesis is that *C. perfringens* isolates from broilers with NE significantly produces more alpha-toxin than isolates from broilers without NE (Hofshagen and Stenwig, 1992). Studies of broilers' immune response to NE also suggest that alpha-toxin is an important virulence factor in the pathogenesis of NE. Broilers afflicted with NE have significantly lower antibody levels against alpha-toxin than healthy controls (Lovland et al., 2004; Lee et al., 2012). Higher levels of toxin-reactive antibodies in healthy chickens when compared to chickens with NE symptoms, indicate that these antibodies may protect birds from developing NE.

The role of alpha-toxin in producing NE in broilers is a very controversial topic. Interpretation of early results can be disputed because most of the studies which reported alpha-toxin as the main virulence factor in the pathogenesis of NE used crude supernatant instead of purified alpha-toxin (Al-Sheikhly and Truscott,1976; Hofshagen and Stenwig, 1992). Conclusions of these studies were based on alpha-toxin being the most dominant

protein present in crude supernatants, and did not consider the presence of other toxins in crude supernatants that might be collaborating or even responsible for the development of NE (Immerseel et al., 2009). The most convincing evidence that alpha-toxin is not the main virulence mediator in the development of NE comes from Keyburn et al. (2006) study where an alpha-toxin negative mutant of a *C. perfringens* strain from an NE outbreak was still able to produce NE in broilers. Other factors of NE pathogenesis also indicate that alpha-toxin is not the main virulence factor of NE. One of the hallmarks of NE in broilers is granulocyte migration from the tissue to the intestinal lumen (Olkowski et al., 2006). This inflammatory reaction is very different from the leukostasis and lack of inflammatory response induced by alpha-toxin in gas gangrene (Bryan et al., 2006), indicating that NE lesions are probably mediated by toxins other than alpha-toxin.

NetB toxin. NetB is a pore-forming toxin, with similarity to *C. perfringens* beta-toxin (38% identity), *C. perfringens* gamma-toxin (40% identity), *Staphylococcus aureus* alpha-hemolysin (30% identity), and *S. aureus* gamma-toxin (23% identity) (Keyburn et al., 2010). These toxins form pores in the cellular membrane causing an influx of ions (Ca, Na, Cl, etc) that eventually lead to osmotic cell lysis. There is strong evidence showing that netB is an essential virulence mediator for the development of NE. Initial screening of poultry NE isolates found that the majority of the isolates (77%) were netB positive (Keyburn et al., 2008 and 2010). In addition, non-necrotic enteritis *C. perfringens* isolates were analyzed for the presence of the netB gene, and most of these isolates (91.2%) were found to be netB negative (Keyburn et al., 2010). Keyburn et al. (2008) reported that *C. perfringens* netB knock out isolates were not able to cause NE, while the original netB positive isolates were

able to cause disease. Therefore, although there is a clear association between netB and NE development, since not all *C. perfringens* isolates from NE outbreaks were netB positive, there may be other virulence factors that play an important role in the onset and development of NE. The presence of netB gene in isolates from healthy birds also suggests that netB's presence is not sufficient to cause disease, which reveals the importance of pre-disposing factors for the development of NE.

Clinical Signs and Pathology

Necrotic enteritis clinical signs are common to enteritis in general: depression, anorexia, diarrhea, dehydration, and ruffled feathers (Immerseel et al., 2004; Opengart, 2008; Cooper and Songer. 2009). The classic acute form of NE is characterized by a sudden increase in flock mortality without any warning clinical signs (Immerseel et al., 2004; Timbermont et al., 2011). The subclinical form of NE is usually mild with no clinical signs or peak mortality. Most of the time, only an overall reduction in bird performance is observed (Hofshagen and Stenwig, 1992; Immerseel et al., 2004). Performance losses are associated with chronic intestinal mucosal damage resulting in poor nutrient digestion and absorption, reduced body weight gain (BWG), and increased feed conversion (FC) ratio (Immerseel et al., 2004; Timbermont et al., 2011). In cases of sub-clinical NE, an increase in liver condemnations at the processing plant is often observed due to cholangiohepatitis (Immerseel et al., 2004; Timbermont et al., 2011). Therefore, the subclinical form of NE is harder to diagnose and birds are not treated, resulting in greater economic losses (Timbermont et al., 2011).

Macroscopic lesions are usually restricted to the jejunum and ileum, but may extend to the duodenum and ceca (Opengart, 2008; Cooper and Songer, 2009; Timbermont et al., 2011). The small intestine is usually distended and filled with gas; intestinal walls are thin and friable (Opengart, 2008; Timbermont et al., 2011). There is necrosis of the intestinal mucosa and presence of a green to yellow diphtheritic membrane that is adherent to the mucosa (Opengart, 2008).

Histopathology reveals a severe inflammatory response to *C. perfringens*. Inflammatory infiltrate is characterized by the presence of heterophils, lymphocytes, macrophages, and plasma cells (Olkowski et al., 2006; Timbermont et al., 2011). Diffuse and severe coagulative necrosis of the mucosa is also observed (Olkowski et al., 2006; Timbermont et al., 2011). Masses of tissue fragments, necrotic cells, cell debris and bacterial colonies comprise the diphteric membrane characteristic of NE (Olkowski et al., 2006; Timbermont et al., 2011). Blood vessel congestion can be observed in the lamina propria and submucosa.

Prevention, Treatment and Vaccination

Necrotic enteritis prevention is usually associated with management practices that minimize the effects of the pre-disposing factors that contribute to disease development. Removing dietary ingredients that may lead to NE, such as fish meal, oats, barley and rye, has been a noteworthy solution in reducing NE incidence (Cooper and Songer, 2009). The use of AGP in feed has also played an important role in the control of NE. The introduction of AGP in the diet assists with coccidiosis management, and modifies the intestinal microbial populations, which both result in a reduction in the incidence of NE (Immerseel et al., 2004;

Cooper and Songer, 2009). However, government bans, bacterial resistance to antimicrobials, and consumers' preferences regarding a medication-free final product have pushed the poultry industry towards reducing the use of AGP in poultry feed (Immerseel et al., 2004; Williams, 2005; Opengart, 2008). Other methods used to control coccidiosis, such as vaccination, may also have an indirect impact in reducing the incidence of NE (Immerseel et al., 2004).

The reduction in the use of antimicrobials in poultry diets resulted in an increase of antibiotics used in treatment of flocks with NE. Necrotic enteritis has been treated by administering lincomycin, bacitracin, oxytetracycline, penicillin, and tylosin in water (Opengart, 2008; Cooper and Songer, 2009). Bacitracin, lincomycin, virginiamycin, penicillin, avoparcin, and nitrovin can also be used in the feed to treat NE (Opengart, 2008; Cooper and Songer, 2009).

Vaccination studies as an effective method of NE prevention show inconsistent results. Most vaccination efforts have been directed to producing toxoid vaccines by using culture supernatant, which alpha-toxin is the major component (Immerseel et al., 2009; Keyburn et al., 2010; Lee et al., 2012). However, findings suggesting that alpha-toxin might not be the main virulence factor in the pathogenesis of NE, could explain why vaccination trials have been so inconsistent with results. There is strong evidence suggesting that netB could be used as a toxoid and offer a better protection (Keyburn et al., 2010; Lee et al., 2012). Though, since netB has been recently discovered, further studies on its mechanism of action and of immune response induction need to be performed before major advances can be made.

Phytate and Phytase

Phytate (myo-inositol hexakiphosphate) is a naturally occurring molecule in grains and seeds, and it is the main organic source of P in animal feedstuffs of plant origin (Sebastian et al., 1996b; Tamim et al., 2004; Selle et al., 2009). In addition to its role in P storage, phytate may also function as an antioxidant in seeds binding to iron, and preventing the combination of free iron and unsaturated fatty acids (Mullaney et al., 2000). Phytate is found in most vegetable feed ingredients at concentrations from 5-25g/kg (Cowieson et al., 2011). However, P bound to phytate is not available for absorption unless it is released from the inositol ring (Sandberg et al., 1993; Tamim et al., 2004). Researchers estimate that from 60 to 80% of the P present in seeds and grains is in the form of phytate phosphorus (PP) (Pirgozliev et al., 2007; Manangi and Coon, 2008). Phytate is usually chelated to dietary minerals such as manganese, sodium, potassium and Ca (Tamim et al., 2004; Selle et al., 2009; Powell et al., 2011). Other molecules such as small peptides, amino acids and sugars can also be indirectly bound to phytate by their association with the previously mentioned minerals (Tamim et al., 2004). Since minerals (especially divalent cations) and other nutrients (small peptides, amino acids, sugars) bind to phytate, the presence of high levels of phytate in the diet is usually related to reduced nutrient availability and absorption, which usually results in poor bird performance (Sebastian et al., 1996a,b; Tamim et al., 2004; Selle et al., 2009).

In order to minimize the detrimental effects of phytate, phytase supplementation has been largely used by poultry nutritionists since 1991 when formulating diets for broilers (Bedford, 2003). Phytases are phosphatases that hydrolyze phosphate from phytate

(Sebastian et al., 1996a; Tamim et al., 2004). There are two classes of commercial phytases that differ in the first phosphate group on the phytate molecule that undergoes phytase attack. The 3-phytases attack the carbon in the third position, whereas 6-phytases attack the carbon in the sixth position (Rutherfurd et al., 2012). Exogenous microbial phytases are mainly active in the stomach of pigs and forestomach of poultry (crop, proventriculus, and gizzard). The acidic pH of these organs increase phytate solubility, making phytate more susceptible to phytase attack (Selle et al., 2009).

Dietary PP is unavailable for the bird unless released from the inositol ring of phytate by endogenous or supplemented phytases (Sandberg et al., 1993). The use of phytase to hydrolyze phytate is well established, and has been extensively reviewed in the literature (Selle and Ravindran, 2007). Benefits from phytase supplementation are not restricted to improvements in P digestibility and absorption, but also include improvements in FC, BW and BWG, as well as nutrient utilization and bone mineralization in broilers (Cowieson et al., 2004, 2006). Performance improvements associated with phytase supplementation are consequent to the release of minerals from the phytate-mineral complex, the utilization of inositol by animals, and increased carbohydrate and protein digestibility (Sebastian et al., 1996a).

Improvements in bird performance are not only associated with greater P availability. Research has shown that diet supplementation with exogenous phytases improves birds' ability to metabolize protein and energy through the release of amino acids, small peptides and carbohydrates that are indirectly bound to the phytate molecule (Ravindran et al., 1995; Cowieson et al., 2006; Cowieson et al., 2011). In addition, nutrient release does not seem to be the only mechanism in which phytase supplementation improves bird performance. It has

been shown that the ingestion of phytic acid can negatively influence amino acid, energy and mineral excretion by broilers (Cowieson et al., 2004). The mechanisms by which phytate ingestion alters gastrointestinal physiology are not completely understood, however it seems to involve the reactive nature of phytic acid and the electrostatic aggregation of dietary protein in the gastric phase of digestion (Cowieson et al., 2009; Selle et al., 2009). Also, high phytate levels in the diet are known to increase endogenous losses by interacting with endogenous enzymes or gastrointestinal mucin, increasing the excretion of endogenous amino acids and minerals (Cowieson et al., 2004).

The beneficial effects of phytase supplementation are likely to be a direct consequence to the negative effects of phytate, which are mediated by a reduction in endogenous losses, and improvements in the digestion, absorption and retention of nutrients. However, there are also indirect benefits from phytase supplementation that go beyond animal performance. Phosphorus in chicken litter is a major pollutant subject to strict governmental regulations. Phosphorus pollution is a hazard to aquatic ecosystems because P is the primary cause of eutrophication in fresh-water reserves (Selle et al., 2009). Fecal P consists of undigested portions of phytate-bound, and nonphytate P from plant sources, undigested portions of P from animal by-products and mineral supplements, and available P that exceeded animals' needs (Waldroup, 1999). Since phytase improves P availability, poultry nutritionists are able to formulate diets supplemented with lower levels of inorganic P, which reduces the P concentration in poultry litter by up to 30% (Perney et al., 1993; Ferguson et al., 1998; Salarmoini et al., 2008). Another approach to reducing dietary P levels, and minimizing excreted P is to develop grains that have reduced levels of phytate (Waldroup, 1999).

It is estimated that over half of the pig and poultry diets formulated worldwide are supplemented with an exogenous source of phytase (Bedford, 2003; Selle et al., 2009). However, less than 35% of phytate within broiler diets is hydrolyzed by phytase as measured by ileal disappearance (Powell et al., 2011). The incomplete hydrolysis of phytate offers the opportunity to evaluate how different ingredients used in broiler diets affect phytase efficiency, and Ca appears to be a major nutrient that affects phytase efficacy**.**

Calcium, Phytate and Phytase

Calcium is the most abundant mineral (by mass) in most animals, making its requirement higher than any other mineral. Therefore, Ca is a major mineral to be supplemented in the diet of many production animals by the use of inorganic sources (Sebastian et al., 1996b). The most recent published guidelines for nutrient requirements for poultry (NRC, 1994) suggest that the Ca requirements for growing broilers are 1.0% from d 0 to 21, 0.9% from d 21 to 42, and 0.80% from d 42 to 56. However, the experiments used to determine Ca requirement for broilers were not conducted with the supplementation of phytase in the diet (NRC, 1994).

Calcium must be soluble in the intestinal lumen in order to be absorbed. Calcium solubility in the intestine is closely related to small intestinal pH (around 6.0). When pH is close to neutral, phytate forms mineral chelates that are highly insoluble (Sebastian et al., 1996b; Tamim et al., 2004; Plumstead et al., 2008). In addition, a high ratio of dietary Ca to P reduces the digestibility and absorption of Ca and P due to increased precipitation of Ca-P complexes (Plumstead et al., 2008; Selle et al., 2009). Selle et al. (2000) have reported that maximum insolubility of phytate-mineral chelates occurs between pH of 4 and 7. Calcium is

one of the divalent cations with lowest affinity for phytate. However, since Ca is the mineral added in highest concentrations to poultry diets, it has a greater impact on forming mineral-phytate chelates than other dietary minerals, making both, Ca and P, unavailable for absorption (Sebastian et al., 1996b; Maenz et al., 1999; Tamim et al., 2004). The phytate molecule can carry up to twelve negative charges, thus having the potential to chelate six Ca atoms (Selle et al., 2009). Therefore, phytase supplementation improves digestibility, absorption and retention of Ca, which is consequent to the release of Ca from Ca-phytate complexes (Sebastian et al., 1996a; Selle et al., 2009; Rutherfurd et al., 2012).

Calcium source has also a significant impact on Ca-phytate precipitation in the gastrointestinal tract of poultry. Limestone, the most commonly used source of Ca in poultry diets, tends to increase digesta pH along the gastrointestinal tract due to its extremely high acid binding capacity (Shafey et al., 1991; Lawlor et al., 2005; Selle et al., 2009). The increase in digesta pH reduces the solubility of Ca and phytate, which tend to form complexes that precipitate and become unavailable for the bird (Sebastian et al., 1996b; Tamim et al., 2004; Plumstead et al., 2008).

Several authors have reported that elevated levels of Ca in the diet decreased phytase efficacy (Applegate et al., 2003; Tamim et al., 2004; Yan et al., 2006). Applegate et al. (2003) reported that a dietary Ca level commonly used in broiler diets (0.9%) resulted in a reduced intestinal phytase activity and reduced apparent ileal PP hydrolysis compared with lower Ca level (0.4%). A high Ca and total P (tP) ratio appeared to exacerbate this effect, by increasing the formation of mineral-chelate complexes and decreasing phytase activity (Qian et al., 1997; Tamim et al., 2004; Selle et al., 2009). Therefore, it has been postulated that high levels of Ca, and high Ca:P ratios have a negative impact on phytase efficacy in poultry diets.

Poultry possess effective phytase/phosphatases activity in the intestinal mucosa, blood, and liver, and can readily dephosphorylate phytate (Maenz et al., 1999; Cowieson et al., 2011). However, phytases can only hydrolyze phosphate groups from the inositol ring when phytate is in solution (Wise, 1983). As previously mentioned, mineral-phytate chelates are most insoluble between pH 4 and 7. Therefore, in the small intestine, mineral-phytate complexes tend to precipitate, becoming unavailable to phytase activity. Thus, the problem with phytate digestion is not a lack of compatible endogenous enzymes but poor substrate solubility in the small intestine. This effect is increased when high amounts of Ca are present (Tamim et al., 2004; Cowieson et al., 2011). Shafey et al. (1991) reported that increased dietary Ca and available P (avP) levels reduced the proportion of soluble minerals and thereby further decreased the availability of these minerals due to higher precipitation of mineral-phytate complexes. This effect was confirmed by Maenz et al. (1999) when they introduced a phytate competitor (EDTA) in broiler diets. The addition of EDTA prevented the inhibitory effect of Ca in phytate hydrolysis. In summary, this effect is described as precipitation of Ca-phytate complexes due to high availability of Ca (in high Ca diets) in the intestinal lumen (pH around 6), because when the substrate precipitates, the enzyme (phytase) cannot hydrolyze it.

Calcium and Animal Performance

In the past, since inorganic sources of Ca and P were inexpensive when compared to other mineral sources, little effort was made to determine the exact Ca and P requirements in broiler diets (Selle et al., 2009; Powell et al., 2011). Therefore, the poultry industry still utilizes NRC (1994) standards for Ca to formulate broiler diets. Calcium and phosphorus are

usually supplemented in poultry diets by the inclusion of limestone, dicalcium phosphate, and meat-and-bone meal (where permitted). Inorganic sources of Ca and P are escalating in price, and the cost of supplementing diets with exogenous phytases is decreasing (Selle et al., 2009). In addition, inorganic sources of Ca and P reserves are not renewable, and their depletion can be delayed by reducing the inclusion levels of Ca and P in animal feed (Mullaney et al., 2000; Selle et al., 2009).

Benefits from supplementing broiler diets with lower levels of Ca are supported by performance results. Anderson et al. (1984) reported that BWG, FC, and bone ash were all significantly reduced by increasing dietary Ca from regular industry standards (0.9%) to 1.5% in the diet. Sebastian et al. (1996) were the first to challenge NRC (1994) recommended Ca levels in diets supplemented with phytase. In their study, efficacy of supplemental phytase was significantly affected by dietary Ca levels, and the optimal growth performance, and retention of P and Ca were achieved at the lowest level of dietary Ca tested (0.6%). Selle et al. (2009) reported that over a 30-day feeding period, high levels of Ca depressed BWG (32%), FI (16.2%), and FC (18.8%) in pigs receiving phytase in the diets.

Benefits from phytase supplementation in nutrient digestibility and bird performance have been extensively reported in the literature. However little information on the effects of enzyme supplementation during disease is available. Additionally, the introduction of new feed additives to poultry diets in the past 20 years, such as exogenous enzymes, significantly impacted nutrient availability and digestibility. However, nutrient requirements for poultry have not been revisited since the last NRC (1994) was published. Thus, the poultry industry needs to re-evaluate nutrient requirements, and research the influence of these new feed additives during disease.

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Chapter III: Influence of calcium, phosphorus and phytase on bird performance, intestinal morphology, mineral digestibility and bone ash during a natural necrotic enteritis episode

ABSTRACT

Diet composition and nutrient balance have a critical impact on intestinal integrity during exposure to enteric pathogens. The objective of this study was to evaluate dietary Ca, P, and phytase on performance, intestinal morphology, bone ash, and Ca and P digestibility during a necrotic enteritis (NE) outbreak. Day-old, Cobb 500 male broilers were weighed and randomized into 8 treatment groups (9 pens/treatment; 32 birds/pen). The 35 d trial was designed as a 2 x 2 x 2 factorial, which included 2 levels (0.6% and 0.9%) of a highly soluble Ca source, 2 levels of available P (0.3% and 0.45%), and 2 levels of an *E. coli* phytase (0 and 1,000 FTU/kg). Birds were placed on litter from a previous flock that exhibited clinical signs of NE. Birds and feed were weighed on d 12, 19 and 35, and body weight (BW), BW gain (BWG), feed intake (FI), and feed conversion (FC) were calculated for each of these periods and cumulatively. Mortality was recorded daily, and pH of the gizzard, duodenum and jejunum was measured, and tibias and ileal digesta were collected. Birds began exhibiting clinical signs of NE on d 9 and elevated NE-associated mortality persisted until d 26. Dietary Ca supplemented at 0.9% or 1000 FTU/kg phytase significantly increased mortality compared to 0.6% Ca or 0 FTU/kg phytase, respectively. FI and FC were affected by a Ca x P interaction. From d 0-19, birds fed diets with 0.9% Ca and 0.3% avP had decreased FI and improved FC compared to birds fed 0.9% Ca and 0.45% avP, while FI and FC were similar in birds fed diets with 0.6% Ca regardless of P level. Calcium x P x phytase influenced BW or BWG from d 0-12. In general, birds fed 0.9% Ca and 0.45% avP with phytase were

heavier compared to birds fed 0.6% Ca, 0.45% avP, and phytase. Calcium at 0.9% increased gizzard (d 19) and jejunum (d 12) pH. Dietary Ca supplemented at 0.9%, P supplemented at 0.45%, and 1,000 FTU/kg phytase significantly increased tibia ash weight compared to 0.6% Ca, 0.3% avP, and 0 FTU/kg phytase, respectively, on d 12. Tibia ash % was influenced by a 3-way interaction on d 35; birds fed diets supplemented with 0.9% Ca and 0.45% avP had a significant increase in tibia ash percentage, regardless of phytase supplementation. A 3-way interaction was also observed for Ca and P digestibility on d 35. Phytase supplementation significantly increased Ca digestibility regardless of Ca and P levels of the diets. In addition, diets containing 0.6% Ca and 1,000 FTU/kg of phytase resulted in a significant increase in P digestibility, regardless of P levels. The results suggest a high level of soluble Ca in the diet may influence NE associated mortality. In addition, bird performance was affected by interactions of Ca, P and phytase during the exposure to *C. perfringens* and subsequent NE outbreak. Results showed improvements in Ca and P digestibility in birds fed lower dietary levels of Ca and P and phytase, which was likely consequent to improved performance when feeding lower Ca diets during the NE episode.

INTRODUCTION

Necrotic enteritis (**NE**) is an important enterotoxemia that affects poultry and is caused by *Clostridium perfringens* types A and C (Wages and Opengart, 2003; Williams, 2005). Although the pathogenesis of NE is still not completely understood, alpha toxin is considered to be one of the main contributors to lesion formation associated with the disease (Cooper and Songer, 2009; Lee et al., 2011). The alpha toxin is a phospholipase that acts on the enterocyte membrane causing extensive damage to the intestinal lining and inducing a severe inflammatory response (Titball et al., 1999; Immerseel et al., 2004; Sakurai et al.,

2004). However, the participation of the alpha toxin in disease production is a controversial topic, since NE can be reproduced by inoculating birds with *C. perfringens* isolates that do not produce alpha toxin (Keyburn et al., 2006). NetB is another toxin that has been associated with NE. NetB is a toxin that forms pores in the cellular membrane causing an influx of ions (Ca, Na, Cl, etc) that eventually lead to osmotic cell lysis (Keyburn et al., 2010).

Damaged intestinal mucosa, which is unable to digest and absorb nutrients, is one of the hallmarks of NE. Therefore, performance losses can be associated with a reduction in feed conversion (**FC**), feed intake (**FI**), body weight (**BW**) and body weight gain (**BWG**). It has been estimated that losses in performance, treatment, prevention and control of the disease cost the poultry industry approximately 2 billion dollars annually (Cooper and Songer, 2009; Lee et al., 2011). The use of growth promoting antibiotics in poultry feed has helped control this disease in commercial production settings (Immerseel et al., 2004). However, with impending antimicrobial bans, and consumer preferences for medication free production systems, the incidence of NE has significantly increased over the last few years (Williams, 2005).

Pre-disposing factors for NE development are extremely important, given that *C. perfringens* is a naturally occurring bacterium in the intestinal tract of poultry. Several factors have been identified that influence the onset of NE including: coccidiosis (Williams, 2005), dietary crude protein level and source (Drew et al., 2004; Palliyeguru et al., 2010), and high dietary Ca (Titball et al., 1999). These factors usually favor disease onset by producing intestinal lesions and promoting clostridial growth in the gut. One of the predisposing factors identified in the past is excess Ca in the diet, but the mechanism by which Ca favors *C. perfringens* is unknown. However, it is believed that excess Ca may

increase intestinal pH (Selle et al., 2009; Walk et al., 2012b) and favor *C. perfringens* growth in a more neutral environment (Wages and Opengart, 2003; Williams, 2005). Additionally, Ca is required for the synthesis and activity of the alpha toxin, and excess dietary Ca may exacerbate damage to the intestinal lining (Titball et al., 1999) during a *C. perfringens* outbreak.

The most recent published guidelines for nutrient requirements for poultry (NRC, 1994) suggest that the Ca requirement for growing broilers is 1.0% from d 0 to 21, 0.9% from d 21 to 42, and 0.8% from d 42 to 56. However, the experiments used to determine Ca requirement for broilers were not conducted with the supplementation of phytase in the diet (NRC, 1994). In the intestinal lumen, Ca must be soluble in order to be absorbed. Phytate can precipitate with Ca, particularly at conditions within the small intestine, creating insoluble Ca-phytate complexes (Selle et al., 2009). Phytate is a naturally occurring anti-nutrient in plants, with the highest concentration found in grains and seeds (Tamim et al., 2004). When pH is close to neutrality, phytate forms mineral chelates that are highly insoluble (Sebastian et al., 1996; Tamim et al., 2004; Selle et al., 2009) and maximum insolubility of phytate-mineral chelates occurs between pH 4 and 7 (Wise and Gilburt, 1981). Calcium is one of the divalent cations with lowest affinity for phytate (Tamim et al., 2004). However, since Ca is the mineral added at the highest concentration in poultry diets, it has a greater impact on forming phytate-mineral chelates due to its plentiful availability in the intestinal environment (Sebastian et al., 1996; Maenz et al., 1999; Selle et al., 2009), making both Ca and P unavailable for absorption.

In order to minimize the detrimental effects of phytate, phytases have been used by poultry nutritionists since 1991 to formulate diets for broilers (Bedford, 2003). Phytase is a

phosphatase that hydrolyzes the phosphate groups from the inositol ring of phytate (Tamim et al., 2004). As phytase hydrolyzes phytate, it increases nutrient availability and digestibility, which results in improved bird performance (Cowieson et al., 2006). Several authors have reported that elevated levels of Ca in the diet decreased phytase efficacy (Applegate et al., 2003; Tamim et al., 2004; Yan et al., 2006; Plumstead et al., 2008). Applegate and collaborators (2003) reported that a dietary Ca level commonly used in broiler diets (0.9%) resulted in a reduced intestinal phytase activity and apparent ileal phytate phosphorus hydrolysis compared with a lower level of Ca (0.4%). A high Ca to total P ratio appeared to exacerbate this effect, by increasing the formation of mineral-chelate complexes and decreasing phytase activity (Qian et al., 1997; Tamim et al., 2004).

Benefits from formulating broiler diets with lower levels of Ca are indicated by improvements in performance of healthy birds. Sebastian et al. (1996) were one of the first groups to challenge the NRC (1994) recommended Ca levels in diets supplemented with phytase. In their study, efficacy of supplemental phytase was significantly affected by dietary Ca levels, and the optimal growth performance and retention of P and Ca was achieved at the lowest level of dietary Ca tested (0.6% total Ca). Similar performance benefits were observed by several other authors (Tamim et al., 2004; Selle et al., 2009; Powell et al., 2011). Considering the improved performance with lower Ca levels and the proposed involvement of Ca in NE pathogenesis, we designed an experiment to evaluate the effects of dietary Ca, P and phytase levels on broiler performance, gastrointestinal morphology, mineral digestibility and bone mineralization during a natural NE outbreak.

MATERIALS AND METHODS

Animals and Animal Husbandry

Cobb 500 male broilers (n = 2,304) were acquired from a commercial hatchery and placed on used litter from a previous flock that had presented clinical signs of NE. Prior to the start of this trial, litter was removed from each pen and mixed together. Mixed litter was equally distributed to pens, and fresh pine shavings were layered on top of the mixed used litter. At day-of-hatch, birds were randomized, weighed by pen, and placed into floor pens relative to the different treatment groups from d 0 to 35. Feed and water were administered *ad libitum* throughout the study. The birds were housed in an environmentally controlled building under a lighting program recommended for Cobb 500 broilers.

Experimental Diets

Diets were formulated to meet or exceed NRC (1994) recommended nutrient levels, with the exception for Ca and P. Experimental diets constituted of a 2 x 2 x 2 factorial design with 2 total Ca levels (0.9% or 0.6%), 2 levels of available P (**avP**; 0.45% or 0.3%), and 2 levels of phytase (0 or 1,000 FTU/kg). Each dietary treatment was replicated by 9 pens (n=32 birds per pen). The phytase used was an *Escherichia coli* 6-phytase expressed in *Trichoderma reesei* with an expected activity of 5,000 FTU/g (Quantum Blue, AB Vista Feed Ingredients, Marlborough UK). Calcium was supplemented in the diets as a combination of dicalcium phosphate and a highly soluble calcified marine seaweed (Vistacal, AB Vista Feed Ingredients, Marlborough, UK). Diets were analyzed for Ca, total P, and recovered phytase activity, and results were comparable to formulated diets (Table 1). Birds were fed crumbled starter diets from d 0-19 and pelleted grower diets from d 19-35.

Animal Performance

On d 12, 19, and 35, birds and feed were weighed by pen to calculate average BW, BWG, FI, and FC for the periods of d 0-12, d 0-19, and d 0-35. Mortality was recorded daily and used to adjust FI and FC according to bird days.

Intestinal Morphology

On d 12, 19, and 35, tissue samples from the duodenum, jejunum and ileum from 1 bird per pen (n=9 per treatment) were collected for villus height and crypt depth measurement. Intestinal segments of approximately one inch were rinsed using phosphate buffered saline and fixed in 10% neutral buffered formalin. Each intestinal segment was cut into five pieces, stored in 70% ethanol, processed, embedded in paraffin, and mounted on slides. Slides were then stained using hematoxylin eosyn protocol (Luna, 1968). A total of three cross-sections per slide and four measurements per cross-section were evaluated for villus height and crypt depth. Cross-sections were evaluated using a bright-field microscope, and measurements were made using NIS-Elements 3.0 software (Nikon Instruments Inc., Melville, NY).

Ca and P Digestibility

On d 12 (n=6 birds/pen), 19 (n=5 birds/pen), and 35 (n=5 birds/pen), birds were sacrificed by cervical dislocation, and ileal digesta (defined as Meckel's diverticulum to the ileal cecal junction) was sampled, pooled per pen, and frozen at -80°C. Frozen digesta samples were freeze dried and ground (mm screen) prior to mineral analysis. Diet and digesta samples were wet ashed using nitric and perchloric acids. Phosphorus was determined by the alkalimeter ammonium molybdate method, and color intensity was read using a spectrophotometer measuring absorbance at 410 nm (AOAC International, 2000). Calcium

was determined using flame atomic absorption spectroscopy (AOAC International, 2000). Titanium oxide was added to the experimental diets as an indigestible marker, and was determined using a colorimetric method previously described by Short et al. (1996). All samples (Ca, P and titanium) were assayed in duplicates. Calcium and P apparent illeal digestibility (AID) was calculated using the following equation:

$$AID = [(nutrient/TiO_2)_{diet} - (nutrient/TiO_2)_{ileum}]/ (nutrient/TiO_2)_{diet}$$

Bone Ash

On d 12, 19, and 35, left tibias from 3 birds/pen were collected, and kept frozen (-20°C) with flesh intact until further analysis. Before analysis, bones were cleaned of all adherent tissue, wrapped in cheese-cloth, and dried at 100°C for 24 h. Using a Soxhlet extraction apparatus, fat was extracted from bones for 48 h in ethyl ether. Fat-extracted tibias were oven dried at 100°C for 24 h, then ashed at 600°C in a muffle furnace for 24 h for bone ash determination (weight and percentage).

pH

On d 12, 19, and 35, 2 birds/pen of average BW were killed to obtain measurements of pH from the gizzard, duodenum, and jejunum. Following cervical dislocation, pH measurements were obtained directly from the digesta contents in the lumen using a digital pH meter (Mettler-Toledo, Columbus, OH) and spear-tip electrode (Sensorex, Garden Grove, CA). The pH values from each gastrointestinal section/bird were then pooled to obtain a mean pH value/pen.

Statistical Analysis

Data were analyzed as a 2 x 2 x 2 factorial using the fit model platform in JMP 9.0 (SAS Institute Inc., Cary, NC) and means were separated using Tukey's honestly significant

difference test. Pen served as the experimental unit for performance measurements, nutrient digestibility, and gastrointestinal pH, and bird was the experimental unit for histological measurements. Data were analyzed for main effects (Ca, P, and phytase) and for the interaction of main effects. Statistical significance was accepted at $P < 0.05$. P-values of mortality data are reported as squared arcsine transformed percent mortality data.

RESULTS

Performance

The flock first presented clinical signs of NE on d 9 and no signs of NE were observed after d 26. Broilers fed diets supplemented with phytase had significantly higher mortality than broilers fed diets not supplemented with phytase for all periods (Table 2). In addition, birds fed diets containing industry standard levels of Ca (0.9%) presented significantly higher mortality than birds fed lower levels of Ca (0.6%) from d 0-19 and 0-35 but no effect of Ca was reported from d 0-12 (Table 2). Mortality was not influenced by dietary levels of P, regardless of feeding period, and no interactions were seen.

A 3-way interaction (Ca x P x phytase) occurred for BW and BWG from d 0-12 (Table 3). There was no difference in BW or BWG when birds were fed 0.45% avP with or without phytase, regardless of Ca level. However, when diets were formulated with 0.3% avP, supplementation with phytase significantly improved BW and BWG compared to diets without phytase (Table 3). Additionally, 0.9% Ca, 0.45% avP, and phytase diets significantly improved BW and BWG compared to those with 0.6% Ca, 0.45% avP, and phytase, while responses were similar across Ca levels with all other treatments (Table 3). No differences in BW or BWG were observed from d 0-19 or d 0-35.

A 2-way interaction (Ca x P) was observed for FI from d 0-12 and d 0-19 (Table 4). In these feeding periods, when broilers were fed 0.9% Ca diets, P was a limiting nutrient for FI. The same effect was not observed for broilers fed 0.6% Ca diets, where no differences in FI were observed between broilers fed 0.3 or 0.45% avP in the diet. No differences in FI related to P levels were observed from d 0-35. Phytase had no effect on FI for the different feeding periods.

Feed conversion was significantly affected by Ca or phytase from d 0-12, but P had no effect during that period (Table 4). A six-point improvement in FC was observed for broilers fed diets supplemented with phytase compared to no phytase supplementation, and a two-point improvement in FC was observed for birds fed 0.9% Ca diets compared to birds fed 0.6% Ca diets. A 2-way interaction between Ca and P occurred for FC from d 0-19. When birds were fed 0.6% Ca diets, FC was similar regardless of P levels (0.3 or 0.45%); however, when birds were fed 0.9% Ca diets, broilers fed 0.3% avP were significantly more efficient than those fed 0.45% avP. Phytase supplementation had no significant impact on FC from d 0-19 or d 0-35.

Histology

Duodenum. On d 12, dietary levels of Ca influenced villus height (VH) and crypt depth (CD) in the duodenum. Villi were significantly longer and crypts were significantly shallower when birds were fed 0.6% Ca diets compared to birds fed 0.9% Ca diets (Table 5). Villus height to crypt depth ratio (VCR) was influenced by a Ca x P interaction. Broilers fed 0.6% Ca and 0.45% avP diets had significantly greater VCR than those fed 0.9% Ca diets, regardless of P levels (Table 5).

On d 19, VH was not influenced by Ca, P, or phytase. However, a 3-way interaction of Ca, P, and phytase influenced CD and VCR. Broilers fed 0.9% Ca had significantly deeper crypts than those fed 0.6% Ca, regardless of P levels or phytase supplementation (Table 5), with the exception of those fed 0.6% Ca and 0.3% avP diets that were not supplemented with phytase. Within the 0.6% or 0.9% Ca dietary treatments, VCR was similar regardless of P levels or phytase supplementation. VCR was also similar between the 0.6% and 0.9% Ca diets, with the exception of the birds fed 0.6% Ca, 0.3% avP with phytase and 0.6% Ca, 0.45% avP without phytase, which had significantly higher VCR than those respective P and phytase groups fed 0.9% Ca (Table 5).

On d 35, birds fed diets supplemented with phytase had significantly shorter villi compared to birds fed diets without phytase (Table 5). Broilers fed 0.9% Ca diets had significantly deeper crypts than broilers fed 0.6% Ca diets, as did those fed 0.3% avP compared to those fed 0.45% avP diets. A 2-way interaction of Ca and P influenced VCR. Broilers fed 0.6% Ca and 0.45% avP diets had significantly higher VCR than those fed 0.6% Ca and 0.3% avP diets, while there was no difference between these groups when birds were fed 0.9% Ca diets (Table 5).

Jejunum. On d 12, no significant differences in VH, CD or VCR in the jejunum were observed among the different treatments (Table 6). However, on d 19, a 3-way interaction between Ca, P, and phytase influenced CD and VCR (Table 6). Similar to results in the duodenum, broilers fed 0.9% Ca had significantly deeper crypts than broilers fed 0.6% Ca, except when they were fed 0.6% Ca and 0.3% avP diets that were not supplemented with phytase. The VCR was similar between P levels with or without phytase within the dietary

Ca levels, with the exception of a difference between birds fed 0.6% Ca and 0.3% avP where phytase inclusion resulted in a larger VCR is compared to no phytase inclusion (Table 6).

On d 35, VH was significantly influenced by phytase supplementation. Villi of birds fed diets not supplemented with phytase were significantly longer than villi of birds fed diets supplemented with phytase. A Ca x P interaction significantly affected CD on d 35; whereas feeding 0.9% Ca and 0.45% avP diets resulted in significantly deeper crypts than birds fed 0.6% Ca 0.45% avP diets. In addition, 0.6% Ca diets resulted in greater VCR than 0.9% Ca diets.

Ileum. On d 12 in the ileum, broilers fed diets supplemented with phytase had significantly longer villi than those fed diets not supplemented with phytase (Table 7). Also, feeding 0.45% avP diets resulted in significantly greater VCR than 0.3% avP diets. No differences in CD were observed in the ileum on d 12.

On d 19, dietary levels of Ca significantly affected ileal CD. Broilers fed 0.6% Ca diets had significantly shallower crypts than those fed 0.9% Ca diets (Table 7). A Ca x P interaction significantly influenced VCR on d 19. Broilers fed 0.6% Ca and 0.45% avP diets had significantly greater VCR than broilers fed 0.9% Ca diets regardless of P levels (Table 7). No significant differences in ileal VH were observed among different treatments on d 19.

On d 35, a Ca x phytase interaction significantly affected VH and CD (Table 7). When diets were not supplemented with phytase, broilers fed 0.6% Ca diets had significantly higher villi than those fed 0.9% Ca. However, feeding 0.6% Ca diets supplemented with phytase resulted in significantly shallower crypts than broilers fed diets not supplemented with phytase, regardless of Ca levels. Dietary levels of P and phytase supplementation had a significant impact on VCR. Broilers fed 0.45% avP had significantly greater VCR than

broilers fed 0.3% avP in the diet, and broilers fed diets supplemented with phytase had significantly greater VCR than those fed diets not supplemented with phytase.

Ca and P Digestibility

On all three sampling days, phytase supplementation in 0.6% Ca diets significantly improved P digestibility regardless of P levels in the diet when compared to the diets not supplemented with phytase (Table 8). However, when diets were formulated with 0.9% Ca, improvements from phytase supplementation were not as consistent. On d 12, phytase supplementation had no effect on P digestibility in diets with 0.9% Ca and 0.45% avP (Table 8). On d 19 and 35, no differences in P digestibility were seen with phytase supplementation in broilers fed diets with 0.9% Ca and 0.3% avP.

On d 12, an interaction of P and phytase influenced Ca digestibility. Phytase supplementation to 0.3% avP diets significantly improved Ca digestibility, but a similar improvement was not seen in diets with 0.45% avP with phytase supplementation (Table 8). On d 12 and 19, a Ca and phytase interaction influenced Ca digestibility (Table 8). On both days, Ca digestibility was significantly lower when broilers were fed 0.6% Ca diets without phytase compared to those with phytase. In contrast, phytase supplementation did not improve Ca digestibility when broilers were fed 0.9% Ca. On d 35, a 3-way interaction (Ca, P and phytase) influenced Ca digestibility. When birds were fed 0.6% Ca diets, phytase supplementation resulted in significantly higher Ca digestibility when diets contained 0.3% avP, but phytase supplementation did not resulted in any difference when diets were formulated with 0.45% avP. In contrast, when diets were formulated with 0.9% Ca, phytase supplementation significantly improved Ca digestibility with the 0.45% avP diets, but not with the 0.3% avP diets.

Tibia Ash

On d 12, no interactions influenced tibia ash weight or percentage. However, Ca and phytase independently significantly affected tibia ash. Broilers fed 0.9% Ca diets had greater ash weight and ash percentage when compared to broilers fed 0.6% Ca diets (Table 9). In addition, phytase supplementation significantly improved ash weight on d 12, but not ash percentage. On d 19, Ca, P or phytase had no effect on tibia ash or tibia ash weight. A 3-way interaction (Ca, P and phytase) influenced tibia ash percentage on d 35. Maximum tibia ash percentage was observed when birds were fed 0.9% Ca and 0.45% avP in the diets, regardless of phytase supplementation. Phytase supplementation improved tibia ash percentage in broilers fed 0.9% Ca and 0.3% avP. However, the improvements on tibia ash percentage from phytase supplementation were not observed with the 0.9% Ca and 0.45% avP diets, or any 0.6% Ca diets. On d 35, tibia ash weight was influenced only by dietary levels of Ca with 0.9% Ca in the diet resulting in significantly greater tibia ash weight than 0.6% Ca in the diet.

pH

The gizzard of broilers fed 0.6% Ca diets was significantly more acidic than the gizzard of broilers fed 0.9% Ca on d 19 (Table 10). Phytase supplementation increased gizzard pH on d 35. Phosphorus had no impact on gizzard pH, regardless of the day.

Dietary Ca or phytase had no impact on duodenal pH regardless of sampling day. However, on d 35, the duodenum of broilers fed diets formulated with 0.3% avP was significantly more acidic than the duodenum of broilers fed 0.45% avP diets (Table 10).

The jejunum of birds fed 0.6% Ca diets was significantly more acidic on d 12 and 35 than the jejunum of birds fed 0.9% Ca diets (Table 10). Phosphorus or phytase had no effect on jejunal pH regardless of sampling day.

DISCUSSION

Dietary Ca or phytase had a significant impact on mortality due to NE. Dietary Ca levels might be affecting mortality by two different mechanisms. The first mechanism is that Ca levels affect intestinal pH, especially jejunal pH. In this study, on d 12 and 35, feeding broilers 0.9% Ca diets increased jejunal pH. Previous research showed that increasing dietary levels of Ca in the diet significantly increased small intestine pH (Shaffey et al., 1991; Walk et al., 2012b). This increase in pH due to Ca supplementation likely creates a more favorable environment for clostridial growth (Williams, 2005). Therefore, standard industry level of Ca in the diet (0.9%) might be increasing mortality due to NE by favoring clostridial growth as a consequence to changes in lower intestine pH.

The second mechanism relates to alpha-toxin and netB synthesis and activity. Alpha-toxin is a phospholipase C sphingomyelinase that is mineral dependant. The catalytic site of the alpha-helical amino-terminal part of alpha toxin is zinc dependent (Guillouard et al., 1997). However, the beta-sandwich carboxy-terminal part is homologous to eukaryotic C2 domains, which are involved in membrane interaction and phospholipid binding through a Ca-dependent mechanism (Guillouard et al., 1997; Petit et al., 1999). Possibly, birds fed 0.9% Ca had increased mortality as a result of an increased activity of alpha-toxin. NetB activity may also be influenced by the industry standard dietary Ca levels. NetB causes cell lysis by creating pores in the cell membrane resulting in an influx of Ca ions to the cytoplasm (Keyburn et al., 2010). In addition to promoting cell lysis through an osmotic imbalance, the

Ca influx caused by netB may also lead to a special type of programmed cell death. Kennedy et al. (2009) reported that the pore-forming alpha-toxin of *C. septicum* forms Ca permeable pores, which increase intracellular Ca. What was surprising about their findings was the fact that the Ca influx did not induce apoptosis. Instead, the Ca influx induced a cascade of events consistent with programmed necrosis since it was associated with calpain activation and release of cathepsins from lysosomes. In addition, they also observed deregulation of mitochondrial activity leading to an increase in reactive oxygen species and dramatically decreasing ATP levels (Kennedy et al., 2009). These findings strongly suggest that cellular death from pore-forming toxins is related to a programmed cellular necrosis.

Histology data from this trial strongly support these mechanisms leading to a higher mortality associated with NE in broilers fed 0.9% Ca in the diet. Generally, higher levels of Ca in the intestinal lumen led to decreases in VH and VCR, and increases in CD, especially on d 19, which was the peak of NE outbreak. This suggests that higher levels of Ca in the intestinal lumen exacerbates the damage to the intestinal lining during NE episodes. The increase in Ca concentration is not only associated with higher dietary levels of Ca. Even when feeding broilers lower levels of Ca, an increase in the inflammatory response (supported by increased CD and decreased VH and VCR) was observed, which was probably related to phytase supplementation. In diets without phytase, Ca tends to bind to the phytate molecule. Birds cannot absorb phytate bound Ca, which accumulates in the intestinal lumen, and consequently has a negative impact on intestinal morphology.

Histology data from this trial also indicated that phytase supplementation might have a negative impact on intestinal morphology, but the negative impact may be dependant on the pathophysiological state of the bird. Exogenous phytases release nutrients from the phytate

molecule regardless of the birds' health status. When the bird is healthy, nutrients released from the phytate molecule can be promptly digested and absorbed. However, when broilers' ability to digest and absorb nutrients is compromised, the nutrients released from the phytate molecule by phytase will be available to enteric pathogens. An increase in nutrient availability to these enteric pathogens will likely favor the colonization of the intestinal environment by undesirable microbial populations. Imbalance in the microbial population usually results in enteric disease, which is consistent with these histological findings.

Increased mortality due to phytase supplementation is also likely secondary to an increase in nutrient availability for *C. perfringens*. Intestinal damage promoted by factors other than NE, such as coccidiosis, negatively influence the birds' ability to utilize nutrients making them available to be utilized by microorganisms in the gastrointestinal tract (including *C. perfringens*). As previously described, once phytase releases nutrients from the phytate molecule, phytase serves as a nutrient delivery system. Nutrients not utilized by the bird become readily available to microorganisms in the intestines. This nutrient shuttle promotes unbalanced growth of *C. perfringens,* resulting in higher mortality due to NE in birds fed diets supplemented with phytase.

Differences in BW and BWG were observed in the initial phase of the growout period. More important than the levels of Ca and P alone is the relationship between them. Optimal BW and BWG were obtained when their ratio was maintained at 2:1, and as long as the Ca:P ratio (2:1) was respected there were no differences in BWG between birds fed lower levels of Ca and birds fed higher levels of Ca.

Performance benefits of phytase supplementation have been exhaustively reported by previous research (Ravindran et al., 1995; Selle et al., 2009; Rutherfurd et al., 2012). The

importance of phytase supplementation becomes more evident when lower levels of Ca and P are used in the diets. In this study, improvements in animal performance and nutrient digestibility consequent to feeding broilers 0.6% Ca were only observed when these diets were supplemented with phytase. Additionally, in this trial when broilers were fed 0.6% Ca diets not supplemented with phytase, the worst BW, BWG and mineral digestibility were observed. Results from this trial also indicate that phytase supplementation is essential when using highly soluble Ca sources. Otherwise, most of the supplemented Ca binds to phytate, forming mineral-phytate chelates that readily precipitate and become unavailable for digestion and absorption (Plumstead et al., 2008; Selle et al., 2009). It is not surprising that after d 19 no differences in bird performance were observed. NE causes extensive damage to intestinal mucosa, which results in birds that are unable to digest and absorb nutrients.

Phytase supplementation improved P digestibility. Optimal P digestibility was observed when feeding birds low levels of Ca in diets supplemented with phytase, regardless of P levels. The reduction in P digestibility observed when birds were fed 0.9% was probably consequent to excess Ca binding to P and precipitating. When Ca and P precipitate, they become unavailable for digestion and absorption. This effect is exacerbated in the absence of phytase, since excess Ca tends to form mineral-phytate chelates that precipitate in the intestinal environment and become unavailable for the bird (Sebastian et al., 1996; Tamim et al., 2004; Plumstead et al., 2008). In addition, when broilers were fed 0.6% Ca diets in the absence of phytase, a reduction in P digestibility was observed. This could be attributed to the Ca source used in this experiment. Calcium was supplemented using calcified seaweed that has higher solubility than regular sources of Ca in pHs close to neutrality (Walk et al.,

2012a). In the absence of phytase, available Ca from calcified marine seaweed binds to phytate reducing Ca and P availability to the bird.

Similar to P digestibility, phytase supplementation improved Ca digestibility. Improvements in Ca digestibility by phytase supplementation were more evident when lower levels of Ca and P were used in the diet. This probably results from the absence of excess mineral that would compensate for Ca and P bound to the phytate molecule. Results from this trial support the fact that phytase supplementation is especially important when feeding broilers low levels of Ca in the diet. Once again, when feeding highly soluble sources of Ca, in the absence of phytase, Ca binds to phytate and becomes unavailable for the bird. The improvements in Ca digestibility due to feeding lower levels of Ca in the diet may also be attributed to an increase in phytase activity. Tamim et al. (2004) reported significant decreases in phytase activity with increasing levels of Ca concentration.

Reports on the influence of dietary Ca on gastrointestinal pH are inconsistent, which is probably due to variability in gastrointestinal pH of broilers. The pH is highly influenced by physiological state. Fasting is one of the factors that affect physiological state, which consequently affects gastrointestinal pH (Winget et al., 1962; Ford, 1974). During sampling, selecting birds that are on the same physiological state is not logistically possible, which would cause variation. In this trial, dietary Ca levels significantly affected gastrointestinal pH of broilers. The gizzard and jejunum of birds fed 0.6% Ca were significantly more acidic than the same gastrointestinal segments of birds fed standard levels of Ca in the diet. Walk et al. (2012b) reported the same effects of Ca on gizzard pH, but they did not observe differences in jejunal pH. Phytase supplementation also had a significant impact on raising gizzard pH. Walk et. al (2012b) reported the same effect on gizzard pH, and attributed this

effect to a reduction in the electrolyte balance due to phytate. When phytate chelates nutrients from the diet it creates a more acidic environment in the gastrointestinal tract.

Higher mineral content in the diet resulted in increased ash weight and ash percentage. However, no signs of impaired skeletal development were observed in this flock among different treatments. A hypothesis for the increase in bone ash is that broilers utilize excess mineral from the diet for storage and not development. That would mean that the actual mineral requirement for appropriate skeletal development is lower than what has been suggested by NRC (1994). Bradbury et al. (2012) reported that feeding birds lower levels of Ca (0.77%) improved their leg strength. Leg health was accessed by the latency-to-lie test which is highly correlated with gait scores. Therefore, although a significant decrease in bone ash was observed when feeding birds low levels of Ca in the diet, it does not necessarily mean a reduction in bone health, or impairment in skeletal development.

No differences in bone mineralization on d 19 were observed, which could be consequent to reduced digestibility due to extensive mucosal damage. The lack of differences in bone ash on d 19 may also support the idea that excess mineral in the diet results in mineral storage. During disease there is a shift in nutrient partitioning that prioritizes keeping the animal alive. Therefore, mineral storage is not a priority at that point and excess mineral is directed to other physiological activities such as immune response.

An overall reduction in Ca and P digestibility was observed on d 19. This reduction in Ca and P digestibility could be explained by the destruction of the intestinal lining during NE, which impairs nutrient digestion and absorption (Immerseel et al., 2004; Timbermont et al., 2011). However, Ca and P digestibility was improved when birds were fed lower levels of Ca and P in the diets, and diets were supplemented with phytase (regardless of

physiological state). Therefore, feeding broilers lower levels of Ca and P might be extremely advantageous under a nutrient utilization perspective during NE outbreaks. Histology data from this trial indicated that less damage to the intestinal mucosa was observed when feeding broilers lower mineral levels in the diet, which would ultimately result in improvements in nutrient utilization.

In conclusion, this trial showed the importance of Ca supplementation as a factor in NE pathogenesis. Lower levels of Ca in the diet ultimately resulted in reduced mortality. In addition, birds fed diets containing lower levels of Ca and P performed equally to birds fed industry standard levels of Ca and P. Improvements in Ca and P digestibility may be attributed to a reduction in indigestible mineral in the intestinal lumen due to mineral precipitation. In addition, improvements in mineral digestibility may also be correlated to a healthier small intestine that would be more able to digest and absorb nutrients when birds are fed lower levels of mineral in the diet. The improvement in intestinal health due to a reduction in mineral contents of the diet is probably related to the involvement of Ca in the pathogenesis of NE, which was reflected in bird mortality. Benefits of diets formulated with lower levels of Ca and P are not restricted to bird performance. They can be extended to improvements in other aspects of poultry production such as waste management, which would benefit growers when trying to meet environmental regulations.

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Table 3.1: Calculated and analyzed composition and nutrient content of experimental starter and grower diets

Ingredients	Starter				Grower			
	Low Ca		Standard Ca		Low Ca		Standard Ca	
	Low P	High P	Low P	High P	Low P	High P	Low P	High P
	%							
Corn	63.32	62.99	61.45	61.12	71.10	70.57	68.98	68.61
Soybean meal, 48%	32.33	32.35	32.47	32.50	24.66	24.75	25.04	25.10
Poultry Fat	1.18	1.31	1.90	2.02	1.51	1.70	2.26	2.39
Salt	0.41	0.41	0.41	0.41	0.33	0.33	0.33	0.33
DL-Methionine	0.30	0.30	0.30	0.30	0.24	0.25	0.25	0.25
L-Lysine HCl	0.25	0.25	0.25	0.25	0.24	0.24	0.23	0.23
L-Threonine	0.07	0.07	0.07	0.07	0.08	0.07	0.07	0.07
HSC[1]	0.68	0.03	1.68	1.03	0.57	0.00	1.57	0.92
Dicalcium Phosphate	0.97	1.79	0.97	1.80	0.77	1.59	0.77	1.59
Mineral Premix	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Titanium dioxide[2]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Phytase[3]	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Calculated Composition								
Crude Protein	21.20	21.20	21.20	21.20	18.20	18.20	18.20	18.20
Ca %	0.60	0.60	0.90	0.90	0.52	0.52	0.80	0.80
Total P %	0.64	0.81	0.64	0.81	0.56	0.73	0.56	0.73
Available P%	0.30	0.45	0.30	0.45	0.25	0.40	0.25	0.40
Nutrient Composition								
Energy (ME; kcal/kg)	3,060	3,060	3,060	3,060	3,150	3,150	3,150	3,150
Analyzed Composition								
Dry Matter	88.70	88.65	88.40	88.40	88.10	88.80	88.00	87.80
Crude Protein	22.45	22.90	23.25	23.30	19.95	19.80	19.95	20.15
Ca	0.70	0.67	0.91	0.98	0.6	0.56	0.92	0.92
Total P	0.62	0.78	0.65	0.80	0.6	0.66	0.58	0.76

[1] High solubility calcium source (Vistacal, AB Vista Feed Ingredients, Marlborough, UK).
[2] Titanium dioxide was added to diets as an indigestible marker in order to calculate apparent ileal digestibility.
[3]Sand was supplemented in place of phytase in diets containing 0 FTU/kg phytase to equal 100%.

Table 3.2: Calcium, Phosphorus and phytase effect on broiler mortality (%) for d 0-12, 0-19, and 0-35.

	Mortality (%)		
Treatments	D 0-12	D 0-19	D 0-35
Ca			
0.60	3.29	5.72[B]	10.15[B]
0.90	3.73	13.62[A]	19.96[A]
SEM	0.93	1.91	2.21
P			
0.30	4.34	10.50	16.40
0.45	2.69	8.85	13.71
SEM	0.93	1.91	2.21
Phytase			
0	1.90[B]	5.03[B]	8.76[B]
1000	5.12[A]	14.32[A]	21.35[A]
SEM	0.93	1.91	2.21
P values			
Ca	0.8693	0.0252	0.0147
P	0.2550	0.4854	0.3805
Phytase	0.0409	0.0069	0.0006
Ca x P	0.9285	0.9879	0.5828
Ca x Phytase	0.8693	0.1803	0.5738
P x Phytase	0.5802	0.2982	0.1557
Ca x P x Phytase	0.9285	0.5511	0.4206

[a-b] Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).

Table 3.3: Calcium, phosphorus and phytase effect on body weight and body weight gain for d 0-12, 0-19, and 0-35.

Treatments			Body Weight (kg)			Body Weight Gain (kg)		
			D 12	D 19	D 35	D 0-12	D 0-19	D 0-35
Ca	P	Phytase						
0.60	0.30	0	0.3799^{C}	0.7981	2.2212	0.3351^{C}	0.7533	2.1764
		1000	0.4082^{A}	0.8485	2.2928	0.3632^{A}	0.8035	2.2478
	0.45	0	0.3911^{ABC}	0.8255	2.2982	0.3464^{ABC}	0.7808	2.2535
		1000	0.3839^{BC}	0.8118	2.2354	0.3391^{BC}	0.7670	2.1905
0.90	0.30	0	0.3817^{C}	0.7842	2.2335	0.3370^{C}	0.7395	2.1888
		1000	0.4006^{AB}	0.8007	2.2402	0.3563^{AB}	0.7565	2.1959
	0.45	0	0.3920^{ABC}	0.8077	2.2513	0.3474^{ABC}	0.7630	2.2066
		1000	0.4060^{A}	0.8129	2.2697	0.3616^{A}	0.7685	2.2252
SEM			0.0042	0.0164	0.0351	0.0042	0.0164	0.0352
Ca	P							
0.60	0.30		0.3941^{AB}	0.8233	2.2570	0.3492^{AB}	0.7784	2.2121
	0.45		0.3875^{B}	0.8186	2.2668	0.3427^{B}	0.7739	2.2220
0.90	0.30		0.3911^{AB}	0.7925	2.2369	0.3467^{AB}	0.7480	2.1924
	0.45		0.3990^{A}	0.8103	2.2605	0.3545^{A}	0.7657	2.2159
SEM			0.0030	0.0116	0.0241	0.0030	0.0116	0.0234
Ca	Phytase							
0.60	0		0.3855	0.8118	2.2597	0.3407	0.7671	2.2150
	1000		0.3961	0.8301	2.2641	0.3512	0.7852	2.2192
0.90	0		0.3869	0.7959	2.2424	0.3422	0.7512	2.1977
	1000		0.4033	0.8068	2.2550	0.3590	0.7625	2.2106
SEM			0.0030	0.0116	0.0234	0.0030	0.0116	0.0234
P	Phytase							
0.30	0		0.3808^{C}	0.7912	2.2274	0.3360^{C}	0.7464	2.1826
	1000		0.4044^{A}	0.8246	2.2665	0.3598^{A}	0.7800	2.2219
0.45	0		0.3916^{BC}	0.8166	2.2748	0.3469^{BC}	0.7719	2.2301
	1000		0.3950^{AB}	0.8123	2.2525	0.3504^{AB}	0.7677	2.2079
SEM			0.0030	0.0116	0.0234	0.0030	0.0116	0.0234
P values								
Ca			0.1554	0.0962	0.5804	0.1269	0.1014	0.5891
P			0.8282	0.5739	0.4860	0.8164	0.5712	0.4856
Phytase			<0.0001	0.2129	0.7234	<0.0001	0.2098	0.7218
Ca x P			0.0191	0.3363	0.7722	0.0202	0.3400	0.7756
Ca x Phytase			0.3336	0.7482	0.8637	0.2921	0.7649	0.8565
P x Phytase			0.0013	0.1097	0.2023	0.0013	0.1092	0.2026
Ca x P x Phytase			0.0135	0.2606	0.1297	0.0141	0.2629	0.1311

[a,c] Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).

Table 3.4: Calcium, phosphorus and phytase effect on feed intake and feed conversion for d 0-12, 0-19 and 0-35.

Treatments		Feed Intake (kg)			Feed Conversion (kg:kg)		
		D 0-12	D 0-19	D 0-35	D 0-12	D 0-19	D 0-35
Ca	P						
0.60	0.30	0.4362^{AB}	1.2536^{AB}	3.5729	1.2512	1.6109^{AB}	1.6147
	0.45	0.4318^{AB}	1.2217^{AB}	3.6107	1.2601	1.5769^{B}	1.6252
0.90	0.30	0.4222^{B}	1.1836^{B}	3.4562	1.2190	1.5814^{B}	1.5772
	0.45	0.4415^{A}	1.2693^{A}	3.6078	1.2460	1.6638^{A}	1.6296
SEM		0.0046	0.0252	0.0559	0.0095	0.0278	0.0206
Ca	Phytase						
0.60	0	0.4363	1.2377	3.6157	1.2810	1.6135	1.6326
	1000	0.4317	1.2376	3.5679	1.2304	1.5743	1.6074
0.90	0	0.4355	1.2378	3.5441	1.2728	1.6493	1.6128
	1000	0.4282	1.2153	3.5198	1.1921	1.5959	1.5904
SEM		0.0046	0.0252	0.0543	0.0095	0.0278	0.0200
P	Phytase						
0.30	0	0.4289	1.2002	3.5067	1.2769	1.6089	1.6065
	1000	0.4296	1.2372	3.5224	1.1934	1.5834	1.5854
0.45	0	0.4429	1.2752	3.6531	1.2769	1.6539	1.6389
	1000	0.4303	1.2157	3.5653	1.2292	1.5868	1.6160
SEM		0.0046	0.0252	0.0543	0.0095	0.0278	0.0200
Ca							
	0.60	0.4340	1.2376	3.5918	1.2557^{A}	1.5939	1.6200
	0.90	0.4319	1.2265	3.5320	1.2325^{B}	1.6226	1.6034
SEM		0.0032	0.0178	0.0384	0.0067	0.0197	0.0141
P							
	0.30	0.4292	1.2187	3.5145	1.2351	1.5961	1.5960
	0.45	0.4366	1.2455	3.6092	1.2531	1.6204	1.6274
SEM		0.0032	0.0178	0.0384	0.0067	0.0197	0.0143
Phytase							
	0	0.4359	1.2377	3.5799	1.2769^{A}	1.6314	1.6227
	1000	0.4299	1.2265	3.5439	1.2113^{B}	1.5851	1.6007
SEM		0.0032	0.0178	0.0384	0.0067	0.0197	0.0141
P Values							
Ca		0.6469	0.6619	0.2821	0.0180	0.3069	0.4192
P		0.1180	0.2936	0.0910	0.0647	0.3874	0.1271
Phytase		0.2048	0.6571	0.5162	<0.0001	0.1015	0.2844
Ca x P		0.0134	0.0235	0.3062	0.3447	0.0408	0.3063
Ca x Phytase		0.7749	0.6572	0.8329	0.1195	0.8007	0.8749
P x Phytase		0.1589	0.0611	0.3515	0.0659	0.4577	0.9643
Ca x P x Phytase		0.3046	0.5973	0.9307	0.1581	0.8440	0.2320

[a,c] Means within a column lacking a common superscript are significantly different from each other (P<0.05).

Table 3.5: Effects of dietary calcium, phosphorus and phytase on duodenal morphology during a necrotic enteritis episode

Treatments[1]			Day 12			Day 19			Day 35		
			VH[2]	CD	VCR	VH	CD	VCR	VH	CD	VCR
Ca	P	Phytase					μm				
0.6	0.30	0	198.53	20.39	9.76	225.09	26.97^{ABCD}	8.72^{ABC}	236.79	24.17	10.01
		1000	193.11	19.83	9.79	227.17	21.73^{CD}	10.68^{A}	243.16	22.89	10.98
	0.45	0	201.26	19.13	10.55	221.69	20.48^{D}	10.98^{A}	259.49	21.13	12.73
		1000	207.73	17.36	12.52	225.05	23.58^{BCD}	9.89^{AB}	239.35	18.95	12.91
0.9	0.30	0	197.94	22.57	8.90	227.97	29.11^{ABC}	7.95^{BC}	254.84	25.12	10.19
		1000	186.97	21.97	8.92	211.15	34.58^{A}	6.49^{C}	238.64	23.83	10.08
	0.45	0	196.63	23.52	8.46	228.86	30.40^{AB}	7.74^{BC}	251.06	24.81	10.25
		1000	175.28	22.52	8.26	231.92	32.41^{A}	7.30^{BC}	236.74	23.46	10.12
SEM			7.2782	1.2619	0.6490	8.2217	1.7747	0.5740	6.6828	1.3078	0.6663
Ca	P										
0.60	0.30		195.82	20.11	9.77^{AB}	226.13	24.35	9.70	239.98	23.53	10.49^{B}
	0.45		204.50	18.24	11.53^{A}	223.37	22.03	10.44	249.42	20.04	12.82^{A}
0.90	0.30		192.46	22.27	8.91^{B}	219.56	31.84	7.22	246.74	24.47	10.13^{B}
	0.45		185.95	23.02	8.36^{B}	230.39	31.40	7.52	243.90	24.13	10.19^{B}
SEM			5.1464	0.6567	0.4589	5.3071	1.2935	0.4538	4.8913	0.8954	0.4561
Ca											
0.60			200.16^{A}	19.18^{B}	10.65^{A}	224.75	23.19^{B}	10.07^{A}	244.70	21.78^{B}	11.66^{A}
0.90			189.20^{B}	22.64^{A}	8.64^{B}	224.98	31.62^{A}	7.37^{B}	245.32	24.30^{A}	10.16^{B}
SEM			3.6391	0.6374	0.3245	3.6092	0.9541	0.3086	3.3546	0.6141	0.3128
P											
0.30			194.14	21.19	9.34	222.84	28.10	8.46	243.36	24.00^{A}	10.31^{B}
0.45			195.22	20.63	9.95	226.88	26.72	8.98	246.66	22.09^{B}	11.50^{A}
SEM			3.6391	0.6374	0.3245	3.6092	0.9541	0.3086	3.3081	0.6055	0.3085
Phytase											
0			198.59	21.40	9.42	225.90	26.74	8.85	250.54^{A}	23.80	10.79
1000			190.77	20.42	9.87	223.82	28.07	8.59	239.47^{B}	22.28	11.02
SEM			3.6391	0.6374	0.3377	3.6092	0.9541	0.3086	3.3081	0.6055	0.3085
P values											
Ca			0.0436	0.0004	<0.0001	0.9637	<0.0001	<0.0001	0.8944	0.0043	0.0010
P			0.8386	0.5453	0.2034	0.4195	0.2976	0.2287	0.4792	0.0277	0.0079
Phytase			0.1458	0.2874	0.3384	0.6775	0.3142	0.5534	0.0201	0.0779	0.6004
Ca x P			0.1578	0.1609	0.0182	0.1764	0.4773	0.6075	0.1904	0.0680	0.0109
Ca x Phytase			0.1213	0.8424	0.2524	0.3375	0.0725	0.1059	0.3701	0.8099	0.4233
P x Phytase			0.9435	0.6646	0.3664	0.2910	0.3571	0.2355	0.1892	0.7777	0.6457
Ca x P x Phytase			0.2979	0.8265	0.2597	0.3530	0.0285	0.0199	0.1312	0.8024	0.6615

[1] Calcium and Phosphorus levels are expressed in %; Phytase levels are expressed in FTU/kg.
[2] Villus height (VH), crypt depth (CD), and villus height:crypt depth ratio (VCR).
[A-D] Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).

Table 3.6: Effects of dietary calcium, phosphorus and phytase on jejunal morphology during necrotic enteritis episode

Treatments[1]			Day 12			Day 19			Day 35		
			VH[2]	CD	VCR	VH	CD	VCR	VH	CD	VCR
Ca	P	Phytase					μm				
0.6	0.30	0	116.05	18.41	6.31	137.46	21.71^{ABCD}	6.40^{BC}	168.69	21.91	7.73
		1000	111.72	17.78	6.43	136.52	17.27^{CD}	8.10^{A}	150.70	23.12	6.71
	0.45	0	109.33	17.83	6.36	120.16	16.85^{D}	7.14^{AB}	172.38	22.98	7.55
		1000	108.94	18.22	6.18	132.41	18.81^{BCD}	7.17^{AB}	152.44	19.92	7.68
0.9	0.30	0	110.33	17.75	6.27	138.69	22.11^{ABC}	6.27^{BC}	163.39	24.09	6.80
		1000	111.60	18.78	6.08	135.20	26.15^{A}	5.35^{C}	156.02	21.77	7.22
	0.45	0	117.17	19.07	6.36	138.32	26.51^{A}	5.25^{C}	167.90	25.75	6.61
		1000	110.30	16.42	6.73	129.33	23.25^{AB}	5.65^{BC}	165.59	25.09	6.69
SEM			5.0070	1.0448	0.4495	6.7498	1.1773	0.3727	6.7510	1.2348	0.3530
Ca	P										
0.60	0.30		113.89	18.09	6.37	136.99	19.49	7.25	159.70	22.51^{AB}	7.22
	0.45		109.14	18.03	6.27	126.29	17.83	7.15	162.41	21.45^{B}	7.61
0.90	0.30		110.97	18.26	6.18	136.95	24.13	5.81	159.70	22.93^{AB}	7.01
	0.45		113.74	17.75	6.54	133.82	24.88	5.45	166.74	25.42^{A}	6.65
SEM			3.6647	0.7153	0.3290	4.1334	0.8325	0.2635	4.5007	0.8232	0.2353
Ca											
0.60			111.51	18.06	6.32	131.64	18.66^{B}	7.20^{A}	161.05	21.98^{B}	7.42^{A}
0.90			112.35	18.00	6.36	135.39	24.50^{A}	5.63^{B}	163.22	24.18^{A}	6.83^{B}
SEM			2.5913	0.5058	0.2326	3.0421	0.6127	0.1939	3.1260	0.5717	0.1634
P											
0.30			112.43	18.18	6.27	136.97	21.81	6.53	159.70	22.72	7.11
0.45			111.44	17.89	6.41	130.05	21.36	6.30	164.58	23.44	7.13
SEM			2.5913	0.5058	0.2326	3.0421	0.6127	0.1939	3.1260	0.5717	0.1634
Phytase											
0			113.22	18.27	6.32	133.66	21.79	6.27	168.09^{A}	23.68	7.17
1000			110.64	17.80	6.35	133.36	21.37	6.57	156.19^{B}	22.48	7.07
SEM			2.5913	0.5058	0.2326	3.0421	0.6127	0.1939	3.1260	0.5717	0.1634
P values											
Ca			0.9377	0.9011	0.9011	0.3715	<0.0001	<0.0001	0.6221	0.0081	0.0134
P			0.6823	0.6859	0.6859	0.1023	0.5915	0.3910	0.2701	0.3753	0.9349
Phytase			0.5140	0.9282	0.9282	0.9438	0.6165	0.2583	0.0086	0.1372	0.6559
Ca x P			0.7517	0.4809	0.4809	0.3666	0.1552	0.6211	0.6233	0.0303	0.1023
Ca x Phytase			0.6282	0.8510	0.8510	0.1585	0.3364	0.0381	0.1123	0.7290	0.1342
P x Phytase			0.3534	0.8439	0.8439	0.6461	0.7886	0.7463	0.8600	0.4162	0.3861
Ca x P x Phytase			0.1039	0.5173	0.5173	0.2664	<0.0001	0.0067	0.6900	0.0694	0.1105

[1] Calcium and Phosphorus levels are expressed in %; Phytase levels are expressed in FTU/kg.
[2] Villus height (VH), crypt depth (CD), and villus height:crypt depth ratio (VCR).
[A-D] Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).

Table 3.7: Effects of dietary calcium, phosphorus and phytase on ileal morphology during a necrotic enteritis episode

Treatments[1]		Day 12			Day 19			Day 35		
		VH[2]	CD	VCR	VH	CD	VCR	VH	CD	VCR
Ca	P					μm				
0.60	0.30	63.26	18.55	3.45	73.49	19.02	3.94^{AB}	93.85	18.89	5.01
	0.45	68.18	17.54	4.05	72.44	17.04	4.31^{A}	97.68	17.13	5.88
0.90	0.30	69.17	17.57	3.99	73.87	21.03	3.55^{BC}	91.42	18.15	5.09
	0.45	70.75	16.90	4.24	68.35	21.99	3.17^{C}	89.74	16.78	5.38
SEM		2.4581	0.8149	0.2048	2.3575	0.7606	0.1596	2.9749	0.6170	0.1769
Ca	Phytase									
0.60	0	62.12	17.66	3.60	72.65	17.58	4.24	99.70^{A}	20.15^{A}	5.04
	1000	69.32	18.43	3.90	73.27	18.49	4.01	91.83^{AB}	15.87^{C}	5.85
0.90	0	65.98	17.10	3.93	69.64	21.90	3.24	88.77^{B}	18.15^{AB}	4.92
	1000	73.94	17.37	4.30	72.57	21.12	3.48	92.39^{AB}	16.78^{BC}	5.55
SEM		2.4581	0.8149	0.2048	2.3575	0.7606	0.1596	3.0483	0.6323	0.1813
Ca										
0.60		65.72	18.04	3.75	72.96	18.03^{B}	4.12^{A}	95.77	18.01	5.44
0.90		69.96	17.24	4.11	71.11	21.51^{A}	3.36^{B}	90.58	17.46	5.24
SEM		1.7689	0.5660	0.1424	1.6736	0.5457	0.1149	2.0231	0.4196	0.1203
P										
0.30		66.21	18.06	3.72^{B}	73.68	20.03	3.75	92.64	18.52^{A}	5.05^{B}
0.45		69.47	17.22	4.15^{A}	70.39	19.52	3.74	93.71	16.95^{B}	5.63^{A}
SEM		1.7689	0.5660	0.1424	1.6504	0.5457	0.1149	2.0231	0.4196	0.1203
Phytase										
0		64.05^{B}	17.38	3.76	71.15	19.74	3.74	94.24	19.15^{A}	4.98^{B}
1000		71.63^{A}	17.90	4.10	72.92	19.80	3.75	92.11	16.32^{B}	5.70^{A}
SEM		1.7689	0.5660	0.1424	1.6504	0.5457	0.1149	2.0231	0.4196	0.1203
P values										
Ca		0.0930	0.3148	0.0739	0.4291	<0.0001	<0.0001	0.0675	0.3491	0.2125
P		0.1953	0.2953	0.0380	0.1638	0.5140	0.9678	0.7001	0.0089	0.0009
Phytase		0.0034	0.5144	0.0955	0.4492	0.9349	0.9717	0.4487	<0.0001	<0.0001
Ca x P		0.5028	0.8294	0.3639	0.3413	0.0617	0.0235	0.3256	0.7329	0.0855
Ca x Phytase		0.8776	0.7539	0.8465	0.6217	0.2773	0.1448	0.0435	0.0145	0.5857
P x Phytase		0.3425	0.3755	0.8750	0.6985	0.2352	0.4471	0.7347	0.8259	0.2421
Ca x P x Phytase		0.9640	0.3945	0.5604	0.3688	0.8100	0.2020	0.6937	0.0545	0.0623

[1] Calcium and Phosphorus levels are expressed in %; Phytase levels are expressed in FTU/kg.
[2] Villus height (VH), crypt depth (CD), and villus height:crypt depth ratio (VCR).
[A-C] Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).

Table 3.8: Effects of dietary calcium, phosphorus and phytase on calcium and phosphorus apparent ileal digestibility on d 12, 19 and 35

Treatments[1]			Day 12		Day 19		Day 35	
			Ca	P	Ca	P	Ca	P
Ca	P	Phytase			%			
0.6	0.30	0	0.4517	0.5754^{D}	0.3146	0.5210^{D}	0.4457^{BC}	0.5869^{CD}
		1000	0.5945	0.8324^{A}	0.4836	0.7825^{A}	0.6193^{A}	0.8237^{A}
	0.45	0	0.5209	0.7375^{B}	0.4239	0.7193^{B}	0.4465^{BC}	0.6358^{C}
		1000	0.5641	0.8193^{A}	0.4816	0.8101^{A}	0.4563^{ABC}	0.7869^{A}
0.9	0.30	0	0.5422	0.6366^{C}	0.3945	0.5989^{C}	0.5643^{AB}	0.6004^{CD}
		1000	0.5483	0.7257^{B}	0.3974	0.6606^{BC}	0.4972^{ABC}	0.6840^{BC}
	0.45	0	0.5498	0.7004^{B}	0.4310	0.6274^{C}	0.3611^{C}	0.5300^{D}
		1000	0.5236	0.7380^{B}	0.4203	0.7090^{B}	0.5411^{AB}	0.7312^{AB}
SEM			0.0169	0.0117	0.0223	0.0139	0.0358	0.0205
Ca	P							
0.60	0.30		0.5231	0.7039^{BC}	0.3991	0.6518^{BC}	0.5325	0.7053
	0.45		0.5425	0.7784^{A}	0.4528	0.7647^{A}	0.4514	0.7114
0.90	0.30		0.5452	0.6811^{C}	0.3960	0.6298^{C}	0.5308	0.6422
	0.45		0.5367	0.7192^{B}	0.4257	0.6682^{B}	0.4511	0.6306
SEM			0.0119	0.0083	0.0158	0.0098	0.0261	0.0149
Ca	Phytase							
0.60	0		0.4863^{B}	0.6564^{C}	0.3693^{B}	0.6201^{C}	0.4461	0.6114
	1000		0.5793^{A}	0.8259^{A}	0.4826^{A}	0.7963^{A}	0.5378	0.8053
0.90	0		0.5460^{A}	0.6685^{C}	0.4128^{B}	0.6132^{C}	0.4627	0.5652
	1000		0.5359^{A}	0.7318^{B}	0.4089^{B}	0.6848^{B}	0.5192	0.7076
SEM			0.0119	0.0083	0.0158	0.0098	0.0261	0.0149
P	Phytase							
0.30	0		0.4970^{B}	0.6060^{C}	0.3546	0.5599^{D}	0.5050	0.5937
	1000		0.5714^{A}	0.7791^{A}	0.4405	0.7216^{B}	0.5583	0.7539
0.45	0		0.5353^{AB}	0.7190^{B}	0.4275	0.6734^{C}	0.4038	0.5829
	1000		0.5438^{A}	0.7786^{A}	0.4509	0.7596^{A}	0.4987	0.7591
SEM			0.0119	0.0083	0.0158	0.0098	0.0261	0.0149
P values								
Ca			0.4985	<0.0001	0.3437	<0.0001	0.9693	<0.0001
P			0.6518	<0.0001	0.0105	<0.0001	0.0032	0.8540
Phytase			0.0010	<0.0001	0.0010	<0.0001	0.0063	<0.0001
Ca x P			0.2472	0.0327	0.4507	0.0004	0.9778	0.5596
Ca x Phytase			<0.0001	<0.0001	0.0004	<0.0001	0.5032	0.0911
P x Phytase			0.0080	<0.0001	0.0526	0.0003	0.4295	0.5981
Ca x P x Phytase			0.1653	0.0005	0.1271	<0.0001	0.0002	0.0013

[1] Calcium and Phosphorus levels are expressed in %; Phytase levels are expressed in FTU/kg.
[A-D] Means within a column lacking a common superscript are significantly different from each other (P<0.05).

Table 3.9: Effects of dietary calcium, phosphorus and phytase on tibia ash (%) and ash weight (g) on d 12, 19 and 35.

Treatments[1]			Day 12		Day 19		Day 35	
			Ash (%)	Weight (g)	Ash (%)	Weight (g)	Ash (%)	Weight (g)
Ca	P	Phytase						
0.6	0.30	0	47.73	0.3104	50.01	0.7500	52.35^{C}	2.1944
		1000	48.72	0.3579	50.78	0.8085	52.62^{C}	2.2794
	0.45	0	47.64	0.3254	51.29	0.7922	52.34^{C}	2.2218
		1000	48.81	0.3490	50.91	0.7922	52.88^{BC}	2.1703
0.9	0.30	0	53.05	0.3520	54.15	1.0222	52.35^{C}	2.3051
		1000	50.91	0.4033	48.92	0.8296	54.04^{AB}	2.4647
	0.45	0	51.18	0.3845	52.31	0.8314	54.13^{A}	2.3611
		1000	52.05	0.4337	52.72	0.9266	54.08^{A}	2.3841
SEM			2.6134	0.0231	2.2334	0.0859	0.2464	0.0571
Ca								
0.60			48.23^{B}	0.3357^{B}	50.74	0.7857	52.55^{B}	2.2165^{B}
0.90			51.80^{A}	0.3934^{A}	52.03	0.9025	53.65^{A}	2.3788^{A}
SEM			1.2652	0.0111	1.1167	0.0429	0.1232	0.0251
P								
0.30			50.10	0.3559	50.97	0.8525	52.84^{B}	2.3109
0.45			49.92	0.3731	51.81	0.8356	53.36^{A}	2.2843
SEM			1.2439	0.0109	1.1167	0.0429	0.1251	0.0260
Phytase								
0			49.90	0.3431^{B}	51.94	0.8489	52.79^{B}	2.2706
1000			50.12	0.3860^{A}	50.83	0.8392	53.41^{A}	2.3246
SEM			1.2439	0.0109	1.1167	0.0429	0.1293	0.0264
P values								
Ca			0.0474	0.0005	0.4218	0.0591	<0.0001	<0.0001
P			0.9168	0.2723	0.5966	0.7812	0.0051	0.4694
Phytase			0.8998	0.0079	0.4857	0.8734	0.0011	0.1440
Ca x P			0.9171	0.3649	0.9320	0.6242	0.0323	0.6977
Ca x Phytase			0.6272	0.6390	0.4116	0.5234	0.2520	0.3113
P x Phytase			0.6520	0.6789	0.4799	0.3490	0.0434	0.0664
Ca x P x Phytase			0.6874	0.7283	0.2870	0.1590	0.0068	0.9998

[1] Calcium and Phosphorus levels are expressed in %; Phytase levels are expressed in FTU/kg.
[A-C] Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).

Table 3.10: Effects of dietary calcium, phosphorus and phytase on gastrointestinal pH of broilers on d 12, 19 and 35

Variables	Gizzard			Duodenum			Jejunum		
	D 12	D 19	D 35	D 12	D 19	D 35	D 12	D 19	D 35
Ca									
0.60	2.1900	1.8947^{B}	2.3312	6.0074	5.7836	5.8470	5.8908^{B}	5.7684	5.6786^{B}
0.90	2.2976	2.1518^{A}	2.3706	6.0042	5.7950	5.8110	5.9486^{A}	5.8129	5.7439^{A}
SEM	0.0669	0.0663	0.0834	0.0229	0.0265	0.0315	0.0140	0.0195	0.0194
P									
0.30	2.2866	2.1108	2.3551	6.0165	5.7780	5.7792^{B}	5.9319	5.7833	5.7015
0.45	2.2010	1.9356	2.3468	5.9950	5.8006	5.8788^{A}	5.9075	5.7981	5.7210
SEM	0.0669	0.0663	0.0863	0.0229	0.0265	0.0326	0.0140	0.0195	0.0201
Phytase									
0	2.1788	1.9727	2.1925^{B}	6.0060	5.7968	5.8277	5.9045	5.7801	5.6842
1000	2.3089	2.0738	2.5093^{A}	6.0056	5.7818	5.8302	5.9350	5.8013	5.7383
SEM	0.0669	0.0663	0.0828	0.0229	0.0265	0.0331	0.0140	0.0195	0.0193
P Values									
Ca	0.2578	0.0070	0.7449	0.9216	0.7607	0.4308	0.0042	0.1102	0.0218
P	0.3678	0.0643	0.9452	0.5097	0.5488	0.0307	0.2214	0.5940	0.4906
Phytase	0.1718	0.2834	0.0097	0.9904	0.6888	0.9561	0.1271	0.4455	0.0563
Ca x P	0.6571	0.4015	0.7923	0.5409	0.0742	0.0845	0.1089	0.2074	0.5133
Ca x Phytase	0.9704	0.8018	0.6375	0.7716	0.6380	0.0778	0.8503	0.6614	0.6120
P x Phytase	0.8516	0.3191	0.4888	0.5377	0.4268	0.6159	0.3078	0.2495	0.3466
Ca x P x Phytase	0.6160	0.5452	0.1978	0.7789	0.9544	0.1289	0.1350	0.9461	0.1113

[a,b] Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).

Chapter IV: Influence of dietary calcium level, calcium source and phytase on bird performance and mineral digestibility during a natural necrotic enteritis episode

ABSTRACT

The poultry industry has little information on the effects of enzyme supplementation in disease settings. The objective of this study was to determine the influence of Ca source (highly soluble calcified seaweed and limestone) phytase supplementation, and dietary levels of Ca on bird performance and mineral digestibility (Ca and P) during a necrotic enteritis outbreak. Cobb 500 male broilers were weighed and randomized into 8 treatment groups (9 pens/treatment; 30 birds/pen) at day of hatch. The 21 d trial was designed as a 2 x 2 x 2 factorial, which included 2 dietary levels of Ca (0.6% and 0.9%), 2 Ca sources (limestone and a highly soluble calcified marine seaweed (HSC)), and 2 levels of an *E. coli* phytase (0 and 1,000 FTU/kg). Birds were placed on used litter from a previous flock that exhibited clinical signs of NE. Birds and feed were weighed on d 7, 14 and 21, and body weight (BW), BW gain (BWG), feed intake (FI), and feed conversion (FC) were calculated for each of these periods and cumulatively. Mortality was recorded daily, and pH of the gizzard and duodenum were measured on d 7, 14 and 21. Ileal digesta (8 birds/pen) was also collected on d 7, 14 and 21. Significance is reported at $P < 0.05$. Birds began exhibiting clinical signs of NE on d 9, and elevated NE-associated mortality persisted until the end of the trial. Mortality was significantly affected by an interaction between Ca source and Ca levels. Significantly higher mortality was observed when broilers were fed 0.9% Ca HSC diets when compared to birds fed 0.6% Ca, regardless of Ca source. Broilers fed 0.6% Ca diets supplemented with phytase were heavier than the other treatments regardless of Ca source. Broilers fed diets formulated with HSC had significantly higher FC then broilers fed diets formulated with

limestone. The gizzard of broilers fed 0.9% Ca in the diet was significantly less acidic than the gizzard of broilers fed 0.6% Ca in the diet. Broilers fed 0.6% Ca in diets supplemented with phytase showed significant improvements in P and Ca digestibility. In conclusion, greater Ca concentrations in the intestinal lumen had a negative effect on mortality associated with NE and on bird performance.

INTRODUCTION

True dietary requirements for Ca and P are of ongoing interest to poultry nutritionists. Diet supplementation with exogenous enzymes, especially phytases, represents a challenge when trying to establish the ideal dietary levels of Ca and P in the diet. The impacts of phytase supplementation on poultry performance (BW, BWG, FI and FC) and mineral digestibility have been extensively reported by several authors (Ravindran et al., 1995; Selle et al., 2009; Rutherfurd et al., 2012). However, little information exists in the literature in regards to the impact of phytases on intestinal health and Ca and P requirements during disease.

The main purpose of phytase supplementation is to increase nutrient availability. Initially, the supplementation of diets with phytases targeted improvements in P availability and digestibility (Perney et al., 1993, Selle et al., 2009). However, research has shown that phytase supplementation also has a significant impact on the availability of other nutrients, such as proteins and amino acids, minerals, and carbohydrates (Cowieson et al., 2006; Cowieson et al., 2009).

Calcium must be soluble in the intestinal lumen in order to be absorbed. Calcium solubility in the intestine is closely related to small intestinal pH (around 6.0) and phytate concentration. When pH is close to neutral, phytate forms mineral chelates that are highly

insoluble (Sebastian et al., 1996; Tamim et al., 2004; Plumstead et al., 2008). In addition, a high ratio of dietary Ca to P reduces the digestibility and absorption of Ca and P due to increased precipitation of Ca-P complexes (Plumstead et al., 2008; Selle et al., 2009). Selle et al. (2000) have reported that maximum insolubility of phytate-mineral chelates occurs between pH 4 and 7. Calcium is one of the divalent cations with lowest affinity for phytate. However, since Ca is the mineral added in highest concentrations in poultry diets, it has a greater impact in forming mineral-phytate chelates than other dietary minerals, making both, Ca and P, unavailable for absorption (Sebastian et al., 1996; Maenz et al., 1999; Tamim et al., 2004). Thus, the problem with phytate digestion is not a lack of compatible endogenous enzymes but poor substrate solubility in the small intestine. This effect is increased when high amounts of Ca are present (Tamim et al., 2004; Cowieson et al., 2011).

Calcium and phosphorus are usually supplemented in poultry diets by the inclusion of limestone, dicalcium phosphate, and meat-and-bone meal (where permitted). Inorganic sources of Ca and P are escalating in price, and the cost of supplementing diets with exogenous phytases is decreasing (Selle et al., 2009). In addition, inorganic sources of Ca and P reserves are not renewable, and their depletion can be delayed by reducing the inclusion levels of Ca and P in animal feed (Mullaney et al., 2000; Selle et al., 2009).

Benefits from supplementing broiler diets with lower levels of Ca are supported by performance results. Anderson et al. (1984) reported that BWG, FC, and bone ash were all significantly reduced by increasing dietary Ca from regular industry standards (0.9%) to 1.5% in the diet. Sebastian et al. (1996) were the first to challenge NRC (1994) recommended Ca levels in diets supplemented with phytase. In their study, efficacy of supplemental phytase was significantly affected by dietary Ca levels, and the optimal growth

performance, and retention of P and Ca was achieved at the lowest level of dietary Ca tested (0.6%). Selle et al. (2009) reported that over a 30 day feeding period, high levels of Ca depressed BWG (32%), FI (16.2%), and FC (18.8%) in pigs receiving phytase supplemented diets.

A healthy intestine is able to digest and absorb nutrients released by phytase from the phytate molecule resulting in improvements in bird performance. However, when the gastrointestinal tract is colonized by enteric pathogens, damage to the intestinal lining impairs function of the intestinal mucosa and birds' utilization of nutrients, and these nutrients may become available to enteric pathogens.

One enteric disease of concern for the commercial poultry industry related to impaired intestinal function is necrotic enteritis (NE). Necrotic enteritis has reemerged in recent years as an important multifactorial enterotoxemia in poultry. The increasing numbers in NE outbreaks are likely consequent to attempts to reduce the use of antimicrobials and coccidiostats in poultry feed by the industry. *Clostridium perfringens* is the etiological agent associated with NE. Although the pathogenesis of NE is not completely elucidated, it is believed that the toxins produced by *C. perfringens* are lesion production. Researchers suggest that the most important toxins in the pathogenesis of NE are netB and alpha-toxin. NetB forms pores in the cellular membrane causing an influx of ions (Ca, Na, Cl, etc) that eventually lead to osmotic cell lysis (Keyburn et al., 2010). Alpha-toxin is a zinc-dependent phospholipase sphingomyelinase that hydrolyses phospholipids and promotes cellular membrane disorganization. In addition to zinc, alpha-toxin also depends on Ca for full activity. Calcium ions are essential for the binding of alpha-toxin to lipid films (Moreau et al., 1988; Petit et al., 1999; Titball et al., 1999). Therefore, there is strong evidence

suggesting that Ca is involved in the pathogenesis of NE. The objective of this study was to evaluate the effects of phytase supplementation, Ca sources and Ca levels on animal performance, gastrointestinal pH, and mineral digestibility during a natural NE outbreak.

MATERIALS AND METHODS

Animals and Animal Husbandry

Cobb 500 male broilers (n = 2,160) were acquired from a commercial hatchery and placed on used litter from a previous flock that had presented clinical signs of NE. Following the previous trial, litter was removed from each pen, and mixed in the center of the grower house. Mixed litter was redistributed to pens, and fresh pine shavings were layered on top of the mixed used litter. Upon arrival at the Virginia Tech farm, birds were randomized, weighed by pen, and placed into floor pens relative to the different treatment groups from d 0 to 21. Feed and water were administered *ad libitum* throughout the study.

Experimental Diets and Animal Performance

Diets were formulated in order to meet or exceed NRC (1994) recommended nutrient levels, with the exception of Ca and P. Experimental diets constituted of a 2 x 2 x 2 factorial design with 2 total Ca levels (0.9% and 0.6%), 2 Ca sources (limestone and calcified marine seaweed), and 2 levels of phytase (0 and 1,000 FTU/kg). Calcium was supplemented in the diets as a combination of dicalcium phosphate, and highly soluble calcified marine seaweed (HSC) (Vistacal, AB Vista Feed Ingredients, Marlborough, UK) or limestone. The phytase used was an *Escherichia coli* 6-phytase expressed in *Trichoderma reesei* and contained an expected activity of 5,000 FTU/g (Quantum Blue, AB Vista Feed Ingredients, Marlborough UK). Diets were analyzed for Ca, total P and recovered phytase activity, and results were comparable to formulated diets (Table 1).

On d 7, 14, and 21, birds and feed were weighed by pen in order to calculate average BW, BWG, average FI, and FC. Mortality was recorded daily and used to adjust FI and FC according to bird days.

Ca and P Digestibility

On d 7 (n=7 birds/pen), 14 (n=6 birds/pen), and 21 (n=6 birds/pen), birds were euthanized by cervical dislocation, and ileal digesta (defined as Meckel's diverticulum to the ileal cecal junction) was sampled. Ileal digesta samples were pooled per pen and frozen (-80°C) until further analysis. Frozen digesta samples were freeze dried and ground (1mm screen) prior to mineral analysis. Digesta and diet samples were wet ashed using nitric and perchloric acids. Phosphorus was determined by the alkalimeter ammonium molybdate method, and color intensity was read using a spectrophotometer measuring absorbance at 410 nm (AOAC International, 2000). Calcium was determined using flame atomic absorption spectroscopy (AOAC International, 2000). Titanium dioxide was included in the diets as an indigestible marker, and it was determined using a colorimetric method previously described by Short et al. (1996). All samples (Ca, P and titanium) were assayed in duplicates. Calcium and phosphorus apparent illeal digestibility (AID) was calculate using the following equation:

$$AID = [(nutrient/TiO_2)_{diet} - (nutrient/TiO_2)_{ileum}]/ (nutrient/TiO_2)_{diet}$$

pH

On d 14 and 21, 2 birds/pen of average BW were euthanized to obtain measurements of pH from the gizzard and duodenum. From each bird, pH measurements were obtained directly from the digesta contents in the lumen using a digital pH meter (Mettler-Toledo, Columbus,

OH) and spear-tip electrode (Sensorex, Garden Grove, CA). The pH values from each gastrointestinal section/bird were then pooled to obtain a mean pH value/pen for analysis.

Statistical Analysis

Data were analyzed as a 2 x 2 x 2 factorial using the fit model platform in JMP 9.0 (SAS Institute Inc., Cary, NC), and means were separated using Tukey's honestly significant difference test. Pen served as the experimental unit. Data were analyzed for main effects (Ca, P, and phytase) and for the interaction of main effects. Statistical significance was accepted at $P < 0.05$. P-values of mortality data are reported as squared arcsine transformed percentage mortality data.

RESULTS

Performance

Birds began exhibiting clinical signs of NE on d 9, and elevated NE-associated mortality persisted until the end of this trial. No significant differences in mortality were observed on d 0-7 or 0-14 (Table 2). However, from d 0-21 mortality was significantly affected by a 2-way interaction between Ca source and Ca levels (Table 2). Mortality was significantly higher when broilers were fed 0.9% HSC diets than broilers fed 0.6% Ca, regardless of Ca source. Calcium source did not significantly affect mortality when broilers were fed equivalent levels of Ca in the diet (Table 2).

From d 0-7, a 3-way interaction (Ca level x Ca source x phytase) significantly affected BW and BWG (Table 3). Broilers fed diets supplemented with phytase were significantly heavier and gained more weight than broilers fed diets that were not supplemented with phytase regardless of Ca level or Ca source, with the exception of those with 0.6% Ca from limestone.

From d 0-14 and 0-21, Ca source or phytase supplementation significantly affected BW and BWG (Tables 3). Broilers fed diets formulated with limestone were significantly heavier than broilers fed diets formulated with HSC. Broilers fed phytase supplemented diets were significantly heavier and gained more weight than those on diets without phytase. Dietary levels of Ca did not affect BW or BWG for the same periods.

A 2-way interaction between Ca level and Ca source significantly affected FI from d 0-7 (Table 4). When broilers were fed diets formulated using HSC as the Ca source, dietary levels of Ca did not significantly affect FI. However, when limestone was used as the Ca source, broilers fed 0.6% Ca diets had higher FI than broilers fed 0.9% Ca diets. A 2-way interaction between Ca levels and phytase also significantly affected FI during the first week of this trial. A significant decrease in FI was observed when broilers were fed 0.9% Ca diets without phytase, but no FI difference due to phytase supplementation was observed with 0.6% Ca diets. From d 0-7, no differences in FC were observed. Phytase significantly increased FI from d 0-14 (Table 4). Broilers fed diets supplemented with phytase consumed more feed than broilers fed diets that were without supplemental phytase. FI was not significantly affected from d 0-21. Calcium source had a significant impact on FC from d 0-14 or 0-21. Diets formulated with limestone as the Ca source resulted in a decrease in FC when compared to diets formulated with HSC as a Ca source.

Ca and P Digestibility

A 3-way interaction (Ca levels, Ca source and phytase) significantly influenced P and Ca digestibility on all sampling days. Optimal P digestibility was observed when broilers were fed 0.6% Ca diets formulated with limestone and supplemented with phytase regardless of feeding period (Table 5). On d 7, phytase supplementation improved P digestibility in

broilers fed 0.6% Ca diets formulated with limestone and 0.9% Ca diets formulated with HSC, but did not affect P digestibility in the 0.6% Ca diets formulated with HSC or 0.9% Ca diet formulated with limestone. This improvement by phytase supplementation in the 0.6% limestone diets was also observed on d 14 and d 21, while it had no effect in other diets regardless of Ca source or level. Overall, a significant decrease in P digestibility was observed when broilers were fed 0.9% Ca, regardless of Ca source and phytase supplementation.

Similar to P digestibility results on d 7, Ca digestibility was improved by phytase supplementation in the 0.6% Ca diets formulated with limestone and 0.9% Ca diets formulated with HSC. However, this response was not seen on d 14 and d 21. On d 14, phytase supplementation only improved Ca digestibility on 0.9% Ca diets formulated with limestone. On d 21, broilers fed 0.6% Ca diets supplemented with phytase and formulated with limestone had higher Ca digestibility than those fed 0.9% Ca diets supplemented with phytase and formulated with limestone. Generally, on d 14 and d 21, 0.6% Ca diets resulted in improved Ca digestibility, regardless of Ca source or phytase supplementation.

pH

Differences in pH measurements were only seen in the gizzard on d 21 (Table 6). The gizzard of broilers fed 0.6% Ca diets was significantly more acidic than the gizzard of broilers fed 0.9% Ca.

DISCUSSION

New regulations concerning antimicrobial use in feeds, and market demand for a product raised without the aid of growth promoters had a significant impact on the increasing incidence of NE in commercial broiler houses. In this experiment, broilers were fed non-

medicated diets, and high mortality due to NE was observed from d 9 until the end of the trial (d 21). Higher mortality due to NE was observed when broilers were fed industry standard levels of Ca (0.9%) in the diet, especially when diets were formulated using HSC. The complete mechanism by which *C. perfringens* leads to NE has not been completely elucidated. *C. perfringens* is an extra cellular pathogen, thus its virulence is mostly associated with its ability to produce toxins, such as alpha-toxin and netB (Songer, 1996). Mucosal damage associated with NE has been correlated with the action of NetB (Keyburn et al., 2010) and alpha-toxin (Petit et al., 1999; Titball et al., 1999) on the intestinal lining. The mechanism of membrane recognition by alpha-toxin is a complex event. This mechanism involves a Ca-mediated phospholipid recognition, which allows alpha-toxin to bind to the enterocyte cellular-membrane (Titball et al., 1999). NetB also depends on Ca to cause cell lysis. The netB mode of action involves the formation of pores in the enterocyte membrane, which results in an influx of ions into the cytoplasm (Keyburn et al., 2010). The influx of ions into the cell cytoplasm eventually leads to osmotic cell lysis. In addition to osmotic lysis, another mechanism involving Ca has been suggested that would induce cell death (Kennedy et al., 2009). Thus, the Ca influx caused by netB may also lead to a special type of programmed cell death. Kennedy et al. (2009) reported that the alpha-toxin of *C. septicum* (pore-forming toxin) forms Ca permeable pores, which increase intracellular Ca. It was believed that this Ca influx induced a cascade of events consistent with programmed necrosis since it was associated with calpain activation and release of cathepsins from lysosomes. In addition, Kennedy et al. (2009) also observed deregulation of mitochondrial activity leading to an increase in reactive oxygen species and dramatically decreasing ATP levels. The increase in mortality due to NE observed when broilers were fed 0.9% Ca as compared to

0.6% Ca in this trial, in association with other researchers findings in regards to Ca participation in the mode of action of alpha-toxin and netB, strongly suggest that dietary Ca level is an important factor in NE pathogenesis. The effect of Ca source also supports this idea. Since HSC is more soluble than limestone (Walk et al., 2012), formulating diets with HSC likely increases Ca availability in the intestinal lumen resulting in more extensive damage to the intestinal mucosa.

In the past, Ca involvement with NE was thought to be associated with an increase in gastrointestinal pH with high levels of Ca in the diet (Williams, 2005). In theory, the increase in gastrointestinal pH would favor the proliferation of *C. perfringens* in the intestinal lumen. Our data do not suggest that higher mortality when broilers were fed 0.9% Ca in the diet was the result of pH changes. Changes in gastrointestinal pH were not observed until d 21, which was well after the initial phase of the NE outbreak. In addition, pH changes were restricted to the gizzard and not lower in the intestinal tract. Therefore, there is strong indication that Ca impact on mortality due to NE was most likely associated with toxin activity.

Phytase supplementation improved BW and BWG regardless of Ca source and Ca levels. Initially, when broilers were fed 0.9% Ca in the diet, phytase supplementation also increased FI, which partially explains the improvements in BW and BWG. However, after d 14, phytase supplementation did not significantly affect FI. Improvements in BW and BWG due to phytase supplementation have been reported by other researchers (Cowieson et al. 2004 and 2006; Selle et al., 2009). These improvements have been associated with extra-phosphoric effects of phytase that result from the release of nutrients, other than P, from the phytate molecule. When these nutrients are released from the phytate molecule they become

available for digestion and absorption, which ultimately results in improvements in animal performance.

After d 7, Ca source had a significant impact on BW, BWG and FC. When broilers were fed diets formulated with HSC, a reduction in BW and BWG was observed. Since there were no differences in FI due to Ca source for the same periods, an increase in FC was expected. HSC is a much more soluble Ca source than limestone under the same pH (Walk et al., 2012). Therefore, when broilers are fed diets formulated with HSC, the concentration of soluble Ca in the intestinal lumen is higher than when broilers are fed diets formulated with limestone. Walk et al. (2012) did not report significant differences in BW, BWG and FI when feeding broilers diets formulated with limestone or HSC. This lack of differences in their study could be consequent to the fact that their birds were not undergoing an intestinal challenge, and Ca concentrations had no impact on the physiological status of the intestinal lining. Therefore, the differences observed in broiler performance due to Ca source in this trial were likely due to the impact of Ca in the pathogenesis of NE. As previously discussed in this manuscript, high Ca concentrations in the intestinal lumen could have a negative impact on the mucosal lining during a NE episode as a result of toxin activity. Therefore, the reduction in BW and BWG may be attributed to the reduction in ability to digest and absorb nutrients by birds fed diets formulated with HSC. This reduction in the ability to digest and absorb nutrients was likely due to more severe damage to the intestinal mucosa when broilers were fed diets formulated with HSC when compared to broilers fed diets formulated with limestone.

In this experiment, dietary Ca levels significantly impacted P digestibility. A high ratio of dietary Ca to P reduces the digestibility and absorption of Ca and P due to increased

precipitation of Ca-P complexes (Plumstead et al., 2008; Selle et al. 2009). This effect is more evident when formulating diets using HSC at standard industry levels (0.9% Ca). Since HSC is more soluble then limestone, there is more Ca in solution that binds to P and precipitates. Phosphorus digestibility was also impacted by exogenous phytase supplementation. Improvements in P digestibility due to phytase supplementation result from phytate P hydrolysis, which releases bound P from the phytate molecule and improves P availability. Optimal P digestibility was observed when feeding diets formulated with phytase and 0.6% Ca. This interaction between Ca and P has been described before, and can be attributed to phytase activity and formation of mineral-phytate chelates. Tamim et al. (2004) reported that phytase activity is decreased with increasing levels of Ca. Calcium is the mineral added in highest concentrations in poultry diets. Therefore, Ca has a greater impact than other dietary minerals in the formation of mineral-phytate chelates (Sebastian et al., 1996; Tamim et al., 2004). The phytate molecule can carry up to twelve negative charges, thus having the potential to chelate six Ca atoms (Selle et al., 2009). When Ca forms mineral-phytate chelates, they precipitate becoming unavailable for digestion and absorption. In addition, when these complexes precipitate they also become unavailable for phytase attack and further decreasing P availability.

Calcium digestibility was also impacted by phytase supplementation. As discussed above, Ca tends to form mineral-phytate chelates that precipitate. Therefore, phytase supplementation improves P and Ca digestibility by reducing the formation of mineral-phytate chelates. In addition, improvements in Ca digestibility were observed when broilers were fed 0.6% Ca in the diet. Similar to P digestibility, excess Ca decreases its digestibility due to the precipitation of Ca and P. Similar to results of Walk et al. (2012), no differences in

Ca digestibility were observed when comparing diets formulated with limestone or HSC, which was surprising due to the greater solubility of HSC when compared to limestone. Walk et al. (2012) suggested that in order to observe improvements in Ca digestibility due to Ca source, further reductions in dietary Ca might be necessary.

In conclusion, Ca appears to be an important factor in NE pathogenesis. Calcium involvement with NE pathogenesis is likely to be associated with netB and alpha-toxin activity. Benefits from decreasing dietary levels of Ca are not restricted to bird mortality due to NE. Feeding lower levels of Ca in the diet also resulted in improvements in bird performance and mineral digestibility. Therefore, NRC (1994) requirements for Ca and P, developed prior to widespread use of phytase, are likely to be inaccurate, and the poultry industry needs to review these requirements especially when supplementing poultry diets with exogenous phytases.

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Table 4.1: Calculated and analyzed composition and nutrient content of experimental starter diets

Ingredients	0 (FTU/kg) Phytase				1000 (FTU/kg) Phytase			
	HSC[1]		Limestone		HSC		Limestone	
	LCa[2]	SCa	LCa	SCa	LCa	SCa	LCa	SCa
	%							
Corn	57.60	55.50	57.93	56.28	58.44	56.35	58.77	57.12
Soybean meal, 48%	36.39	36.73	36.33	36.60	36.25	36.59	36.19	36.46
Poultry Fat	2.51	3.26	2.29	2.98	2.20	2.96	2.09	2.68
Salt	0.04	0.04	0.04	0.04	0.35	0.35	0.35	0.35
DL-Methionine	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32
L-Lysine HCl	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
L-Threonine	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
HSC[2]	0.74	1.74	0.00	0.00	0.74	1.74	0.00	0.00
Limestone	0.00	0.00	0.58	1.37	0.00	0.00	0.58	1.37
Dicalcium Phosphate	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70
Sodium Phosphate	0.93	0.94	0.93	0.94	0.22	0.23	0.22	0.22
Mineral Premix	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Titanium dioxide[3]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Phytase[4]	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.02
Calculated composition								
Crude Protein	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0
Ca %	0.60	0.90	0.60	0.90	0.60	0.90	0.60	0.90
Total P %	0.76	0.76	0.76	0.76	0.60	0.60	0.60	0.60
Available P%	0.45	0.45	0.45	0.45	0.30	0.30	0.30	0.30
Ca:P ratio	1.3:1	2:1	2:1	1.3:1	1.3:1	2:1	1.3:1	2:1
Nutrient composition								
Energy (ME; Kcal/lb)	1382	1382	1382	1382	1382	1382	1382	1382
Analyzed Composition								
Dry Matter	87.9	87.8	87.9	87.5	87.5	87.4	87.1	88.3
Crude Protein	24.5	24.8	22.7	23.3	22.3	23.2	23.4	22.8
Ca	0.63	0.97	0.72	1.01	0.67	0.95	0.62	1.02
Total P	0.82	0.86	0.84	0.84	0.61	0.64	0.64	0.68
Phytase (FTU/kg)	<50	<50	<50	<50	630	643	788	693

[1] High solubility calcified seaweed (Vistacal, AB Vista Feed Ingredients, Marlborough, UK).
[2] LCa = low Ca (0.6%); SCa = industry standard Ca (0.9%)
[3] Titanium dioxide was added to diets as an indigestible marker in order to calculate apparent ileal digestibility.
[4]Sand was supplemented in place of phytase in diets containing 0 FTU/kg phytase to equal 100%.

Table 4.2: Dietary calcium level, calcium source and phytase effect on broiler mortality (%) for d 0-7, 0-14, and 0-21.

Variables		Day 0 – 7	Days 0 - 14	Days 0 - 21
Ca (%)	Ca Source			
0.60	Limestone	2.22	5.18	7.77^{B}
	HSC	1.11	4.07	5.37^{B}
0.90	Limestone	2.22	7.59	11.66^{AB}
	HSC	2.25	9.66	19.96^{A}
SEM		0.6526	2.0964	2.5778
Ca (%)	Phytase			
0.60	0	2.40	6.66	8.51
	1000	2.31	2.59	4.62
0.90	0	2.40	7.40	12.77
	1000	2.07	9.85	18.85
SEM		0.6526	2.0964	2.5778
Phytase	Ca Source			
0	Limestone	2.77	6.48	9.07
	HSC	1.66	7.59	12.22
1000	Limestone	2.03	6.29	10.37
	HSC	1.33	6.14	13.11
SEM		0.6526	2.0964	2.5778
Ca (%)				
	0.60	1.66	4.62	6.57^{B}
	0.90	2.24	8.62	15.81^{A}
SEM		0.4615	1.4824	1.8227
Ca Source				
	Limestone	2.22	6.38	9.72
	HSC	1.68	6.87	12.66
SEM		0.4615	1.4824	1.8227
Phytase				
	0	2.40	7.03	10.64
	1000	1.50	6.22	11.74
SEM		0.4615	1.4824	1.8227
P Values				
Ca levels		0.1514	0.0713	0.0018
Ca source		0.3608	0.6999	0.1393
Phytase		0.0919	0.1915	0.5229
Ca levels x Ca source		0.4460	0.3698	0.0383
Ca levels x Phytase		0.4903	0.0658	0.0636
Ca source x Phytase		0.3770	0.8382	0.9576
Ca levels x Ca source x Phytase		0.5109	0.9542	0.7645

[a-b] Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).

Table 4.3: Dietary calcium level, calcium source and phytase effect on body weight and body weight gain d 0-7, 0-14 and 0-21.

Treatments			Body Weight (kg)			Body Weight Gain (kg)		
			D 7	D 14	D 21	D 0-7	D 0-14	D 0-21
Ca (%)	Ca Source	Phytase						
0.60	Limestone	0	0.1680^{A}	0.4370	0.8739	0.1230^{A}	0.3920	0.8289
		1000	0.1688^{A}	0.4576	0.9170	0.1234^{A}	0.4123	0.8717
	HSC	0	0.1571^{B}	0.4147	0.8094	0.1117^{B}	0.3693	0.7640
		1000	0.1710^{A}	0.4548	0.8931	0.1259^{A}	0.4096	0.8480
0.90	Limestone	0	0.1562^{B}	0.4212	0.8645	0.1110^{B}	0.3760	0.8193
		1000	0.1708^{A}	0.4625	0.8774	0.1255^{A}	0.4172	0.8321
	HSC	0	0.1527^{B}	0.4052	0.7786	0.1072^{B}	0.3598	0.7331
		1000	0.1708^{A}	0.4546	0.8805	0.1257^{A}	0.4095	0.8355
SEM			0.0016	0.0085	0.5196	0.0015	0.0085	0.0262
Ca (%)								
	0.60		0.1662^{A}	0.4410	0.8734	0.1210^{A}	0.3958	0.8282
	0.90		0.1626^{B}	0.4359	0.8502	0.1174^{B}	0.3906	0.8050
SEM			0.0008	0.0042	0.0131	0.0007	0.0042	0.0131
Ca Source								
	Limestone		0.1659^{A}	0.4446^{A}	0.8832^{A}	0.1207^{A}	0.3994^{A}	0.8380^{A}
	HSC		0.1629^{B}	0.4323^{B}	0.8404^{B}	0.1176^{B}	0.3871^{B}	0.7951^{B}
SEM			0.0008	0.0042	0.0131	0.0007	0.0042	0.0131
Phytase								
	0		0.1585^{B}	0.4195^{B}	0.8316^{B}	0.1132^{B}	0.3743^{B}	0.7863^{B}
	1000		0.1703^{A}	0.4574^{A}	0.8920^{A}	0.1251^{A}	0.4122^{A}	0.8468^{A}
SEM			0.0008	0.0042	0.0131	0.0007	0.0042	0.0131
P values								
Ca levels			0.0022	0.3968	0.2209	0.0015	0.3921	0.2199
Ca source			0.0087	0.0468	0.0253	0.0059	0.0449	0.0250
Phytase			<0.0001	<0.0001	0.0020	<0.0001	<0.0001	0.0019
Ca levels x Ca source			0.2591	0.9593	0.9398	0.2311	0.9539	0.9376
Ca levels x Phytase			0.0002	0.2186	0.8735	<0.0001	0.2137	0.8762
Ca source x Phytase			0.0005	0.2600	0.0879	0.0002	0.2402	0.0849
Ca x Ca source x Phytase			0.0352	0.6424	0.5196	0.0288	0.6387	0.5196

[a,c] Means within a column lacking a common superscript are significantly different from each other (P<0.05).

Table 4.4: Dietary calcium level, calcium source and phytase effect on feed intake and feed conversion d 0-7, 0-14 and 0-21.

Variables		Feed Intake (kg)			Feed Conversion (kg:kg)		
		D 0 – 7	D 0 - 14	D 0 - 21	D 0 – 7	D 0 – 14	D 0 – 21
Ca (%)	Ca Source						
0.60	Limestone	0.1216^{A}	0.4973	1.1603	0.9867	1.2372	1.3656
	HSC	0.1154^{AB}	0.4857	1.1395	0.9758	1.2497	1.4166
0.90	Limestone	0.1146^{B}	0.4785	1.1372	0.9709	1.2101	1.3925
	HSC	0.1186^{AB}	0.4996	1.1619	1.0208	1.3101	1.4973
SEM		0.0024	0.0075	0.0242	0.0218	0.0246	0.0347
Ca (%)	Phytase						
0.60	0	0.1176^{A}	0.4781	1.1122	1.0030	1.2576	1.3997
	1000	0.1194^{A}	0.5048	1.1876	0.9594	1.2293	1.3825
0.90	0	0.1103^{B}	0.4715	1.1418	1.0130	1.2878	1.4891
	1000	0.1228^{A}	0.5066	1.1573	0.9788	1.2324	1.4006
SEM		0.0024	0.0075	0.0242	0.0218	0.0246	0.0347
Phytase	Ca Source						
0	Limestone	0.1156	0.4760	1.1289	0.9891	1.2414	1.3794
	HSC	0.1123	0.4998	1.1685	1.0269	1.2059	1.5095
1000	Limestone	0.1205	0.4737	1.1251	0.9686	1.3040	1.3787
	HSC	0.1217	0.5116	1.1764	0.9697	1.2558	1.4044
SEM		0.0024	0.0075	0.0242	0.0218	0.0246	0.0347
Ca (%)							
	0.60	0.1185	0.4915	1.1499	0.9812	1.2434	1.3911
	0.90	0.1166	0.4890	1.1495	0.9959	1.2601	1.4449
SEM		0.0016	0.0053	0.0171	0.0154	0.0174	0.0245
Ca Source							
	Limestone	0.1181	0.4879	1.1487	0.9788	1.2236^{B}	1.3790^{B}
	HSC	0.1170	0.4926	1.1507	0.9983	1.2799^{A}	1.4569^{A}
SEM		0.0016	0.0053	0.0171	0.0154	0.0174	0.0245
Phytase							
	0	0.1140^{B}	0.4748^{B}	1.1270	1.0080	1.2727	1.4444
	1000	0.1211^{A}	0.5057^{A}	1.1724	0.9691	1.2308	1.3916
SEM		0.0016	0.0053	0.0171	0.0154	0.0174	0.0245
P Values							
Ca levels		0.4318	0.7465	0.9874	0.5046	0.5005	0.1296
Ca source		0.6538	0.5312	0.9348	0.3754	0.0257	0.0296
Phytase		0.0043	0.0001	0.0676	0.0797	0.0942	0.1361
Ca levels x Ca source		0.0378	0.0528	0.3553	0.1691	0.0801	0.4450
Ca levels x Phytase		0.0294	0.5801	0.2249	0.8310	0.5841	0.3119
Ca source x Phytase		0.3535	0.3500	0.8111	0.4028	0.7986	0.1407
Ca (%) x Ca source x Phytase		0.9823	0.9794	0.7080	0.4018	0.7367	0.3360

[a,b] Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).

Table 4.5: Effects of dietary calcium level, calcium source and phytase supplementation on Ca and P apparent ileal digestibility (AID) on d 07,14 and 21.

Variables			Phosphorus AID			Calcium AID		
			Day 07	Day 14	Day 21	Day 07	Day 14	Day 21
Ca (%)	Ca Source	Phytase (FTU/kg)	%					
0.6	Limestone	0	0.5682^{DE}	0.6333^{BC}	0.5812^{CD}	0.4897^{BC}	0.5766^{ABC}	0.4957^{ABC}
		1000	0.7427^{A}	0.7531^{A}	0.7223^{A}	0.6451^{A}	0.6656^{A}	0.6282^{A}
	HSC	0	0.6316^{BC}	0.6866^{B}	0.6898^{AB}	0.5370^{ABC}	0.6205^{A}	0.6070^{AB}
		1000	0.6622^{B}	0.6367^{BC}	0.6433^{ABC}	0.5666^{ABC}	0.5771^{ABC}	0.5397^{ABC}
0.9	Limestone	0	0.5967^{CDE}	0.6213^{C}	0.6102^{BCD}	0.5412^{ABC}	0.5843^{AB}	0.4715^{ABC}
		1000	0.6407^{BC}	0.6132^{CD}	0.5789^{CD}	0.5045^{BC}	0.4856^{CD}	0.4340^{C}
	HSC	0	0.5405^{E}	0.5496^{E}	0.5679^{CD}	0.4438^{C}	0.4508^{D}	0.4456^{C}
		1000	0.6171^{BCD}	0.5626^{DE}	0.5403^{D}	0.5966^{AB}	0.5187^{BCD}	0.4584^{BC}
SEM			0.0143	0.0120	0.0210	0.0278	0.0209	0.0353
Ca								
	0.60		0.6512^{A}	0.6774^{A}	0.6591^{A}	0.5596	0.6100^{A}	0.5676^{A}
	0.90		0.5980^{B}	0.5867^{B}	0.5743^{B}	0.5215	0.5098^{B}	0.4524^{B}
SEM			0.0071	0.0060	0.0107	0.0139	0.0104	0.0176
Ca Source								
	Limestone		0.6371^{A}	0.6552^{A}	0.6231	0.5451	0.5780^{A}	0.5074
	HSC		0.6129^{B}	0.6089^{B}	0.6103	0.5360	0.5418^{B}	0.5127
SEM			0.0071	0.0060	0.0107	0.0139	0.0104	0.0176
Phytase								
	0		0.5843^{B}	0.6227^{B}	0.6123	0.5029^{B}	0.5580	0.5050
	1000		0.6657^{A}	0.6414^{A}	0.6212	0.5782^{A}	0.5618	0.5151
SEM			0.0071	0.0060	0.0107	0.0139	0.0104	0.0176
P values								
Ca			<0.0001	<0.0001	<0.0001	0.0575	<0.0001	<0.0001
Ca Source			0.0198	<0.0001	0.3961	0.6439	0.0174	0.8345
Phytase			<0.0001	0.0320	0.5555	0.0003	0.8031	0.6903
Ca x Ca Source			0.1262	0.0878	0.0708	0.7439	0.3517	0.8095
Ca x Phytase			0.0406	0.0612	0.0131	0.3836	0.2025	0.3764
Phytase x Ca Source			0.0078	<0.0001	0.0033	0.4216	0.5651	0.1430
Ca x Ca Source x Phytase			<0.0001	<0.0001	0.0023	0.0002	<0.0001	0.0157

[a,e] Means within a column lacking a common superscript are significantly different from each other (P<0.05).

Table 4.6: Effects of dietary calcium level, calcium source and phytase supplementation on gastrointestinal pH of broilers on d 14 and d 21

Variables		Day 14		Day 21	
		Gizzard	Duodenum	Gizzard	Duodenum
Ca (%)					
	0.60	1.6520	5.3899	2.6373[B]	5.6822
	0.90	1.8618	5.3763	2.9635[A]	5.7375
SEM		0.0920	0.0415	0.0653	0.0251
Ca Source					
	Limestone	1.7897	5.3606	2.8232	5.7238
	HSC	1.7241	5.4055	2.7776	5.6959
SEM		0.0920	0.0415	0.0653	0.0253
Phytase					
	0	1.6988	5.3990	2.8025	5.7223
	1000	1.8150	5.3671	2.7982	5.6974
SEM		0.0920	0.0415	0.0648	0.0255
P values					
	Ca	0.1080	0.8165	0.0006	0.1231
	Ca Source	0.6139	0.4447	0.6237	0.4344
	Phytase	0.3721	0.5861	0.9633	0.4855
	Ca x Ca Source	0.9890	0.1909	0.7705	0.3243
	Ca x Phytase	0.4389	0.0986	0.6375	0.7378
	Phytase x Ca Source	0.0820	0.7691	0.0675	0.1525
	Ca x Ca Source x Phytase	0.4337	0.1318	0.4099	0.8878

[a,b] Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).

Chapter V: Epilogue

In the past 20 years, several feed additives have been introduced into poultry diets in order to improve nutrient availability and digestibility. However, poultry nutritionists still use NRC (1994) nutrient requirements as reference when formulating broiler diets. Phytase supplementation is probably one of the best examples to illustrate the likely inaccuracy of nutrient requirements for growing broilers. It has been estimated that at least 50% of poultry diets are supplemented with exogenous phytases. NRC experiments that were designed to establish nutrient requirements for growing broilers were not conducted supplementing birds with exogenous phytases, and yet poultry nutritionists still formulate broiler diets using NRC (1994) requirements as reference. Therefore, to say that NRC (1994) nutrient requirements are inaccurate is probably an understatement, and the poultry industry needs to review these requirements especially when supplementing poultry diets with exogenous phytases.

Benefits from phytase supplementation are evident from improvements in nutrient digestibility and bird performance. However, little information is available in peered reviewed literature about the effects of phytase supplementation on gastrointestinal health. Since exogenous phytases improve nutrient availability, phytases have the likelihood to alter the dynamics in the intestinal lumen in disease settings. In this dissertation we had the chance to research some of the consequences of phytase supplementation during a necrotic enteritis outbreak. Our data revealed that while phytase supplementation still improved nutrient digestibility, necrotic enteritis masked benefits in bird performance from phytase supplementation. Results from this trial also suggest that phytase supplementation might be detrimental to intestinal health, since nutrients released from the phytate molecule may be utilized by enteric pathogens when animals are unable to digest and absorb these nutrients.

Information regarding the effects on phytase supplementation on gastrointestinal health could be extremely useful for the poultry industry. When supplementing diets with exogenous phytases, poultry nutritionists are apprehensive with the discrepancy between bird performance of commercial houses and research trials. These disparities in response are probably associated with environmental and managerial differences of commercial houses and experimental settings. During research trials, the environment is more controlled in order to eliminate external factors that would influence the results. In commercial settings, all these factors act in association likely resulting in the discrepancies observed. Therefore, understanding the effects of phytase supplementation in gastrointestinal health could help identify these differences in responses, and ultimately improve efficacy of phytase supplementation by addressing these differences.

Further research needs to be conducted to better elucidate the role of calcium in the pathogenesis of necrotic enteritis. The increase in mortality due to NE observed when broilers were fed 0.9% Ca as compared to 0.6% Ca in this trial, in association with other researchers findings in regards to Ca participation in the mode of action of alpha-toxin and netB, strongly suggest that dietary Ca level is an important factor in NE pathogenesis. It is necessary to research the mechanisms in which calcium affects toxin activity. In addition, further research is needed to determine the true nutrient requirements with exogenous phytase supplementation. Research has revealed several extra-phosphoric benefits from phytase supplementation. The supplementation of poultry diets with other exogenous enzymes, such as glucanases, xylanases and proteases, also has an impact in nutrient requirements. The intestinal lumen is a very dynamic environment giving ample opportunity for enzymes to interact with nutrients and with each other. Additionally, data from this work

supports that exogenous phytases have a significant impact in gastrointestinal health. Therefore, the impact of enzyme supplementation needs to be further explored.