THE ROLE DIETARY CALCIUM PLAYS ON NECROTIC ENTERITIS PATHOGENESIS AND BROILER PERFORMANCE

A Dissertation

by

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ABSTRACT

The objective of this dissertation was to determine if limestone (source and particle size), dietary calcium inclusion rate, or different protein sources have an impact on naturally occurring necrotic enteritis (NE) pathogenesis and resulting broiler performance. Two experiments were conducted. The first experiment was to determine the impact of limestone source and particle size, as well as, dietary calcium levels on the pathogenesis of NE. The second experiment was to evaluate the effects of animal protein, dietary calcium level, and limestone particle size supplementation on the pathogenesis of NE.

Experiment one determined that there was an interaction between particle size and dietary "calcium" levels on grower mortality. Birds fed high levels of fine particle size had increased mortality. Through day 35, there is a 3-way interaction with overall performance of the birds. Birds fed standard levels of coarse particle size of calcium source 1 decreased in body weight and increased feed conversion ratio. According to ileal digestible intake after the outbreak of NE, there was a compromise in absorption of phosphorus and amino acids. It was determined that the largest impact was seen with the standard particle size, therefore, experiment two used standard particle size of limestone source 1 and 2, as well as, both dietary calcium levels.

Experiment two investigated the role dietary protein sources, dietary calcium level, and limestone particle size have on the pathogenesis of NE. Up to d 15, birds fed low dietary levels of limestone source 1 in the veggie diets had a reduced body weight. Additionally, during the same time period, standard dietary levels of calcium in the all-veggie diets reduced the feed conversion ratio. Conclusively, birds fed animal protein in the diet had increased levels of mortality.

During both experiments, limestone had an impact on the overall performance of the birds. Mortality was increased during grower phase due to interactions with the limestone. In conclusion, limestone reduces the overall impact NE has on flocks and presents great potential in additional research moving forward. Findings show that limestone is more than just a dietary filler.

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NOMENCLATURE

NE	Necrotic Enteritis
BW	Body Weight
d	Day
AvP	Available Phosphorus
Asp	Aspartic Acid
Thr	Threonine
Ser	Serine
Glu	Glutamic Acid
Pro	Proline
Gly	Glycine
Ala	Alanine
Val	Valine
Met	Methionine
Ile	Isoleucine
Leu	Leucine
Tyr	Tyrosine
Phe	Phenylalanine
Lys	Lysine
His	Histidine
Arg	Arginine
P	Phosphorus

AGP	Antibiotic growth promoter
CDI	*Clostridium difficile* infections
GI	Gastrointestinal

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1. INTRODUCTION

According to global market analysis, in 2019, poultry production was reported at 218 billion pounds of ready to cook meat worldwide (Foreign Agriculture Service, 2020). In the US, there was approximately 44 billion pounds of ready to cook poultry meat (Foreign Agriculture Service, 2020). Continual improvements in performance have allowed the poultry industry to fulfill an increase in consumer demand for a low-cost protein source that is readily available.

The poultry industry has always been driven by consumer demand. In 2006, the EU banned the use of antibiotics in animal feeds. Historically, when the EU establishes an enactment, the US adopts similar practices shortly after due to the export of poultry meat. In 2017, the US implemented the veterinarian feed directive, resulting in two major changes in poultry production. The first being discontinued use of sub-therapeutic levels of medically important antibiotics, and second, establishing that antibiotics deemed medically important are only allowable to be used with a prescription. Since the veterinarian feed directive was put in place, feeding management programs and resulting changes in intestinal health have been critical areas of challenges for the industry. Compromised intestinal health resulting from non-antibiotic feeding programs has increased the incidence of necrotic enteritis (NE). In the industry, NE naturally occurs throughout the world resulting from different predisposing factors that increase the proliferation of *Clostridium perfringens*.

The impact from NE is evident when the difference in estimated economic losses are shown. There were estimates of $2 billion in 2000 compared to $6 billion in 2015 (Wade and Keyburn, 2016). Estimated escalations in economic losses are due to increased mortality and morbidity present with NE. The morbidity associated with subclinical NE can be attributed with decreases in broiler performance, due to necrosis of the small intestine and reduced absorption of

nutrients in the bird. Clinical levels of NE can produce up to 1% daily mortality, which reduces the profitability of poultry flocks. Overall, reduced nutrient absorption results in an increase in feed conversion of the birds, lighter overall body weight, and increased flock mortality.

Little is known about the exact factors that cause NE, but there are many components that are thought to contribute to the onset of this costly disease. Factors include increased dietary protein, intestinal damage due to coccidiosis, different protein sources, and stress, and a variety of other dietary contributors. Researchers have looked at the role phytase has through the release of the bound cation minerals on the pathogenesis of NE (Paiva et al., 2014; Paiva et al., 2013; Walk et al., 2012). Additionally, research has been done investigating the impact that different calcium (Ca) sources have on the onset of NE. In the research trials with elevated soluble calcium, birds did not need to be administered a high dose of *C. perfringens* by oral gavage to induce NE. There is extensive research on NE and how the disease alters the intestinal environment when a high dose of C. perfringens is administered, but little is known about the naturally occurring NE occurring in the poultry industry. Therefore, there is a prominent need for research investigating different dietary calcium sources (specifically limestone) and different dietary protein ingredients for their contributory role in naturally occurring NE.

2. LITERATURE REVIEW

2.1. Necrotic Enteritis

Necrotic enteritis (NE) is an acute enterotoxemia that significantly impacts the poultry industry. This is a disease that affects broilers (2-5 weeks old) and turkeys (7-12 weeks old) raised on litter and also commercial layer pullets raised in cages (Hargis, 2014). There is a significant economic impact of NE on the broiler industry with an estimated cost of $0.05 per chick, which in 2000, was estimated as a worldwide total of US$2 billion (Wade and Keyburn, 2016). In 2015, this estimated impact increased to US$6 billion worldwide (Wade and Keyburn, 2016).

Antibiotic growth promotors (AGP), ionophores, and anticoccidials have historically been the partial control options for NE (Lee et al., 2011; Prescott, 1979; Williams, 2005). Necrotic enteritis has reemerged as a significant problem as a result of restrictions on in-feed antibiotics. To minimize the incidence of NE, there has been significant pressure for better management because many factors can serve as risk-factors for this disease occurrence.

2.1.1. Predisposing Factors

Clostridium perfringens is a Gram-positive anaerobic bacterium that is able to form spores. It is wide spread in the environment (e.g. in soil and sewage) and is commonly found in the intestinal tract of animals and humans, where it is pathogenic in certain circumstances (Petit et al., 1999). Necrotic enteritis is a disease that could potentially affect all poultry due to the fact that the pathogenic bacterium, *C. perfringens*, is present, however it only becomes a problem when the proper conditions are present for *C. perfringens* to become active and produce toxins needed to cause intestinal damage. Figure 1 demonstrates multiple factors that play a role in a NE outbreak (Moore, 2016).

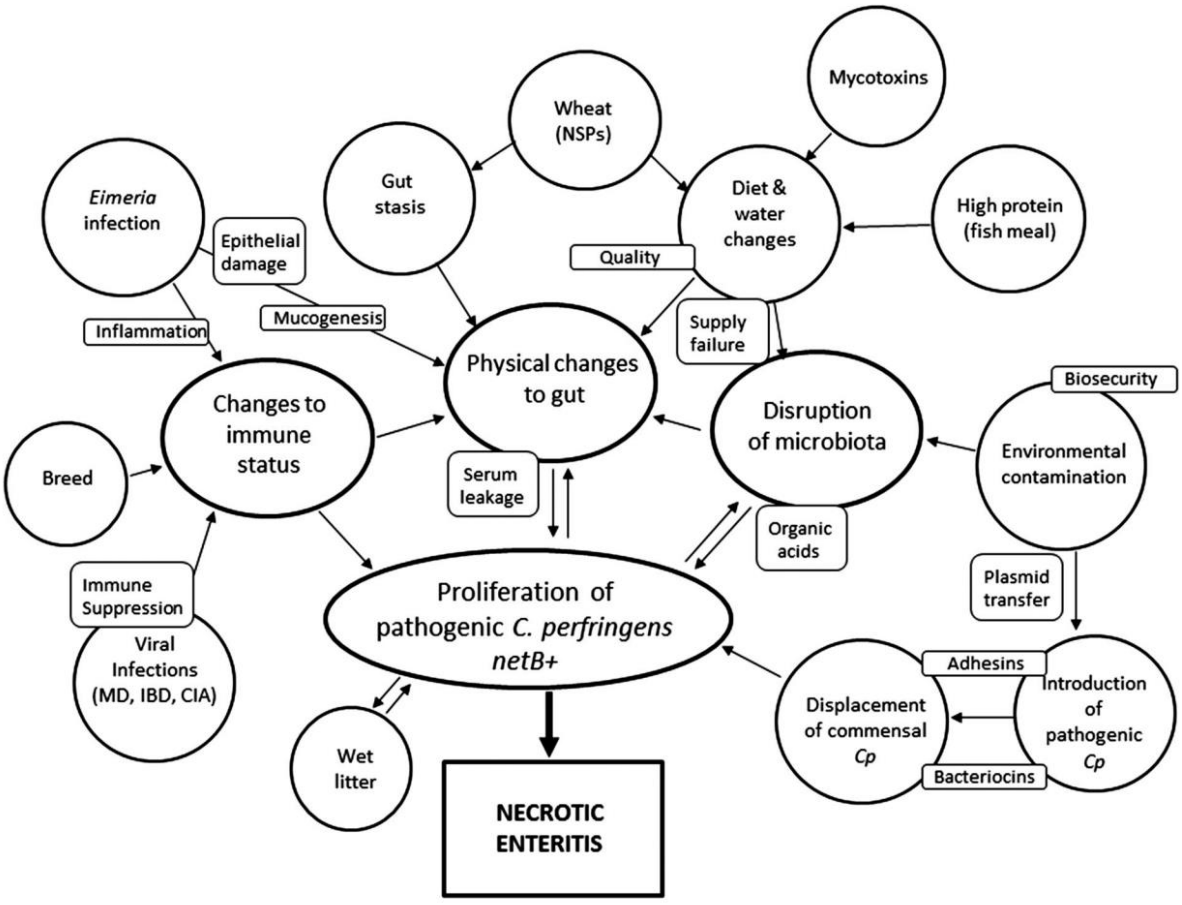


Figure 1. Summary of predisposing factors for NE development in chickens. Predisposing factors shown in circles and the major effects of these factors are shown in ovals. Important factors that may drive the influence of the predisposing factors are shown in the small rectangular boxes (reprinted from Moore, 2016).

2.1.2. Coccidiosis

Due to the similarity in the life cycle timing of coccidiosis, and the time point in which NE mortality typically spikes, there is usually a connection between coccidiosis infection and NE. Coccidiosis is caused by seven different species of *Eimeria*. These include *Eimeria acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox,* and *E. tenella,* which have specific factors that distinguish them from one another. *E. acervulina, E. mivati,* and *E. praecox* can all be found in the upper digestive tract and are distinguishable by macroscopic lesions. *E.*

acervulina presents whitish, round lesions, sometimes in ladder-like streaks in a light infection, but can also result in thickened intestinal walls with a heavy infection. *E. mivati* is similar to that of *E. acervuline* in a heavy infection, but in a light infection there are rounded plaques of oocysts present. To distinguish between these two *Eimeria* species, intestinal scrapings can be observed microscopically for differences in oocyst shape and size. *E. praecox* does not present lesions and can only be observed microscopically. The *E. praecox* oocysts are much larger than the other two *Eimeria* species that infect the upper intestine. *E. tenella* is the easiest of its counterparts to diagnose since it is the only species to infect the avian ceca. All other species affect the mid to lower sections of the intestine, but each has factors that make them diagnosable to a trained technician (Conway and McKenzie, 2007).

Development of the parasite in the host cells involves both asexual and sexual reproduction. Destruction of the intestinal host tissue leads to multiple different lesions, which can be visible during the peak cycling of the oocysts. Visible lesions, as well as oocyst scrapings from the mucosa, can be scored or counted to provide numerical rankings to track the success of preventative measures or severity of infection (Johnson and Reid, 1970). Most control is attempted by vaccination, anticoccidial drugs, or a combination of the two. The majority of issues arise from subclinical coccidiosis, since clinical outbreaks are not as common due to methods of control. Subclinical outbreaks allow for damage to occur in the intestinal lining, which provides potential environments for opportunistic microorganisms, such as *E. coli or C. perfringens,* to cause disease.

2.1.3. Dietary Factors

Specific dietary factors have been shown to influence the proliferation of *C. perfringens* which, consequently, advances the onset of NE. Previously, an overview of cereal content of

diets fed to broilers in Norway from 1969 to 1989 demonstrated that 2 major outbreaks of NE were associated with increased use of barley and wheat in the broiler diet (Kaldhusdal and Skjerve, 1996). More importantly, the ratio of wheat and barley to maize was an important predictor in NE, since cereal grains increase the viscosity of digesta. High levels of animal protein sources have also been reported to increase the occurrence of NE (Drew et al., 2004; Kaldhusdal and Skjerve, 1996; Wilkie et al., 2005; Zanu et al., 2020a). Meat and bone meal has the potential to induce the onset of NE with levels as low as five to six percent throughout the grow out of the bird (Zanu et al., 2020a). Drew et al. (2004) distinguished that the level and source of dietary protein have significant effects on intestinal populations of *C. perfringens* in broiler chickens. At 28 d, increasing the level of fish meal fed from 230 g/kg to 400 g/kg increased counts of *C. perfringens* in ileum and cecum, but no there was no effect observed in birds fed soy protein concentrate (Drew et al., 2004). Wilkie et al. (2005) observed similar results with birds fed animal protein-based diets having elevated numbers of *C. perfringens* compared to those fed plant protein-based diets. The exception was birds fed potato protein concentrate had similar *C. perfringens* counts to the animal-based diets. Dietary protein level and source have a significant impact on the quantity of *C. perfringens* in the lower intestine, but other dietary factors (such as amino acids and calcium) have an impact on the pathogenesis of NE (Wilkie et al., 2005).

Researchers have determined that dietary calcium level and source, along with phytase, have an impact on the occurrence of NE. Unlike most NE studies, the research conducted by Paiva et al. (2013; 2014) was a naturally occurring NE and birds were not challenged with high doses of *C. perfringens*. Birds had increased mortality due to NE when fed 1000 FTU/kg of an *E. coli* 6-phytase expressed in *Trichoderma reesei* (Paiva et al., 2014). Additionally, increased

mortality was observed with industry standard levels of calcium (0.9% fed in all phases) from d 0 to 19 and d 0 to 35 (Paiva et al., 2014). During a naturally occurring NE outbreak birds fed industry levels of a highly calcified marine seaweed had increased mortality from d 0 to 21 (Paiva et al., 2013). In a natural occurring model, dietary calcium level and source have an impact on the proliferation of *C. perfringens* and the severity of NE outbreak (Paiva et al., 2013). Overall, dietary calcium source and level, dietary protein source and level, as well as some dietary additives, affect the pathogenesis of NE.

2.1.4. Immune Status and Stress

C. perfringens is classified as an opportunistic pathogen, this organism is present in the intestinal tract throughout the life of the bird, but only if there is an environment ideal for proliferation, then NE will occur. Coinfection of *C. perfringens* and immunosuppressive viruses, such as infectious bursal disease virus, chick anemia virus, and Marek's disease virus, has been suggested to promote the development of NE (Lee et al., 2011). Most NE research trials are challenge studies where the immune system is compromised with a high dose of *Eimeria* species or commercial bursal disease vaccine (Lee et al., 2011; McReynolds et al., 2007; Stringfellow et al., 2009; Zanu et al., 2020a). However, immunosuppression is not the only physiological alteration that can affect the bird and increase susceptibility to NE.

Rapid growth and increased performance of modern broilers place physiological pressure on the birds, but due to the location in which chickens are raised, there is also the possibility of environmental stressors such as: withdraw of feed, heat stress, cold stress, and many others (Burkholder et al., 2008; Tsiouris et al., 2015; Tsiouris et al., 2018). It was previously reported that cold stress, as well as heat stress, increased *Clostridium* counts in the cecum (Tsiouris et al., 2015; Tsiouris et al., 2018). Cold stress impacted the counts of *C. perfringens* present in the

cecum, rather than visible lesions present in the gastrointestinal (GI) tract (Tsiouris et al., 2015). Heat stress had a larger impact on the gross lesion scores than it did on the cecum *C. perfringens* counts (Tsiouris et al., 2018). Tsiouris et al. (2018) determined that longer term (3 days) temperature stress influenced intestinal lesion scores associated with NE, but Burkholder et al. (2008) determined that shorter term (24 hours) heat stress was enough to alter the intestinal microbial population. The alteration in the intestinal tract microbiota due to temperature stress may allow for opportunistic pathogens (*C. perfringens*) to proliferate and increase chances of NE. A trial with 24-hour fasting of the birds increased the attachment of *Salmonella enteritidis* to ileal tissue, which would allow for opportunistic enteric pathogen (such as *C. perfringens*) to cause disease (Burkholder et al., 2008). During feed changes from starter to grower, possibilities of feed withdraw or absence for extended periods of time may alter the intestinal population for pathogenic *C. perfringens* growth or activation, which can lead to NE. However, similar situations do not always cause NE. Determining the factors that make the intestinal tract favorable for the pathogenic *C. perfringens* to proliferate and cause disease is critical to developing applicable methods of control for the poultry industry.

2.1.5. Intestinal Physiopathology

Less is known of the physiopathology of NE, since the exact conditions needed for highly pathogenic *C. perfringens* to proliferate are not fully understood. What is occurring in the intestine during an outbreak is even less understood, though, there are many visual signs indicating a bird is suffering from clinical NE. Some of the signs present with NE are common to other poultry diseases including: ruffled feathers (Immerseel et al., 2004), reluctance to move (Helmboldt and Bryant, 1971), and depressed activity (lethargy) (Immerseel et al., 2004). There are also signs of somnolence (Al-Sheikhly and Al-Saieg, 1980), pronounced droopiness

(Helmboldt and Bryant, 1971), and diarrhea (Helmboldt and Bryant, 1971). The largest issue with NE is that most of the time the morbidity quickly transitions into daily mortality rates of approximately 1 % (Wade and Keyburn, 2016), which can last for 5 to 10 days with mortality totaling 2 to 50 % (Hargis, 2014). Though the clinical indices are similar to other poultry diseases, there are some intestinal signs that make NE distinguishable from other diseases.

Morbidity present in the birds is due to what is occurring in the lower intestine (jejunum/ileum). Gross lesions are found in the small intestine, which as NE worsens then the small intestine becomes ballooned, friable, and contains a foul-smelling, brown fluid (Hargis, 2014). The mucosa is usually covered in a tan to yellow pseudo membrane often referred to as a "Turkish towel" (Hargis, 2014). Determination of NE can be made with a trained eye, but little is known about what makes the intestinal environment favorable for the proliferation of *C. perfringens*.

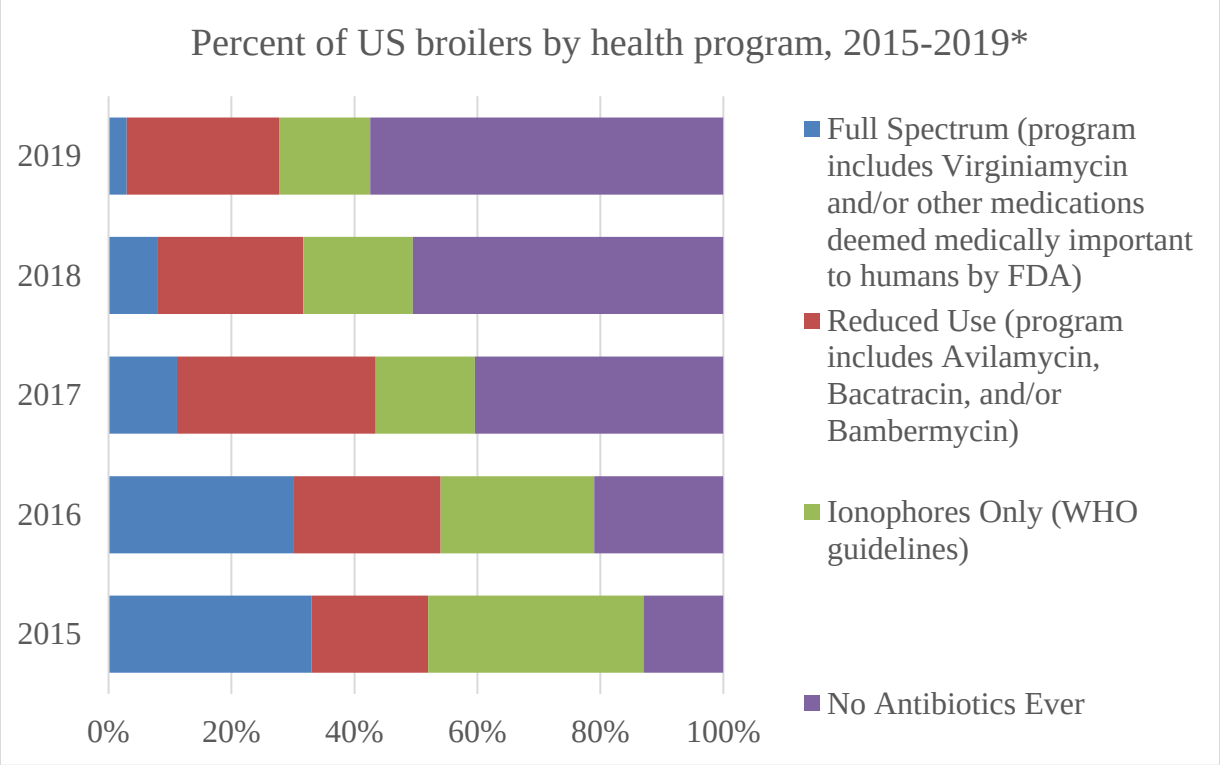
There are intestinal physiopathological circumstances that favor the development of NE, such as intestinal pH, decreased intestinal motility, intestinal mucosal damage, and intestinal obstruction (Cooper and Songer, 2009). The lower gastrointestinal tract normally has a pH of approximately 7.0 to 7.5 (Dharne, 2008), but there are times that the lower intestine can have a pH around 6. *Clostridium perfringens* is able to grow at a pH from 5.0 to 9.0, but ideal pH is from a 6.0 to 7.0 (Juneja et al., 2010). During specific times of the bird's life and based on diet or other factors changing pH, the lower intestine is ideal for the growth of the *Clostridium perfringens*. Wheat based or barley based diets increase mortality up to 2 to 3 times higher due to decreased intestinal motility (Immerseel et al., 2004). This is most likely due to the nutrients being available for longer in the lower intestine. Additionally, *C. perfringens* proliferates *in vitro* at higher rates in ground wheat or barley compared to corn (Cooper and Songer, 2009). In

summary, there are many factors that indicate the birds have NE and physiopathological circumstances that favor the development of NE, but there needs to be further research to determine possible treatments for NE with increases in no antibiotic ever production.

2.1.6. Prevention and Treatment

Necrotic enteritis is most commonly controlled through management practices, since the reduction of stress for the birds can allow for maintenance of an intestinal environment that is not favorable for the proliferation of *C. perfringens*. Control options for NE most commonly include anything that reduces exposure to risk factors such as coccidiosis and unsuitable diets (Cooper and Songer, 2009). Removal of dietary factors, such as, wheat, barley, and animal byproducts can reduce the incidence of NE (Zanu et al., 2020b). For years, NE was controlled through the use of antibiotic growth promoters (AGP), in which many of the antibiotics proved to reduce the incidences of NE. There is evidence that inclusion of narasin at 70 ppm was enough to reduce NE-related mortality in a challenge experiment (Brennan et al., 2001). Many of the other banned AGPs either reduced the numbers of *C. perfringens* in the intestinal tract or would reduce the mortality associated with NE (Watkins et al., 1997). The increase in NE can possibly be associated with the decline in AGP use the past few years resulting from the voluntary withdrawal of antibiotics in the US to move towards no antibiotics ever production, as well as, the veterinarian feed directive (2020). The change in health production programs is illustrated in Figure 2.

Figure 2. Percent of US broilers by health program from 2015 to 2019



†2015 numbers extrapolated from feed tonnage.
* Data by year may not total 100% due to rounding
Source: Rennier Associates, Inc.

The microbial population of the intestinal tract is a complex natural resource that has the possibility to mitigate NE similar to that of antibiotics. Inoculation of germ-free birds with broth culture of *C. perfringens* caused mortality with signs of depression, no appetite, deep breathing, reluctance to move and apathy, while the conventional birds expressed no clinical signs of NE and no mortality. The authors reported this indicated that the intestinal microflora were involved in protecting the birds from the detrimental effect of *C. perfringens* (Fukata et al., 1988). The primary tools for utilizing the native microbial population to combat the pathogenic microorganisms of the intestinal tract of food animals include competitive inclusion, probiotics, and prebiotics. Probiotics also known as direct fed microbials (DFM) are characterized as live

microbial supplements, which are used in the feed to improve the health of animals by balancing the intestinal microbes (Khalique et al., 2020). Competitive exclusion technology is a specific type of probiotic strategy that involves the addition of a non-pathogenic bacterial culture of a single strain or multiple strains, to the intestinal tract of food animals in order to reduce colonization or decrease pathogenic bacteria in the GI tract (Callaway et al., 2008). Whereas, prebiotics are organic compounds that are unavailable to, or undigestible by the host animal. However, prebiotics are available to a specific proportion of the microbial population. They are often described as 'functional foods' or 'nutraceuticals' (Schrezenmeir and De Vrese, 2001). Probiotics and prebiotics have been shown to reduce the severity of NE in birds that have been challenged with *C. perfringens* (Al-Baadani et al., 2016). The use of Aviguard®, which is a competitive exclusion culture, had results similar to the antibiotic bacitracin methylene disalicylate (BMD) which reduced the severity of lesions associated with NE and also reduced mortality compared to the challenged groups without BMD or DFM (Hofacre et al., 2019). Probiotics and prebiotics can improve intestinal health through a variety of mechanisms that are still not understood (Callaway et al., 2008). However, the continued increase in knowledge of the mechanisms involved in balancing the beneficial microbiota allows for the possible use of probiotics and prebiotics through competitive exclusion as possible treatments for NE.

Vaccination with an adjuvant with low doses of different clostridual toxins (alpha, NetB, beta, epsilon) can induce an immunological response against clostridia and clostridial toxins in mice, lambs, pigs, cattle, and poultry (De La Rosa et al., 1997; Keyburn et al., 2013; Schoepe et al., 2001; Springer and Selbitz, 1999; Troxel et al., 1997). The vaccination strategy for inducing most immunological responses is done so by the subcutaneous injection of live clostridia or clostridial toxins, but due to the high throughput of the poultry industry not every bird can be

handled. To accomplish this injection of recombinant NetB at day 7 and day 17 was able to protect birds against disease, but it did not protect the bird from a severe challenge with *C. perfingens* (Keyburn et al., 2013). A more suitable form of vaccination of the birds needs to be looked at due to the high cost associated with handling all the birds. Protection of the progeny chicks has had moderate response to prevention of NE with the maternal transfer of antibodies from vaccinated hens (Crouch et al., 2010; Lovland et al., 2004). Further research is needed to understand how the bird can be protected against NE through different vaccine forms or strategies.

2.1.7. *Clostridium perfringens* Toxins

Clostridium perfringens is a Gram-positive spore-forming anaerobe that is the causative agent of many histotoxic and enterotoxic diseases in humans and animals (Uzal et al., 2014). *Clostridium perfringens* strains have now been proposed to be classified into seven toxinotypes (A, B, C, D, E, F, and G) based on the toxins which they produce (Petit et al., 1999; Rood et al., 2018). The key feature of these bacteria's is that their mechanism of damage is by the production of potent protein toxins, most of which are extracellular. All the toxins are involved in different specific disease symptoms. Major toxins include alpha toxin, beta toxin, epsilon toxin, iota toxin, and NetB toxin (Rood et al., 2018). Among them, the main toxins hypothesized to cause NE in poultry are alpha- and beta-toxin.

2.1.7.1. Alpha-Toxin

Alpha-toxin is a zinc (Zn) dependent phospholipase/sphingomyelinase C and is encoded by the Plc gene (Cooper and Songer, 2009; Petit et al., 1999; Titball et al., 1999a). The alpha-toxin and beta-toxin both interact with the cell membrane to promote membranes disorganization (Petit et al., 1999). The α-helical amino-terminal part contains the zinc-dependent catalytic site,

where as the β-sandwich carboxy-terminal part is homologous to eukaryotic C2 domains, which are involved in membrane interaction and mediate the binding to phospholipids in a calcium-dependent manner (Petit et al., 1999). Hydrolysis of lecithin results in the formation of diacylglycerol, resulting in activation of protein kinase C, and subsequent stimulations of the arachidonic acid cascade (Immerseel et al., 2004; Petit et al., 1999). The arachidonic acid cascade induces the synthesis and release of inflammatory mediators such as leukotrienes, thromboxane, platelet-activating factor and prostacyclin (Petit et al., 1999). This results in blood vessel contraction, increased vascular permeability, platelet aggregation and myocardial dysfunction, all of which contribute to local and systemic clinical manifestations characterized by profound shock and death, leading to increased mortality associated with NE (Petit et al., 1999).

Long and Truscott (1976) proposed that *C. perfringens* type A were the causative agent for NE. Type A has been determined to be positive for alpha toxin and deficient in all other toxins (Petit et al., 1999). Further studies indicated that when germ-free birds were inoculated with type A *C. perfringens,* NE-related mortality was increased compared to conventional birds (Fukata et al., 1988). Interpretation of the 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 the purified alpha-toxin (Fukata et al., 1988; Long and Truscott, 1976). It was also reported that *C. perfringens* type C was a causative agent for NE (Songer, 1996), whereas, the type C *C. perfringens* is positive for alpha and beta toxin (Petit et al., 1999). More recently, there was the discovery of NetB in avian *C. perfringens* type A strains that has changed the understanding of NE development (Keyburn et al., 2008). The NetB toxin is produced by most strains isolated from necrotic lesions, but is less commonly found in *C.*

perfringens isolates from healthy birds (Keyburn et al., 2010). This has led to the conclusion that NetB is the key virulence factor in *C. perfringens* strains that cause avian necrotic enteritis (Keyburn et al., 2010).

2.1.7.2. NetB Toxin

NetB toxin was discovered in type A strain *C. perfringens* (Keyburn et al., 2008) and has been determined to be a key virulence factor in the *C. perfringens* strains that cause avian NE (Keyburn et al., 2010). Since this protein has similarity to *C. perfringens* beta toxin (38% identity), it was designated NE toxin, B-like (NetB) (Keyburn et al., 2008). NetB toxin also has sequence similarities to gamma-toxin (40% identity), *Staphylococcus aureus* alpha-hemolysin (30% identity), and the A, B, and C components of *S. aureus* gamma-toxin (25%, 22%, and 23% identity, respectively) (Keyburn et al., 2010). Similarities in the sequences reveal that only 14 amino acids are identical in these proteins, and 50 residues are conservatively substituted (Keyburn et al., 2010). In most studies, there was a minor fraction of NetB negative isolates from diseased birds (Keyburn et al., 2010; Keyburn et al., 2008; Martin and Smyth, 2008). In a study conducted by Martin and Smyth (2008), NetB negative isolates were recovered from a chicken displaying clear signs of NE. Twenty-five isolates from the same chicken were recovered from different lesions areas, and all were negative for NetB toxin (Martin and Smyth, 2008). These results indicate that there is a clear association between NetB toxin and development of NE, but there may be other yet to be determined virulence factors that are associated with these netB negative disease-producing strains (Keyburn et al., 2010). The indication that there are other toxins or virulence factors that induce NE reveals the importance of pre-disposing factors that lead to this disease occurrence.

2.2. Calcium

Calcium in the diet of broilers influences growth, feed efficiency, bone development, leg health, nerve function, and the immune system (Ross, 2018). The role calcium plays in the avian system is much different than that of the mammalian system with differences in the skeletal metabolism at times of demand. The domestic chicken will respond to hypocalcemia conditions within minutes, whereas, mammals can take over 24 hours (Stanford, 2006). The demand of birds for calcium is due to the bird producing an egg every 25-26 hours as well as their rapid growth rate when birds are young. This is very evident in egg-laying birds where 10% of their calcium reserves can be required for egg production (Stanford, 2006). Due to the high demand for calcium, it is vital that it be supplied in adequate quantities and on a consistent basis for optimal performance. The hen's skeleton contains approximately 20 grams of calcium (Soares, 1984). In a laying hen, the ovulatory cycle is approximately 25-26 hours, and it is estimated that almost 1000 mg calcium/kg body weight per day is needed by the hen for egg shell formation (Soares, 1984). Plasma calcium concentrations of 20 to 35 mg/dl should be present in a laying hen, while they should be half in non-laying birds (Soares, 1984), which is important because calcium quantity must be monitored as it is a possible risk factor for NE (Paiva et al., 2013).

During different phases of grow-out of a bird, the calcium requirement varies. According to the NRC, calcium requirements for the birds range from 0.8 to 2.0% of the diet from young birds to birds producing eggs (National Research Council, 1994). Many improvements have been made in the overall production of broiler birds with the days to market weight declining yearly as well as the feed efficiency of the birds continually improving. With the improvement in production of the birds, there has not been much attention to required change in the calcium

requirements of birds. In 1994, the suggested calcium requirement for broilers was 1% of the diet for a bird up to three weeks, which then decreased with age (National Research Council, 1994). The most recent recommendation for birds is 0.96% (Ross, 2018) for the first 10 days with declining percentage with age. Throughout the years, there has not been a change in the calcium requirements, though there have been improvements in the performance potential of the broiler.

Calcium exists as three fractions in avian serum: 1) the ionized salt, 2) calcium bound to proteins, and 3) as complexed calcium bound to a variety of anions (citrate, bicarbonate, and phosphate) (Stanford, 2006). The protein bound portion is bound to albumin and considered to be physiologically inactive. The anion and the ionized salts are both of significant importance to the birds as well the development of the NE. The ionized salts are the active fraction of serum calcium, with a role in bone homeostasis, muscle and nerve conduction, blood coagulation, and control of hormone secretions such as vitamin D_3 and parathyroid hormone (Stanford, 2006). This is important as there is evidence that calcium present within the GI tract plays a critical role in *C. difficile* germination, colonization, and pathogenesis in humans (Kochan et al., 2017). The data provide a mechanism as to why the individuals with insufficient calcium absorption are more prone to *C. difficile* infections (CDI), and modulation of the free intestinal calcium is a potential strategy to reduce CDI (Kochan et al., 2017). The ionized salts possibly play an important role in the pathogenesis of NE, as previous research has determined increased dietary calcium levels resulted in NE-related mortality (Paiva et al., 2013). As for the anions, they are important as they are easily bound by the phytate within the intestinal tract and rendered indigestible by the bird, but the common practice of the addition of phytase to the feed can release these bound calcium increasing digestible calcium in the intestine.

2.2.1. Phytate and Phytase

Phytic acid, myoinositol hexakis dihydrogen phosphate, is an ubiquitous compound that is abundant in all seeds serving as the chief storage from of phosphorus (P) (Maga, 1982). Phytate-phosphorus accounts for 50 to 80% of the total phosphorus present in cereals, grain legumes, and oilseeds (Ravindran, 1995). Since a major portion of poultry diets consist of plant-derived ingredients, phytate assumes a considerable nutritional factor. The ability for poultry to utilize phytate-phosphorus is generally assumed to be poor, presenting producers with two problems 1) the need to add inorganic P to the diets, and 2) the excretion of large quantities of P in the manure (Nelson, 1967). Phytic acid is, in its less complexed state, highly reactive and easily forms complexes with Zn, calcium, magnesium (Mg), and iron (Fe) ions, carbohydrates and proteins (Selle et al., 2000; Singh et al., 2018). Since minerals and other nutrients bind to phytate, the presence of high levels of phytate in the diet is usually related to reduced nutrient availability and absorption, which can result in poor bird performance (Selle et al., 2009). To reduce the antinutritive factors of phytate, phytase is commonly used as an enzyme to hydrolyze phytic acid to phosphoric acid and myo-inositol (Singh et al., 2018).

Walk et al. (2012) discovered that high levels (5000 FTU/kg) of phytase increased NE occurrence and resulting mortality in non-challenged Cobb 500 male broilers. Phytates are salts of phytic acid, which consist of a myo-inositol ring associated with up to 6 phosphate anions. Hydrolysis of the ester bonds between the phosphate groups and the inositol ring is necessary before the minerals become available for absorption from the GI tract (Cowieson et al., 2004). Since poultry do not possess effective levels of endogenous phytases the bird's ability to utilize the phytate-P and chelated minerals is limited (Cowieson et al., 2004). Addition of phytase allows for the release of bound minerals for digestion (Cowieson et al., 2004). There are two

classes of commercial phytases that differ in the first phosphate group of the phytate molecule that is cleaved. The 3-phytases targets the carbon in the third position, whereas 6-phytases release the carbon in the sixth position (Singh et al., 2018). Exogenous microbial phytases are mainly active in the upper digestive tract of poultry and pigs due to the acidic pH of these organs that increases phytate solubility, thereby making phytate more susceptible to phytase activity (Selle et al., 2009).

The use and inclusion rate of phytase in diets has increased to promote maximal performance of birds. The release of minerals, amino acids and energy bound by the phytate allow for increases in nutrient availability and performance of birds (Amerah et al., 2014). The addition of 500 FTU/kg of phytase significantly increased feed intake (FI), body weight (BW), and feed conversion ratio (FCR) in birds fed nutritionally marginal diets (Liu et al., 2008). The increase of phytase from 500 FTU/kg to 1000 FTU/kg increased phytate digestibility as well as improved performance of the birds (Li et al., 2016; Liu et al., 2008). Phytase has proven to be a benefit to the poultry industry to reduce the excretion of P and increase the performance of the birds, but the increased release of calcium could allow for the production of the *C. perfringens* toxins.

2.2.2. Sources of Calcium

As feed accounts for 70% of the cost of commercial poultry production, ways to reduce the cost of feed are always being investigated. Supplying the birds with the proper amount of calcium has always been an easy task as limestone is an easily accessible and low-cost product. While formulating diets, maximum calcium levels must be set as the low cost of limestone will result in it being included in the diet at levels higher than needed. There has been extensive research on the use of calcium sources other than limestone, which are calcium lactate, calcium

gluconate, oyster shell, scallop shells, egg shells, and many others (Reid and Weber, 1976; Watkins et al., 1977; Xing et al., 2020). Of all the tested samples it is common practice in the industry to use limestone as the main source of calcium in the diet of broiler birds. Laying hens will commonly have the addition of oyster shell for longer retention time in the gizzard, which allows the calcium to be more slowly released into the intestine during the time of maximum need for shell calcification (Watkins et al., 1977). The main concerns of utilizing limestone and oyster shell include the difference in composition of the minerals as well as the differences in solubility of the varying particle size, which can result in differences in calcium availability to the birds.

There are many locations throughout the United States in which limestone is extracted and crushed for use in animal feeds. Due to the different soil composition of the mine area from which limestone is extracted, the mineral composition of the limestone will vary. Limestone is going to be compromised of mostly calcite, which is the most stable form of calcium carbonate. Reid and Weber (1976) reported that limestone can vary from 36 to 40 % calcium and can have as much as 2% Mg in its composition. Of the 5 limestones sampled in their trial, it was determined that calcium availability ranged from 74.9 to 95.7% (Reid and Weber, 1976). In laying hens, it was suggested that a larger particle size could be stored in the gizzard and "metered out" for maintained supply of calcium for egg shell formation (Watkins et al., 1977). Therefore, with the variability of limestone composition and the possibility of slowly releasing calcium, the particle size of limestone could have an effect on dietary requirements. McNaughton (1981) determined that birds fed a more coarse (20 to 60 United States Bureau of Standards (USBS) particle sized $CaCO_3$) limestone during the first 3 weeks of growth had

increased body weights and bone ash compared to a 12 to 20 or 100 to 200 USBS particle-sized $CaCO_3$ source.

Dietary calcium has an impact on the performance of birds, whether it be the composition of the limestone, the particle size, or the dietary inclusion rate. However, there has been little research on the role calcium plays in pathogenesis of intestinal disease, specifically NE. There is reported evidence of *C. perfringens* needing calcium for full activation of alpha-toxin (Titball et al., 1999b). The different insolubility of limestone sources may change the calcium availability for the birds, which in turn will play a role in the pathogenesis of NE. Therefore there is a need for more research on how dietary calcium specifically limestone geographic source, particle size, and inclusion level in different poultry diets, alters the intestinal environment to favor the proliferation of *C. perfringens*.

3. THE ROLE OF DIETARY CALCIUM LEVEL AND LIMESTONE SOURCE AND PARTICLE SIZE IN NATURAL NECROTIC ENTERITIS PATHOGENESIS AND BIRD PERFORMANCE

3.1. Introduction

Necrotic enteritis (NE) has been estimated to cost the poultry industry from US$2 to 6 billion annually from losses in production and increased mortality (Wade and Keyburn, 2016). First reported by Parish in 1961, NE was minimized by sub-therapeutic levels of antibiotics in commercial poultry diets. However, consumer demand for the reduction of antibiotics and the Veterinarian Feed Directive (2020) have necessitated the poultry industry move towards no antibiotic ever or antibiotic-free production. Reduction in the use of antimicrobials and coccidiostats in feeding programs have caused a shift in production practices with more strict requirements on bird management. All management strategies must be of optimal performance to ensure that intestinal health is maintained to prevent risk for the proliferation of *C. perfringens* and subsequent NE.

Elucidating predisposing factors or contributors to NE development are important since *C. perfringens* is naturally present in the intestinal tract of poultry. Alterations in the intestinal environment (pH, tissue damage, dysbacteriosis), result in opportunity for the *C. perfringens* to proliferate and produce toxins, which destroy the intestinal mucosa resulting in NE. Several factors have been identified to influence the onset of NE, including coccidiosis (William, 2005), dietary crude protein (CP) level and source, (Drew et al., 2004; Palliyeguru et al., 2010), high dietary calcium (Ca) (Titball et al., 1999), and calcium source (Paiva et al., 2013). *Clostridium perfringens* must produce extracellular toxins to cause damage to the intestine. Two toxins have been reported to cause intestinal damage, alpha and NetB toxin. Alpha toxin is a phospholipase

C with enzymatic activity that is dependent upon Zn^{2+} and Ca^{2+} for full activity, which causes lysis of the erythrocytes (Titball et al., 1999). NetB is a pore-forming toxin that has been shown to cause NE in chickens. These toxins form pores that disrupt the phospholipid membrane bilayer of both human and animal cells, causing an influx of ions (i.e., Na^{+}, Cl^{-}, Ca^{2+}) that may lead to osmotic cell lysis (Keyburn et al., 2010).

Calcium in the diet of broilers influences growth, feed efficiency, bone development, leg health, nerve function, and the immune system (Ross, 2018). During different phases of grow-out of a bird, the calcium requirement varies. According to the NRC, calcium requirements for the birds range from 0.8 to 2.0% of the diet from young birds to breeder birds producing eggs (National Research Council, 1994). Many improvements have been made in the overall production of broiler birds with the days to market weight declining yearly as well as the feed efficiency of the birds continually improving. With the improvement in production of the birds, there has not been much attention to required change in the calcium requirements of birds. In 1994, the suggested calcium requirement for broilers was 1% of the diet for a bird up to three weeks, which then decreased with age (National Research Council, 1994). The most recent recommendation for birds is 0.96% (Ross, 2018) for the first 10 days with declining percentage with age. The addition of limestone as a flow conditioner in premixes and soybean meal, which is often not accounted for in dietary formulations often increases dietary calcium levels above recommended levels (Zanu et al., 2020c). Increased dietary calcium levels have been demonstrated to result in natural NE-occurrence and mortality (Paiva et al., 2014).

There are many locations throughout the United States in which limestone is extracted and crushed for use in animal feeds. Due to the different soil composition of the mine area from which limestone is extracted, the mineral composition of the limestone will vary. Limestone is

going to be compromised of mostly calcite, which is the most stable form of calcium carbonate. Reid and Weber (1976) reported that limestone can vary from 36 to 40% calcium and can have as much as 2% Mg in its composition. Of the 5 limestones sampled in their trial, it was determined that calcium availability ranged from 74.9 to 95.7% (Reid and Weber, 1976). In laying hens, it was suggested that a larger particle size could be stored in the gizzard and slowly absorbed for maintained supply of calcium for egg shell formation (Watkins et al., 1977). Therefore, with the variability of limestone composition and the possibility of slowly releasing calcium, the particle size of limestone could influence dietary requirements and availability to the birds.

Particle size would play a role in dietary requirement, since particle size of calcium source could affect the formation of complexes and reduce the ability of phytase to hydrolyze phytate (Zanu et al., 2020c). Kim et al. (2018) reported that birds fed larger (402 μm) particle size had no difference in gizzard pH regardless of dietary calcium level, but when a smaller (<75 μm) particle size was fed gizzard pH increased linearly with calcium concentration in the presence and absence of phytase. In a related study, diets with a particle size of 2 mm resulted in a reduction in calcium and phosphorus (P) solubility compared to 1 mm particle size (Walk et al., 2012). It could be supported that solubility is related, to some extent, to particle size. Therefore, the reduction in particle size would increase calcium solubility reduce the digestibility of nutrients, and increase the incidence of NE. This is supported by Paiva et al, (2013) as they observed that high dietary calcium from a readily soluble calcium source led to increased incidences of NE-related mortality.

Therefore, the objective of the current research was to evaluate dietary calcium level and limestone source as well as particle size involvement in NE pathogenesis and resulting performance associated with natural NE occurrence.

3.2. Materials and Methods

3.2.1. Animal and Housing

All animal care procedures were approved by Texas A&M University Institutional Animal Care and Use Committee. A total of 2,880-day old Ross 708 by-product males were allocated to 8 dietary treatments of 9 replicate pens per treatment. On arrival, all birds received a commercial (1x) dose of Coccivac B52 (Merck) and were allowed to preen for approximately 1 hour. After preening, birds were placed in 3 foot by 6-foot floor pens (0.9 sq ft/bird at termination) with new pine shavings of approximately 3 to 4 inches in depth. Birds were reared in a temperature and light controlled barn in which the Ross management manual (Ross, 2018) was followed during the 35 day (d) grow-out period. Lighting schedules were 23 hours of light for the first 7 days and 6 hours of darkness throughout the remainder of the experiment. Feed and water were provided *ad libitum* throughout the duration of the trial (d 0-35). Mortality were checked twice a day, and all mortality were necropsied for observation of NE lesions.

3.2.2. Experimental Design and Diets

Experimental diets consist of a corn and soybean meal-based diet set up in a randomized complete block design (RCBD). Diets were arranged in a 2×2×2 factorial arrangement. There were 2 dietary calcium levels (Standard vs Low), 2 limestone sources (1 vs 2), and 2 limestone particle sizes (Coarse vs Fines). Dietary calcium levels were reduced as the birds aged with inclusion levels of 1.05% vs 0.75%, 0.95% vs 0.70%, and 0.85% vs 0.65% for the starter (d 0-14), grower (d15-28), and finisher (d 28-35), respectively. A 2:1 ratio of Ca:P was maintained

through all dietary phases. The two limestone particle sizes were 120 micron (Coarse), similar to limestone used in the poultry industry, and as 200 micron (Fine), used as a highly soluble source of limestone. Diets were as described in Table 1.

Table 1. Arrangement of dietary treatments with two dietary calcium levels, two particle sizes, and two limestone sources

Treatment	Calcium Level	Particle Size	Limestone Source
1	Standard	Coarse	1
2	Standard	Fine	1
3	Standard	Coarse	2
4	Standard	Fine	2
5	Low	Coarse	1
6	Low	Fine	1
7	Low	Coarse	2
8	Low	Fine	2

The limestone sources were acquired from two different mines within the United States. Source 1, which was acquired from a mine in Alabama, was a harder more mature (smaller, rounder, and more well sorted) stone with reduced solubility. Source 2, which was acquired from a mine in Florida, had higher surface area and was an immature stone with increased solubility. To create the two different particle sizes, large stones of each source were crushed, dried, milled, screened, and air classified to get an average of 200 microns for the fine and an average of 120 microns for the coarse. Limestone samples were analyzed by X-ray diffraction to obtain the composition of the limestone as well as were analyzed to determine the distribution of particle size. Particle size determination was done with standard methods of 13 sieve with agitators and

flow agent for 10 minutes. For composition, limestone samples were prepared and analyzed by an outside laboratory with the Malvern/Panalytical Emperian XRD with a Pixcell 1D detector and scanned from 5 °2Θ to 75 °2Θ at 45kV and 40mA. Semiquantitative results were achieved using Rietveld analysis. Composition of the two limestone sources is described in Table 2.

Table 2. Mineral composition of two limestone sources and two particle size

Source	1		2	
Particle Size	Coarse	Fine	Coarse	Fine
Calcite, $CaCO_3$, (%)	97	98	100	100
Dolmite, $CaMg(CO_3)_2$, (%)	3	2	0	0
Silicia, SiO_2, (%)	Trace	Trace	Trace	Trace

Diets were formulated on a least-cost basis and were composed primarily of corn, soybean meal and corn dried distiller's grains with solubles (DDGS) (Table 3). Composite samples of each feed ingredient were analyzed by an outside laboratory for proximate analysis, calcium, and phosphorus (P), and values were used to formulate all dietary treatments. Isocaloric diets were fed in three dietary phases: starter (d 0-14), grower (d 15-28), and finisher (d 29-35). Diets for each phase were mixed using a 2-ton horizontal double-ribbon Scott mixer, pelleted using a 1-ton/hr California Pellet Mill equipped with a 4.4-mm diameter die and conditioner, and crumbled using a roller when appropriate. Starter was fed as a crumble, and all other phases were fed in pellet form. Feed samples were collected in triplicate and sent to a third-party laboratory for analysis of proximates and dietary minerals. All diets had inclusion of 1500 FTU/kg of phytase with a nutrient credit of 0.16% calcium and 0.15% available P (AvP) (Quantum Blue, AB Vista Feed Ingredients, Marlborough, UK).

Table 3. Ingredient and nutrient composition (calculated and analyzed) of variable dietary calcium levels of starter, grower, and finisher diets

	Starter[1]		Grower[2]		Finisher[3]	
Ingredient	Standard Ca	Low Ca	Standard Ca	Low Ca	Standard Ca	Low Ca
Corn	54.03	56.03	57.63	59.30	61.54	62.50
Soybean Meal, 48% CP	35.6	35.40	30.00	29.85	24.80	24.70
DDGS	4.00	4.00	6.00	6.00	8.00	8.00
Soybean Oil	1.89	1.19	2.36	1.76	2.50	2.16
Limestone	1.45	0.90	1.35	0.90	1.25	0.98
Monocalcium Phosphate	1.13	0.58	0.85	0.38	0.60	0.35
Methionine	0.36	0.35	0.30	0.30	0.25	0.25
L-Lysine	0.26	0.26	0.25	0.25	0.24	0.25
Sodium Bicarbonate	0.22	0.22	0.21	0.21	0.22	0.22
Salt	0.23	0.23	0.23	0.23	0.21	0.21
Vitamins[4]	0.13	0.13	0.13	0.13	0.13	0.13
Threonine	0.11	0.11	0.10	0.10	0.08	0.08
Choline	0.09	0.09	0.09	0.09	0.09	0.09
Valine	0.04	0.04	0.03	0.03	0.05	0.05
Trace Mineral[5]	0.05	0.05	0.05	0.05	0.02	0.02
Phytase[6]	0.03	0.03	0.03	0.03	0.03	0.03
Xylanase[6]	0.01	0.01	0.01	0.01	0.01	0.01
Titanium Dioxide	0.40	0.40	0.40	0.40	0.00	0.00
Total	100.0	100.00	100.00	100.00	100.00	100.00
Predicted Nutrient Composition						
AME, kcal/kg	3031.00	3031.25	3108.69	3108.41	3163.26	3163.06
Crude Protein, %	22.640	22.710	20.897	20.963	19.334	19.367
Fat, %	4.600	3.780	4.988	4.480	5.286	4.996
Calcium, %	1.050	0.752	0.949	0.698	0.851	0.704
Available P, %	0.502	0.390	0.449	0.352	0.402	0.351
Analyzed Nutrient Composition						
Dry Matter, %	88.70	88.40	88.13	88.27	87.84	88.58
Protein, %	21.90	22.20	19.98	20.70	18.85	20.30
Calcium, %	0.85	0.63	0.92	0.54	0.91	0.55
Total P, %	0.55	0.49	0.45	0.39	0.44	0.41

[1]Starter phase (d 0-14)

[2]Grower phase (d 15-28)

[3]Finisher phase (d 29-35)

[4]Vitamin premix added at this rate yields 11,463 IU vitamin A, 4,012 IU vitamin D3, 47.8 IU vitamin E, 0.02 mg B12, 6.21 mg riboflavin, 47.8 mg niacin, 21.01 mg d-pantothenic acid, 135.8 mg choline, 1.53 mg menadione, 1.82 mg folic acid, 3.06 mg thiamine, 0.57 mg biotin. The carrier is ground rice hulls.

[5]Trace mineral premix added at this rate yields 60.0 mg manganese, 60 mg zinc, 60 mg iron, 7 mg copper, 0.4 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium per kg of diet. The carrier is calcium carbonate and the premix contains less than 1% mineral oil.

[6]AB Vista Feed Ingredients, Marlborough, UK

3.2.3. Measured Variables

3.2.3.1. Live Performance

Pen body weight (BW) and feed consumption were recorded at d 14, 28, and 35 to determine average BW and average BW gain (BWG). Feed consumption and daily mortality were used to determine mortality corrected feed conversion ratio (FCR). On d 14 and 25, 3 birds/pen were necropsied to determine the development of intestinal lesions for coccidiosis and NE (Conway and McKenzie, 2007).

3.2.3.2. Bone Ash

On d 14, 28, and 35, left tibias from three birds/pen (of average weight and free of defects) were randomly selected and frozen (-20°C) until further analysis. Before analysis, bones were cleaned of all adhering tissues, wrapped in wing bands to label, and dried at 100°C for 24 h. Using a Soxhlet extraction apparatus, fat was extracted from bones for 16 h in ethyl ether. Fat-extracted tibias were oven dried at 100°C for 24 h, and then ashed at 600°C in a muffle furnace for 24 h for bone ash percentage determination (Atteh and Leeson, 1985).

3.2.3.3. Ileal Digestibility

On d 14 and 23, six birds/pen and four birds/pen, respectively, were euthanized by cervical dislocation for digesta collection. Digesta samples were collected from entire ileum (defined as Meckel's diverticulum to ~2 cm cranial of ileocecal junction), pooled/pen, and immediately frozen (-20°C) until further analysis. Samples were freeze dried via lypholizer (Labconco FreeZone Freeze Dry Systems, 8811 Prospect Ave, Kansas City, MO 64132) and ground to pass a 1 mm screen. Dried, ground ileal digesta and experimental diets were sampled for amino acids (method 982.30 E(a)), calcium (method 975.03 B(b)), and P (method 968.08)

according to AOAC (2006) and titanium dioxide (TiO_2) according to Journal of Animal Science (2004) at University of Missouri Agriculture Experiment Station (Columbia, MO).

3.2.3.3.1. Digestibility Calculations

Apparent ileal amino acid digestibility was calculated using the following equation (Paiva et al., 2014):

AID= [(nutrient/TiO2)diet – (nutrient/TiO2)ileum] / (nutrient/TiO2)diet

where: (nutrient/TiO2)diet= the ratio of nutrient to titanium dioxide in the diet

(nutrient/TiO2)ileum= the ratio of nutrient in the ileal digesta

To calculate digestible nutrient intake in g/d, the following equation was used (Walk and Rama Rao, 2018):

Digestible nutrient intake (g/day) = [(diet nutrient, %) x (AID nutrient, % / 100)] / 100 x daily intake (g)

where: Diet nutrient = the analyzed nutrient concentration of the diet

AID nutrient = the calculated apparent ileal nutrient digestibility

3.2.3.4. pH

On d 14 and 25, three birds/pen were randomly selected of average weight and were used to obtain measurements of pH from duodenum, jejunum, and ileum. Following cervical dislocation, pH measurements were obtained directly from the lumen of each section with digesta present. Incisions were made at the midpoint of the duodenum, mid-way thru the jejunum, and mid-way thru the ileal section. The spear-tip electrode was inserted in both the cranial and caudal direction to obtain readings using the digital pH meter (Professional Portable Meat pH Meter, Hanna Instruments, Woonsocket, RI) (Walk et al., 2012). The pH readings from each bird were pooled to get an average for each pen.

3.2.4. Statistical Analysis

A 2 (calcium dietary levels) × 2 (limestone particle sizes) × 2 (limestone sources) factorial arrangement of treatments within a RCBD was utilized analyzed by pen. Data were subjected to an ANOVA using the GLM procedure of SAS 9.4 (SAS institute, Inc., 2020), with significance level set as $p \leq 0.05$; Tukey's HSD test was used to further explore differences between treatment means.

3.3. Results

3.3.1. Performance

Beginning at d 16, numerous birds were clinically morbid, and mild mortality (<0.5% daily mortality) resulting from NE (confirmed by necropsy) increased and persisted through d23 (Table 4). A significant interaction between limestone particle size and calcium source during the grower phase indicated that the higher level of fine particle size limestone resulted in increased mortality compared to the lower calcium level of this particle size. However, no difference was seen between dietary calcium level with the larger particle size. Throughout the remainder of the trial, there were no significant differences in mortality.

Randomization of the birds at d 0 was achieved, and there were no differences in BW at placement (Table 5). As the birds aged, dietary calcium had an effect on BW whether it was the limestone source, particle size, or dietary inclusion level (Table 4). At d 14, there was a 2-way interaction (limestone source x particle size) with birds fed course particle size of limestone source 1 having reduced BW compared to the other treatments. At d 28, there was a 2-way interaction of calcium level and limestone source on BW, with birds fed standard levels of dietary calcium source 1 having reduced BW compared to other treatments. A 3-way interaction

(calcium level x limestone source x particle size) at d 35, indicated birds fed standard dietary calcium levels of coarse particle limestone source 1 had reduced BW of approximately 300 grams compared to all other treatments, with the exception of this same calcium level and source of the smaller particle size. Birds fed the higher dietary calcium level of fine particle source 1 was intermediate in BW to all other treatments.

The differences in BWG followed a similar trend to that of BW, with 2-way interactions through the grower phase and 3-way interactions during the finisher phase and cumulative trial period (d 0-35). During the starter (d 0-14) phase, there was a significant limestone source and particle size interaction with birds fed coarse particle size of source 1 having reduced BWG (Table 6). During the grower (d 14-28) phase and from d 0-28, there was a dietary calcium level by limestone source interaction with source 1 at higher dietary calcium level reducing BWG. During the finisher phase (d28-35) and cumulatively for the trial (d0-35), there were similar calcium level x limestone source x particle size interactions. In both periods, birds fed the higher dietary calcium level of source 1 coarse particle size had reduced BWG compared to other treatments with the exception of the group with higher calcium level of fine particle size of this same source being intermediate to all groups during the cumulative grow-out period.

The differences in FCR were similar to that of the BW and BWG in that as the birds aged, significant differences shifted from 2-way interactions to 3-way interactions (Table 7). During the starter phase, there was a limestone source and particle size interaction with birds fed coarse particles of source 1 having less efficient FCR, while birds fed fine particles of source 2 had FCR similar to all others. During the grower phase and d 0-28, there was a calcium level and limestone source interaction with birds fed higher dietary calcium levels of source 1 having less efficient FCR compared to other groups. During the finisher phase and d 0-35, there were three-

way interactions. At the lower inclusion level of dietary calcium, there were no differences in FCR resulting from limestone particle size or source. However, at the high calcium inclusion level during the finisher period, source 1 course particles of limestone resulted in less efficient FCR than source 2 large particles, while other groups were intermediate to these two. Results were similar for d 0-35, with both particle sizes of source 1 resulting in less efficient FCR than source 2.

Table 4. Dietary calcium levels and limestone source and particle size effect on mortality during natural occurring necrotic enteritis.

Treatment		Percent Mortality						
		Starter	Grower	Finisher	D 0-28	D 0-35	D 14-21	D 21-28
Level	Size							
Standard	120	5.833	1.957[ab]	0.000	7.222	7.222	1.574[ab]	0.397
Standard	200	4.583	3.647[b]	0.519	7.222	7.500	3.072[b]	0.613
Low	120	5.556	2.733[ab]	0.811	7.500	7.917	1.963[ab]	0.791
Low	200	5.972	0.185[a]	0.494	6.111	6.389	0.185[a]	0.000
	Pooled SEM	0.935	0.692	0.285	1.089	1.090	0.581	0.265
	P Value							
	Level	0.5574	0.0778	0.2381	0.7039	0.8503	0.0514	0.7280
	Size	0.6597	0.5695	0.7597	0.5269	0.5716	0.8246	0.3622
	Source	0.3797	0.0725	0.1990	0.8991	0.7531	0.1689	0.0957
	Level * Size	0.3797	0.0063	0.2096	0.5269	0.4145	0.0114	0.1131
	Level * Source	0.8832	0.3375	0.7830	0.7039	0.6598	0.3205	0.7280
	Size * Source	0.1453	0.8363	0.0807	0.2563	0.4145	0.4828	0.3622
	Level * Size * Source	0.3059	0.1234	0.8421	0.103	0.1191	0.0969	0.6730

a-b Means within column with different superscripts differ at $p \leq 0.05$
n=9

Table 5. Evaluation of limestone source, particle size, and dietary calcium level on body weight of broilers during naturally occurring necrotic enteritis.

Treatment			Body Weight			
			D 0 (g)	D 14 (kg)	D 28 (kg)	D 35 (kg)
Level	Size	Source				
Standard	120	1	35.55	0.447	1.173	1.647[b]
Standard	200	1	35.66	0.455	1.236	1.811[ab]
Standard	120	2	35.54	0.459	1.366	1.974[a]
Standard	200	2	35.62	0.456	1.358	1.945[a]
Low	120	1	35.70	0.439	1.336	1.938[a]
Low	200	1	35.89	0.456	1.335	1.902[a]
Low	120	2	35.85	0.459	1.326	1.919[a]
Low	200	2	35.65	0.456	1.329	1.943[a]
Pooled SEM			0.140	0.004	0.027	0.041
Level	Size					
Standard	120		35.55	0.453	1.270	1.810
Standard	200		35.64	0.456	1.297	1.878
Low	120		35.77	0.449	1.331	1.929
Low	200		35.77	0.456	1.332	1.923
Pooled SEM			0.098	0.003	0.022	0.034
Level		Source				
Standard		1	35.61	0.451	1.204[b]	1.729[b]
Standard		2	35.58	0.458	1.362[a]	1.960[a]
Low		1	35.79	0.447	1.336[a]	1.920[a]
Low		2	35.75	0.457	1.328[a]	1.931[a]
Pooled SEM			0.098	0.003	0.019	0.030
	Size	Source				
	120	1	35.63	0.443[b]	1.254	1.792
	120	2	35.70	0.459[a]	1.346	1.947
	200	1	35.77	0.455[a]	1.285	1.856
	200	2	35.63	0.456[a]	1.343	1.944
	Pooled SEM		0.100	0.003	0.022	0.033
	P Value					
	Level		0.0803	0.5153	0.0144	0.0070
	Size		0.6683	0.1392	0.4704	0.2980
	Source		0.7400	0.0064	0.0003	0.0001
	Level * Size		0.6385	0.5067	0.5049	0.2126
	Level * Source		0.9449	0.6368	<0.0001	0.0004
	Size * Source		0.2953	0.0145	0.3856	0.2633
	Level * Size * Source		0.358	0.4953	0.3398	0.0345

a-b Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).
n=9

Table 6. Evaluation of limestone source, particle size, and dietary calcium level on body weight gain of broilers during naturally occurring necrotic enteritis.

Treatment			Body Weight Gain (kg)				
			Starter	Grower	Finisher	D0-28	D0-35
Level	Size	Source					
Standard	120	1	0.411	0.726	0.474[b]	1.137	1.611[b]
Standard	200	1	0.419	0.781	0.575[a]	1.200	1.775[ab]
Standard	120	2	0.424	0.907	0.608[a]	1.331	1.938[a]
Standard	200	2	0.421	0.901	0.587[a]	1.322	1.910[a]
Low	120	1	0.403	0.897	0.602[a]	1.300	1.902[a]
Low	200	1	0.420	0.880	0.566[a]	1.300	1.866[a]
Low	120	2	0.423	0.867	0.593[a]	1.291	1.883[a]
Low	200	2	0.420	0.873	0.614[a]	1.294	1.908[a]
	Pooled SEM		0.015	0.029	0.032	0.017	0.016
Level	Size						
Standard	120		0.418	0.816	0.541	1.234	1.775
Standard	200		0.420	0.841	0.581	1.261	1.842
Low	120		0.413	0.882	0.597	1.295	1.893
Low	200		0.420	0.877	0.590	1.297	1.887
	Pooled SEM		0.011	0.027	0.025	0.016	0.015
Level		Source					
Standard		1	0.415	0.753[b]	0.524[b]	1.169[b]	1.693[b]
Standard		2	0.422	0.904[a]	0.598[a]	1.326[a]	1.923[a]
Low		1	0.412	0.888[a]	0.584[a]	1.300[a]	1.884[a]
Low		2	0.422	0.870[a]	0.604[a]	1.292[a]	1.896[a]
	Pooled SEM		0.011	0.021	0.025	0.012	0.012
	Size	Source					
	120	1	0.407[b]	0.812	0.538	1.219	1.757
	120	2	0.423[a]	0.887	0.600	1.311	1.911
	200	1	0.419[a]	0.830	0.571	1.250	1.820
	200	2	0.420[a]	0.887	0.601	1.308	1.909
	Pooled SEM		0.010	0.027	0.026	0.016	0.015
	P Value						
	Level		0.4740	0.0089	0.0151	0.0147	0.0071
	Size		0.1387	0.6148	0.2104	0.4715	0.2986
	Source		0.0057	0.0008	0.0008	0.0003	0.0001
	Level * Size		0.4923	0.4273	0.0743	0.5062	0.2131
	Level * Source		0.6315	<0.0001	0.0449	<0.0001	0.0004
	Size * Source		0.0148	0.6235	0.2251	0.3883	0.2647
	Level * Size * Source		0.5089	0.2747	0.0012	0.3373	0.0342

a-b Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).
n=9

Table 7. Evaluation of limestone source, particle size, and dietary calcium level on feed conversion ratio (FCR) of broilers during naturally occurring necrotic enteritis.

Treatment			Feed Conversion Ratio				
			Starter	Grower	Finisher	D0-28	D 0-35
Level	Size	Source					
Standard	120	1	1.668	1.817	1.814[b]	1.747	1.774[c]
Standard	200	1	1.628	1.736	1.665[ab]	1.684	1.679[b]
Standard	120	2	1.629	1.542	1.648[a]	1.576	1.593[a]
Standard	200	2	1.635	1.556	1.674[ab]	1.586	1.605[ab]
Low	120	1	1.687	1.540	1.619[a]	1.596	1.601[a]
Low	200	1	1.634	1.566	1.657[a]	1.591	1.605[ab]
Low	120	2	1.625	1.593	1.636[a]	1.605	1.612[ab]
Low	200	2	1.643	1.571	1.562[a]	1.599	1.589[a]
	Pooled SEM		0.004	0.003	0.018	0.027	0.041
Level	Size						
Standard	120		1.648	1.679	1.726	1.662	1.683
Standard	200		1.632	1.646	1.669	1.635	1.642
Low	120		1.656	1.567	1.627	1.600	1.606
Low	200		1.639	1.568	1.610	1.595	1.597
	Pooled SEM		0.003	0.022	0.015	0.022	0.034
Level		Source					
Standard		1	1.648	1.776[b]	1.735	1.716[b]	1.726[b]
Standard		2	1.632	1.549[a]	1.661	1.581[a]	1.599[a]
Low		1	1.661	1.553[a]	1.638	1.594[a]	1.603[a]
Low		2	1.634	1.582[a]	1.599	1.602[a]	1.600[a]
	Pooled SEM		0.003	0.018	0.014	0.019	0.030
	Size	Source					
	120	1	1.678[b]	1.678	1.710	1.672	1.687
	120	2	1.627[a]	1.568	1.642	1.591	1.602
	200	1	1.631[a]	1.651	1.661	1.638	1.642
	200	2	1.639[ab]	1.564	1.618	1.593	1.597
	Pooled SEM		0.003	0.021	0.014	0.022	0.033
	P Value						
	Level		0.4998	<0.0001	0.0013	0.0002	<0.0001
	Size		0.1145	0.4846	0.1167	0.2214	0.0442
	Source		0.0484	<0.0001	0.0173	<0.0001	<0.0001
	Level * Size		0.9798	0.4369	0.3836	0.4074	0.1871
	Level * Source		0.6340	<0.0001	0.4279	<0.0001	<0.0001
	Size * Source		0.0074	0.5948	0.5425	0.1635	0.1088
	Level * Size * Source		0.5819	0.1154	0.0030	0.1542	0.0078

a-b Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=9

3.3.2. Coccidiosis Lesion Scores

There were no differences in the *Eimeria acervulina, Eimeria maxima,* or *Eimeria tenella* macroscopic scores or *Eimeria maxima* microscopic counts from mucosal scrapings of the midgut at d 14 or 25 ($P>0.05$; Table 8).

3.3.3. Bone Ash

At d 14 or 23, there were no differences in bone ash percentage ($P>0.05$; Table 9).

3.3.4. pH

On d 14 and 25, there were no differences observed in pH in the duodenum or jejunum (Table 10). There were main effect differences in the ileum pH observed between calcium levels and particle size on d14. Birds fed the standard level of calcium had higher pH than those on lower dietary calcium, and birds on fine particle limestone had higher pH than those on larger particle. At d 25, there was a limestone particle size and limestone source interaction with birds fed coarse particle size of calcium source 1 having higher pH than those with source 2, and no difference between sources occured with the smaller particle size.

Table 8. Evaluation of limestone source, particle size, and dietary calcium level on coccidiosis lesion scores and microscopic *E. maxima* score at d 14 and 25 of broilers during naturally occurring necrotic enteritis.

Treatment			Day 14				Day 25			
			EA	EM	ET	Microscopic EM Score	EA	EM	ET	Microscopic EM Score
Level	Size	Source								
Standard	120	1	0.778	0.111	0.000	0.815	0.815	0.222	0.000	0.481
Standard	200	1	0.556	0.000	0.000	0.593	0.630	0.407	0.000	0.625
Standard	120	2	0.630	0.037	0.000	0.556	0.630	0.222	0.000	0.583
Standard	200	2	0.926	0.000	0.111	0.741	0.481	0.296	0.000	0.519
Low	120	1	0.815	0.000	0.111	0.889	0.815	0.444	0.111	0.556
Low	200	1	0.852	0.000	0.000	0.519	0.667	0.185	0.000	0.519
Low	120	2	0.778	0.111	0.074	0.630	0.593	0.222	0.000	0.444
Low	200	2	0.630	0.037	0.074	0.481	0.630	0.481	0.037	0.741
Pooled SEM			0.112	0.098	0.033	0.033	0.103	0.097	0.098	0.014
P Value			0.1442	0.5237	0.5064	0.1313	0.3052	0.2455	0.1344	0.624

n=9

Table 9. Effect of dietary calcium levels and limestone source and particle size on the bone ash percentage at d 14, 28, and 35 during naturally occurring necrotic enteritis.

Treatment			Bone Ash Percentage		
			Day 14 (%)	Day 28 (%)	Day 35 (%)
Level	Size	Source			
Standard	120	1	49.106	52.580	50.917
Standard	200	1	45.958	50.735	49.454
Standard	120	2	49.039	51.302	52.356
Standard	200	2	49.231	51.248	54.806
Low	120	1	48.042	51.537	50.940
Low	200	1	47.398	51.986	50.536
Low	120	2	48.470	52.424	50.588
Low	200	2	47.650	52.313	50.346
	Pooled SEM		0.516	0.437	1.162
	P Value		0.2467	0.0906	0.4748

n=9

Table 10. Evaluation of limestone source, particle size, and dietary calcium level on intestinal pH of broilers during naturally occurring necrotic enteritis.

Treatments			Intestinal pH					
			Day 14			Day 25		
			Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
Level	Size	Source						
	120	1	6.257	5.986	5.838	6.244	5.991	6.169
	120	2	6.238	5.996	5.822	6.229	5.975	5.946
	200	1	6.306	5.988	5.938	6.194	5.960	5.908
	200	2	6.302	6.041	6.005	6.201	6.008	5.992
Pooled SEM			0.047	0.038	0.072	0.041	0.036	0.076
Main Effect Means								
Level								
1.05			6.302	6.015	6.011[a]	6.164	6.013	6.102[a]
0.9			6.249	5.990	5.790[b]	6.271	5.955	5.905[b]
Pooled SEM			0.033	0.029	0.049	0.027	0.026	0.054
Size								
120			6.247	5.991	5.830[b]	6.327	5.983	6.058
200			6.304	6.014	5.971[a]	6.198	5.984	5.950
Pooled SEM			0.033	0.028	0.051	0.029	0.026	0.056
Source								
1			6.281	5.987	5.888	6.219	5.975	6.038
2			6.270	6.019	5.913	6.215	5.992	5.969
Pooled SEM			0.033	0.029	0.052	0.029	0.025	0.055
P Value								
Level			0.2558	0.5273	0.0023	0.0082	0.1320	0.0073
Size			0.2206	0.5666	0.0466	0.3252	0.9845	0.1327
Source			0.8060	0.4316	0.7185	0.9198	0.6689	0.3308
Level * Size			0.7629	0.9663	0.7886	0.0877	0.7392	0.3590
Level * Source			0.0352	0.1066	0.3664	0.6789	0.8211	0.0582
Size * Source			0.8670	0.5993	0.5572	0.7861	0.3934	0.0342
Level * Size * Source			0.2389	0.0663	0.7674	0.9664	0.3737	0.0527

a-b Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).
n=9

3.3.5. Nutrient Intake

Amino acids analyzed from ileal digesta included aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine. There were significant differences in nutrient intake at d 14 (Table 11 and 12) and d 25 (Table 13) for all amino acids. Significant 3-way interactions of calcium dietary level, limestone particle size, and limestone source were observed on d 14 with aspartic acid, threonine, valine, methionine, and tyrosine (Table 12). In general, with limestone source 1 and lower level of dietary calcium, the smaller limestone particle size resulted in higher digestible intake, while the opposite was seen at the higher dietary calcium level. In contrast, these differences were not seen with limestone source 2. At d 14, digestible intake of most amino acids showed a 2-way interaction of limestone particle size and dietary calcium level with low dietary calcium levels of fine particle size increasing digestible amino acid intake as compared to the high dietary calcium level of fine particle size. However, differences were not seen between dietary calcium levels with the larger particle size limestone. There were also two-way interactions of limestone source and calcium dietary level with source 1 inclusion at the higher dietary calcium level resulting in reduced digestible amino acid intake compared to the low calcium level of source 1, while these differences were not observed for source 2 except for methionine. At d 25, for all amino acids, there were 3-way interactions. All amino acids, except for methionine, had increased digestible intake when birds were fed low dietary levels of source 1 limestone of fine particle size. At the low dietary calcium levels, while fine particle size of limestone source 1 increased digestible amino acid intake, this same particle size of source 2 resulted in decreased intake for most amino acids. In contrast to the results at the low level of calcium, at the high dietary calcium levels, fine particle size of source 1 decreased

digestible intake of numerous amino acids compared to the larger particle size of this source, and there were no differences between particle sizes of source 2. Methionine digestible intake was increased when birds were fed higher levels of limestone source 2 in fine particle size and decreased at the lower calcium level with this source and particle size.

At d 14, birds fed higher dietary calcium levels of the coarse particle size of source 1 had an increased digestible intake of calcium compared to the fine particle size of source 1 or either particle size of source 2, however there were no differences between groups at the lower calcium levels. In contrast, at d 25 there were no differences in digestible calcium intake. At d 14 coarse particle size of limestone source 1 resulted in increased digestible intake of phosphorus compared to fine particle size of this source, while there was no difference between particle size results with source 2. At d 25, at the lower level of dietary calcium, coarse limestone of source 1 increased digestible intake of P compared to the fine particle size of this source and either particle size of source 2. However, at the higher dietary calcium level, fine particle size of limestone source 2 increased digestibility compared to the other groups with the fine particle size of source 1 resulting in the lowest digestibility of P.

Table 11. Three-way interaction of limestone source, particle size, and dietary calcium level on digestible intake (g/bird) of broilers during naturally occurring necrotic enteritis at d 14

Treatment			Digestible Intake (g/bird)					
			Aspartic Acid	Threonine	Valine	Methionine	Tyrosine	Calcium
Level	Size	Source						
1.05	120	1	0.822^{bc}	0.331^{bc}	0.407^{bc}	0.235^{g}	0.288^{bc}	0.335^{a}
1.05	200	1	0.777^{d}	0.312^{d}	0.390^{bc}	0.256^{e}	0.277^{c}	0.277^{b}
1.05	120	2	0.831^{bc}	0.330^{bc}	0.420^{ab}	0.246^{f}	0.296^{ab}	0.204^{c}
1.05	200	2	0.811^{c}	0.326^{cd}	0.414^{ab}	0.278^{b}	0.289^{bc}	0.198^{c}
0.75	120	1	0.822^{bc}	0.327^{cd}	0.409^{bc}	0.258^{de}	0.294^{b}	0.174^{c}
0.75	200	1	0.878^{a}	0.352^{a}	0.432^{a}	0.297^{a}	0.309^{a}	0.182^{c}
0.75	120	2	0.828^{bc}	0.332^{bc}	0.415^{ab}	0.272^{c}	0.300^{ab}	0.209^{c}
0.75	200	2	0.853^{ab}	0.345^{ab}	0.421^{ab}	0.262^{d}	0.294^{b}	0.201^{c}
Pooled SEM			0.007	0.004	0.005	0.001	0.003	0.010
P Value								
Level			<0.0001	<0.0001	0.0006	<0.0001	<0.0001	<0.0001
Source			0.2149	0.2744	0.0156	0.0007	0.2665	<0.0001
Size			0.4579	0.157	0.5888	<0.0001	0.2537	0.0377
Level * Size			<0.0001	<0.0001	0.0002	<0.0001	0.0035	0.0433
Level * Source			0.0044	0.1637	0.0026	<0.0001	0.0012	<0.0001
Size * Source			0.8345	0.8109	0.5828	<0.0001	0.0681	0.2544
Level * Size * Source			0.0097	0.0144	0.0397	<0.0001	0.0051	0.0284

a-g Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=9

Table 12. Two-way interaction of limestone source, particle size, and dietary calcium level on digestible intake (g/bird) of broilers during naturally occurring necrotic enteritis at d 14

Treatment		Digestible Intake (g/bird)					
		Serine	Glutamic Acid	Proline	Glycine	Alanine	Isoleucine
Level	Size						
1.05	120	0.351^{b}	1.588^{b}	0.494^{ab}	0.339^{b}	0.426^{b}	0.374^{ab}
1.05	200	0.342^{c}	1.552^{c}	0.477^{c}	0.328^{c}	0.411^{c}	0.362^{c}
0.75	120	0.357^{b}	1.588^{b}	0.486^{bc}	0.345^{b}	0.427^{b}	0.365^{bc}
0.75	200	0.372^{a}	1.627^{a}	0.499^{a}	0.359^{a}	0.447^{a}	0.376^{a}
Pooled SEM		0.003	0.009	0.003	0.003	0.004	0.003
Level	Source						
1.05	1	0.340	1.543^{b}	0.476^{b}	0.327	0.411	0.361^{c}
1.05	2	0.353	1.595^{a}	0.493^{a}	0.340	0.426	0.374^{ab}
0.75	1	0.362	1.620^{a}	0.496^{a}	0.350	0.436	0.376^{a}
0.75	2	0.367	1.596^{a}	0.489^{a}	0.355	0.438	0.366^{bc}
Pooled SEM		0.003	0.009	0.003	0.003	0.004	0.003
Size	Source						
120	1	0.350	1.579	0.486	0.336	0.423	0.370
120	2	0.359	1.597	0.493	0.348	0.430	0.389
200	1	0.353	1.586	0.486	0.341	0.425	0.368
200	2	0.361	1.594	0.490	0.346	0.434	0.370
Pooled SEM		0.003	0.010	0.004	0.004	0.004	0.003
P Value							
Level		<0.0001	<0.0001	0.0167	<0.0001	<0.0001	0.263
Source		0.004	0.0709	0.0815	0.0012	0.0298	0.7182
Size		0.2459	0.7588	0.6202	0.6753	0.3911	0.9149
Level * Size		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Level * Source		0.0722	<0.0001	0.0002	0.1374	0.0957	0.0001
Size * Source		0.7585	0.4361	0.6539	0.1724	0.8597	0.5201
Level * Size * Source		0.7723	0.8431	0.3137	0.0855	0.4815	0.635

a-g Means within a column lacking a common superscript are significantly different from each other ($P<0.05$)
n=9

Table 12 Continued.

Treatment		Digestible Intake (g/bird)					
		Phenylalanine	Lysine	Histidine	Arganine	Phosphorus	Leucine
Level	Size						
1.05	120	0.429[a]	0.620[ab]	0.233[b]	0.598[b]	0.221	0.744[ab]
1.05	200	0.413[b]	0.607[b]	0.225[c]	0.583[c]	0.205	0.716[c]
0.75	120	0.426[a]	0.611[b]	0.231[b]	0.596[b]	0.206	0.733[bc]
0.75	200	0.436[a]	0.632[a]	0.240[a]	0.619[a]	0.181	0.757[a]
Pooled SEM		0.003	0.005	0.002	0.003	0.005	0.006
Level	Source						
1.05	1	0.415[b]	0.593[b]	0.225[b]	0.581[c]	0.215	0.716[b]
1.05	2	0.426[ab]	0.630[a]	0.233[a]	0.599[b]	0.211	0.742[a]
0.75	1	0.433[a]	0.625[a]	0.237[a]	0.611[a]	0.197	0.751[a]
0.75	2	0.429[a]	0.618[a]	0.235[a]	0.604[ab]	0.189	0.739[a]
Pooled SEM		0.003	0.004	0.002	0.004	0.005	0.006
Size	Source						
120	1	0.426	0.608	0.230	0.593	0.232[a]	0.736
120	2	0.429	0.622	0.234	0.601	0.195[b]	0.741
200	1	0.423	0.613	0.231	0.600	0.181[c]	0.733
200	2	0.426	0.626	0.233	0.602	0.204[b]	0.740
Pooled SEM		0.003	0.005	0.002	0.004	0.004	0.006
P Value							
Level		0.0006	0.0232	<0.0001	<0.0001	<0.0001	0.0038
Source		0.2652	0.0005	0.0209	0.0795	0.0179	0.2530
Size		0.3004	0.2174	0.9841	0.1330	<0.0001	0.7095
Level * Size		<0.0001	<0.0001	<0.0001	<0.0001	0.2176	<0.0001
Level * Source		0.0116	<0.0001	0.0003	<0.0001	0.8198	0.0009
Size * Source		0.8303	0.9683	0.4381	0.3409	<0.0001	0.9007
Level * Size * Source		0.1077	0.2079	0.0999	0.4393	0.0720	0.4273

a-g Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=9

Table 13. Evaluation of limestone source, particle size, and dietary calcium level on digestible intake (g/bird) of broilers during naturally occurring necrotic enteritis at d 25

Treatment			Digestible Intake (g/bird)								
			Aspartic Acid	Threonine	Serine	Glutamic Acid	Proline	Glycine	Alanine	Valine	Methonine
Level	Size	Source									
1.05	120	1	1.786^{d}	0.709^{de}	0.747^{d}	3.248^{e}	0.994^{d}	0.697^{d}	0.894^{d}	0.862^{d}	0.536^{e}
1.05	200	1	1.713^{d}	0.690^{e}	0.732^{d}	3.134^{f}	0.942^{e}	0.668^{d}	0.845^{e}	0.821^{d}	0.560^{cd}
1.05	120	2	2.031^{b}	0.788^{ab}	0.849^{b}	3.678^{c}	1.107^{b}	0.780^{bc}	0.971^{bc}	0.961^{b}	0.557^{d}
1.05	200	2	2.036^{b}	0.777^{b}	0.833^{bc}	3.676^{c}	1.102^{b}	0.782^{bc}	0.969^{bc}	0.974^{b}	0.592^{a}
0.75	120	1	2.082^{b}	0.784^{ab}	0.861^{b}	3.755^{bc}	1.131^{ab}	0.801^{b}	0.988^{ab}	0.985^{b}	0.576^{b}
0.75	200	1	2.224^{a}	0.821^{a}	0.902^{a}	3.942^{a}	1.157^{a}	0.842^{a}	1.025^{a}	1.039^{a}	0.569^{bc}
0.75	120	2	2.101^{b}	0.768^{bc}	0.856^{b}	3.804^{b}	1.092^{bc}	0.791^{b}	0.974^{bc}	0.965^{b}	0.556^{cd}
0.75	200	2	1.931^{c}	0.730^{cd}	0.808^{c}	3.502^{d}	1.049^{c}	0.753^{c}	0.939^{c}	0.912^{c}	0.538^{e}
Pooled SEM			0.016	0.008	0.008	0.210	0.009	0.007	0.009	0.009	0.002
P Value											
Level			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.8715
Source			<0.0001	0.0177	<0.0001	<0.0001	<0.0001	<0.0001	0.0005	<0.0001	0.3164
Size			0.0414	0.2162	0.0831	0.0007	0.0085	0.2845	0.0723	0.0857	<0.0001
Level * Size			0.4032	0.2135	0.3026	0.9979	0.1417	0.1785	0.0563	0.7478	<0.0001
Level * Source			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Size * Source			<0.0001	0.0067	0.0002	<0.0001	0.4330	0.0300	0.3557	0.0078	0.5673
Level * Size * Source			<0.0001	0.0011	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

a-g Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=9

Table 13 Continued

Treatment			Digestible Intake (g/bird) at d 25								
			Isoleucine	Leucine	Tyrosine	Phenylalanine	Lysine	Histidine	Arganine	Calcium	Phosphorus
Level	Size	Source									
1.05	120	1	0.789^{d}	1.529^{d}	0.576^{e}	0.897^{e}	1.219^{e}	0.487^{d}	1.250^{f}	0.412	0.384^{b}
1.05	200	1	0.755^{d}	1.452^{c}	0.566^{e}	0.852^{f}	1.197^{e}	0.464^{e}	1.208^{g}	0.366	0.336^{c}
1.05	120	2	0.883^{b}	1.685^{b}	0.653^{bc}	1.008^{bc}	1.372^{c}	0.539^{b}	1.432^{d}	0.378	0.392^{b}
1.05	200	2	0.897^{b}	1.680^{b}	0.646^{c}	1.000^{c}	1.403^{bc}	0.543^{b}	1.421^{d}	0.376	0.466^{a}
0.75	120	1	0.909^{b}	1.710^{ab}	0.665^{bc}	1.024^{bc}	1.417^{ab}	0.552^{b}	1.466^{c}	0.473	0.452^{a}
0.75	200	1	0.953^{a}	1.766^{a}	0.694^{a}	1.081^{a}	1.455^{a}	0.576^{a}	1.565^{a}	0.349	0.406^{b}
0.75	120	2	0.906^{b}	1.702^{ab}	0.671^{b}	1.037^{b}	1.410^{b}	0.553^{b}	1.501^{b}	0.394	0.400^{b}
0.75	200	2	0.830^{c}	1.591^{c}	0.603^{d}	0.946^{d}	1.286^{d}	0.513^{c}	1.359^{e}	0.323	0.397^{b}
Pooled SEM			0.007	0.013	0.005	0.008	0.008	0.004	0.006	0.028	0.008
P Value											
Level			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.9353	0.0034
Source			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.1608	0.0036
Size			0.0207	0.0011	0.0002	0.0003	0.0020	0.0038	<0.0001	0.0103	0.3832
Level * Size			0.6248	0.5031	0.0944	0.4284	0.0002	0.7687	0.6387	0.1111	0.0053
Level * Source			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.3835	<0.0001
Size * Source			0.0014	0.0218	<0.0001	<0.0001	<0.0001	0.0032	<0.0001	0.2833	<0.0001
Level * Size * Source			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.9254	0.0029

a-g Means within a column lacking a common superscript are significantly different from each other (P<0.05)
n=9

3.4. Discussion

As in previous trials conducted by our laboratory, dietary calcium level had a significant effect on mortality due to natural occurrence of NE (Paiva et al., 2014; Paiva et al., 2013). While we have previously reported that reducing dietary calcium levels decreased the incidence of NE, it appears the calcium involvement is even more complex. Interestingly, in this experiment we observed interaction between limestone particle size and dietary calcium level during the grower phase that indicated standard dietary level of fine particle size calcium resulted in increased NE-associated mortality. In contrast, no difference was seen between dietary calcium level with the larger particle size. This difference between particle size is possibly due to calcium solubility and availability in the intestinal tract. While not evaluated in this trial, the very fine particle of limestone may pass more quickly through the gizzard to result in more available calcium in the upper and possibly lower GI tract as well. In previous trials, as in this one, we have seen that higher levels of more soluble calcium resulted in more severe natural occurrence of NE (Paiva et al., 2014; Paiva et al., 2013). Based on these results, it is not only level of calcium, but also particle size and source of limestone that can result in differences in pathology and occurrence of NE. Recent work contradicts the results of the current trial, with coarse particle size increasing mortality during the grower phase. Even with the contradiction, an explanation of the current work could be that smaller particle limestone source of calcium passes more rapidly through the gizzard and is more soluble, it may serve as a risk-factor for NE based on one or more of the hypotheses described below. Among the risk factors for NE, the most discussed is the link between coccidiosis, induced intestinal damage and subsequently *C. perfringens* growth (Williams et al., 2005). During this experiment and others that we have conducted evaluating dietary calcium and NE interaction, there was no difference in macroscopic or microscopic

coccidiosis lesion scores that could be linked to the onset of NE, especially as it relates to dietary calcium. That does not mean that the onset of NE was not contributed to by an increase of mucus or damage to the intestinal mucosa from coccidiosis infection, but we have never observed the response to coccidiosis vaccination to be affected by dietary calcium. While NE-related mortality was not as high in this trial as others we have conducted, but morbid birds were observed during daily observation. These birds exhibited clinical signs of NE including lethargy, ruffled feathers, weakness, dehydration, mucosal damage with central foci of more intense damage and poor performance. This natural occurrence of NE without high mortality allowed for an opportunity similar to industry conditions to evaluate the dietary factors in this trial.

There are multiple hypotheses for the mode of action by which dietary calcium contributes to natural pathogenesis of NE. One possible scenario is an increase in the intestinal pH due to the difference in dietary calcium levels and limestone solubility. Evidence in the current research indicate that birds with increased ileum pH were also the same birds that had increased incidences of NE-related mortality. Previous research showed that increasing dietary levels of calcium in the diet significantly increased small intestine pH (Paiva et al., 2014; Shafey et al., 1991; Walk et al., 2012). The increase in intestinal pH likely makes a more favorable environment for C. perfringens growth (William 2005). Another possibility for the increase in NE-associated mortality resulting from higher dietary calcium levels, would be a connection between dietary calcium level and pathogenesis of *C. perfringens* from increased bacterial activation and toxin production. One of the toxins thought to be involved in NE pathology is alpha-toxin, though the exact role in NE pathogenesis is unknown (Titball et al., 1999a). Keyburn et al. (2006) later reported that NetB or the beta toxin was present, and when tested with avian blood cells there was an increase in pores formed in the lipid bilayer of the cells.

Following pore formation, there is an influx of calcium ions into the cells resulting in lysis of the cell and necrosis of tissue (Keyburn et al., 2010). Therefore, excess available calcium contributed to increased cellular death and tissue necrosis.

In addition to the impact of dietary calcium level, limestone source, and limestone particle size on NE-association morbidity and mortality, there were numerous effects on broiler performance. In general, limestone source 1, particularly the coarse particle size, had more negative impacts on performance (BW, BWG, and FC) at the higher level of inclusion. While the smaller limestone particle size had more impact on NE morbidity and mortality, the larger particle size was more detrimental to performance, particularly with source 1. The higher level of calcium inclusion of this source 1 limestone larger particle size reduced performance significantly compared to the lower level of dietary inclusion. The effects on performance could involve the availability of calcium in the gastrointestinal (GI) tract associated with dietary phytate and chelation of the calcium. Even though there are differences observed in overall performance, there were no differences observed in bone ash percentage. This is similar to reported results of Paiva and collaborators (2013) with lower levels of calcium not compromising bone health.

The most impactful finding of this research was that dietary calcium, in particular limestone particle size, geographic source, and inclusion levels, are complex contributors to broiler performance. While for many years, limestone was largely over-supplemented due to cost and protection of leg health, this research suggests there is a critical need to evaluate the role of calcium on intestinal health parameters and the contribution to performance and NE occurrence. The results suggest that dietary calcium levels could be reduced and possibly counteract the detrimental impact on performance due to NE. Reductions in dietary calcium levels have been

shown to optimize broiler performance. Studies have determined that dietary calcium levels of 0.7% or lower, broiler performance can be optimized (Driver et al., 2005; Hamdi et al., 2015). Therefore, dietary calcium levels can be reduced to increase overall performance of broilers. The role particle size plays on broiler performance has been variable due to the variability in limestone particle size (Majeed et al., 2020). With the current trial, coarse particle size impacted performance, but most of the impact was observed in limestone source 1. Dietary calcium was a potentiating risk factor for NE and limestone characteristics need to be further examined for the influence they have on intestinal health.

Destruction of the intestinal mucosa due to high dietary calcium and the occurrence of NE poses an issue with the digestibility of nutrients. This is evident in the current research with almost all amino acids digestible intake reduced when standard levels of dietary calcium were fed. In research similar to the current around d 14, there is typically a two-way interaction between high dietary calcium levels and another factor observed (particle size and challenge vs. no challenge) that affects digestibility. Research by Zanu et al. (2020d) showed high levels of dietary calcium with an *Eimeria* cocktail (*E. maxima, E. acervulina, and E. Brunetti*) and *C. perfringens* challenge reduced the digestibility of crude protein. Similarly, in the current research, birds fed high levels of the fine particle size experienced significantly increased NE-related mortality, which are also the birds that had the greatest reduction in digestible intake of the amino acids. With most research evaluating calcium on the digestibility of nutrients, the main focus is on calcium and P digestibility. The current trial had results similar to that of Kim et al. (2018) with birds sampled later in life (around d 28) showing no effect on the digestibility of calcium when evaluating particle size and dietary calcium levels. At d 14, birds fed higher dietary calcium levels of the coarse particle size of source 1 had an increased digestible intake of

calcium compared to the fine particle size of source 1 or either particle size of source 2, however there were no differences between groups at the lower calcium levels. This is in contrast to reported results of Paiva et al. (2013) where increased levels of calcium decreased digestibility of calcium. The differences in phytase or no phytase compared to the current research with all diets containing phytase possibly could be the difference observed between the trials. This is evidence a subsequent trial by Paiva et al. (2014) where birds sampled at d 12 had reduced digestibility with low dietary calcium levels and phytase inclusion, but the addition of phytase increased digestibility to levels similar to the birds fed high dietary calcium. calcium digestibility was significantly affected by the particle size of the limestone in previous work. Kim et al., (2018) determined that calcium digestibility was affected by the fine and coarse particle size when 1000 FTU/kg of phytase was included in the diet at d 28. In more recent research, calcium digestibility was increased at d 14 and 35 when birds were fed fine particle size (Majeed et al., 2020). Previous work is variable depending upon the particle size used during the work, therefore it is difficult to get results that correspond to one another.

Interaction of calcium and P, as well as the differences in calcium digestibility, NE should have an impact on the digestibility of P. In the current study, birds had reduced digestible intake of P when fed diets with lower calcium level, which means the birds had an increased digestibility of P when fed low levels of calcium. This result is similar to previous work which indicates low levels of dietary calcium improving P digestibility regardless of P levels in the diet when fed 1000 FTU/kg of phytase (Paiva et al., 2013). With limestone source 1, increased digestible intake of P was observed with the birds consuming larger particle size. Similarly, previous work observed birds fed different particle size with the inclusion of phytase, there is a reduction in digestibility of P when the limestone particle size is changed from coarse to fine

(Kim et al., 2018). As birds age, dietary calcium level, limestone source, and limestone particle size all impacted the digestible intake of P. Which compared to previous research, this is similar to previous work that all the different factors have impacted the digestibility of P, but there has not been research evaluating limestone particle size, source, and dietary calcium levels all in one trial(Kim et al., 2018; Majeed et al., 2020; Paiva et al., 2013).

Previous studies have reported an increased pH in the gizzard with high dietary calcium levels, which could contribute to reduced enzyme activity and less protein digestion (Kim et al., 2018; Paiva et al., 2014; Paiva et al., 2013). Subsequently, excess undigested protein could then potentially pass to the lower intestine as a substrate for *C. perfringens*. In this current trial, and a previously published trial from our laboratory, increased dietary calcium level resulted in increased pH in the lower small intestine (Paiva et al., 2014). With a rise in pH in the lower intestine, this could contribute to a more conducive environment for *C. perfringens* proliferation and overgrowth in the digestive tract. The changes in intestinal pH could also be related to a common observation in our trials with different levels of dietary calcium and different solubility of calcium. The classical NE lesions progress at approximately day 16 in the mid- to lower-small intestine.

The performance results mostly correspond to the nutrient digestibility data with lower levels of dietary calcium, particularly of the small particle limestone resulting in improved digestibility. Source 1 of the large particle limestone at high levels was detrimental to amino acid digestibility, corresponding with reduced performance. Many conclusions can be obtained from the current trial. First, addition of dietary calcium in the formulation needs to be accounted for from all aspects of the diet from limestone, dicalcium phosphate, as well as the addition of the limestone as a flow agent in minerals and soybean meal. Second, there can be a reduction in

inclusion of dietary calcium to increase broiler performance, without detrimental impact in leg health. Finally, limestone needs more focus as a feed ingredient and the variability of particle size between distributors as well as mineral composition could negatively impact broiler performance.

4. THE ROLE OF DIETARY CALCIUM LEVEL AND SOURCE WITH DIFFERENT PROTEIN SOURCES ON THE DEVELOPMENT AND PATHOGENESIS OF NECROTIC ENTERITIS

4.1. Introduction

Enteric diseases, specifically necrotic enteritis (NE), are an issue faced by the global poultry industry. The increase in incidence of NE is financially devastating to the poultry industry. There are performance losses due to subclinical NE, as well as clinical NE leading to increased mortality in the flock. The occurrence of NE has been estimated to cost the poultry industry from $2 to $6 million annually (Wade and Keyburn, 2016). Historically, NE has been partially controlled by antibiotic growth promoters (AGP) and anticoccidial usage in commercial poultry diets (Lee et al., 2011; Prescott, 1979; Williams, 2005). However, the use of prophylactic in-feed medication has declined in the past few years with the voluntary withdrawal of antibiotics in the US to move towards no antibiotics ever production, as well as, the Veterinarian Feed Directive (2017) that requires a script for the use of antibiotics. Therefore, the incidence of NE in the commercial broiler industry has increased in recent years.

calcium is an essential nutrient and has many biochemical functions. It is essential in blood coagulation, eggshell formation, transmission of nerve impulses, muscle contraction, immune function, and energy and fat metabolism (Akbari Moghaddam Kakhki et al., 2019; Kozyreva et al., 2009; Pilvi et al., 2008; Toyoda et al., 2018; Vasin et al., 2010). calcium requirement for optimal performance is lower than that of bone mineralization. Most producers put more emphasis on bone mineralization, therefore diets commonly contain more calcium than required. In addition, the use of limestone as a flow agent in minerals and soybean meal, which is not accounted for in formulations, increases dietary calcium levels above those projected in the

diet. With the increase in NE, several investigators have focused on the role dietary calcium plays on the pathogenesis of NE (Paiva et al., 2014; Paiva et al., 2013; Williams, 2005; Zanu et al., 2020d). Paiva et al. (2013) reported that different dietary calcium sources increased naturally occurring NE-related mortality. Increased mortality was observed with the use of a highly soluble calcified seaweed source of calcium at typical industry (0.9% dietary calcium) levels. Similar results were observed in a subsequent trial with industry levels of dietary calcium increasing NE-related mortality (Paiva et al., 2014). More recent studies have similar results with high levels of calcium increasing mortality in broilers (Zanu et al., 2020d).

To reduce feed cost and increase dietary amino acids, varying levels of meat and bone meal (MBM) have been used in commercial poultry diets (Eagleson et al., 2018; Kratzer and Davis, 1959). Meat and bone meal is used as a valuable source of protein, energy, phosphorus (P), and calcium, due to the increased availability of the nutrients, with the nutritional content varying, but typically containing 48 to 58% protein, 33 to 35% ash, 8 to 10% calcium, 4 to 5% P, 8 to 12% fat, and 4 to 7% water (Zanu et al., 2020a). Meat and bone meal would be a highly soluble form of calcium for the birds. Numerous dietary factors have been reported to contribute as risk factors for occurrence of NE. High levels of animal protein sources have also been reported to increase the occurrence of NE (Drew et al., 2004; Kaldhusdal and Skjerve, 1996; Wilkie et al., 2005; Zanu et al., 2020a). Meat and bone meal has the potential to induce the onset of NE with levels as low as five to six percent throughout the grow out of the bird (Zanu et al., 2020a). Drew et al. (2004) reported that the level and source of dietary protein have significant effects on intestinal populations of *C. perfringens* in broiler chickens. At 28 d of age of broilers, increasing the level of fish meal fed from 230 g kg^{-1} to 400 g kg^{-1} increased counts of *C. perfringens* in ileum and cecum, but no there was no effect observed in birds fed soy protein

concentrate (Drew et al., 2004). Wilkie et al. (2005) observed similar results with birds fed animal protein-based diets having elevating numbers of *C. perfringens* compared to those fed plant protein-based diets. The exception was birds fed potato protein concentrate had similar *C. perfringens* counts to the animal-based diets. Additionally, an overview of cereal content of diets fed to broilers in Norway from 1969 to 1989 demonstrated that 2 major outbreaks of NE were associated with increased use of barley and wheat in the broiler diet (Kaldhusdal and Skjerve, 1996). More importantly, the ratio of wheat and barley to maize was an important predictor in NE, since cereal grains increase the viscosity of digesta. Dietary protein level and source have a significant impact on the quantity of *C. perfringens* in the lower intestine, but other dietary factors (such as amino acids and calcium) have an impact on the pathogenesis of NE (Wilkie et al., 2005). Animal protein, or a highly soluble calcium source, possibly has an influence on the proliferation of *C. perfringens* or the onset of NE.

There is evidence that dietary calcium level plays a role in NE-related mortality, but protein source and calcium solubility could also contribute to the onset of NE. Therefore, the objective of this trial was to evaluate the impact of MBM, dietary calcium levels, and limestone sources on the onset of NE.

4.2. Materials and Methods

4.2.1. Animal and Housing

All animal care procedures were approved by Texas A&M University Institutional Animal Care and Use Committee. A total of 4,320-day old Ross 708 male byproducts were allocated to 8 dietary treatments of 12 replicate pens per treatment. On arrival, all birds received a commercial (1x) dose of Coccivac B52 (Merck) and were allowed to preen for approximately 1 hour. After preening, birds were placed in 3-foot by 6-foot (0.9 sq. ft/bird at d 35) floor pens on

used litter top-dressed with new pine shavings of approximately 3-4 inches in depth. Birds were placed in a temperature and light controlled barn in which the Ross manual (Ross, 2018) was followed for environmental set points during the grow-out of the birds. Lighting was 23 hours of light for the first 7 days and 6 hours of darkness throughout the remainder of the experiment. Feed and water were provided *ad libitum* throughout the duration of the 35-day trial. Mortality was checked twice a day, and all mortality were necropsied for observation of NE lesions. During this experiment, early mortality was increased due to poor chick quality.

4.2.2. Experimental Design and Diets

Experimental diets consisted of a corn and soybean meal-based diet in a randomized complete block design (RCBD). Diets were arranged in a 2×2×2 factorial including two protein sources (all veggie protein source or MBM), 2 dietary calcium levels (Standard or Low) and 2 limestone sources (1 or 2). Diets were set up in a fashion described in Table 14.

Table 14. Factorial arrangement of dietary treatments with two protein sources, two dietary calcium levels, and two limestone sources

Treatment	Protein Source	Calcium Level	Limestone Source
1	All-Veggie	Standard	1
2	All-Veggie	Low	1
3	All-Veggie	Standard	2
4	All-Veggie	Low	2
5	Animal Protein	Standard	1
6	Animal Protein	Low	1
7	Animal Protein	Standard	2
8	Animal Protein	Low	2

The two limestone sources were the same as those in Experiment 1 (Chapters 3 and 4), with source 1 being a more mature stone from Alabama and source 2 a higher surface area, less

mature stone from Florida. Dietary calcium levels were the same as Experiment 1 with 1.05% or 0.75%, 0.95% or 0.70%, and 0.85% or 0.65% for the starter (d 0-14), grower (d15-28), and finisher (d 28-35) respectively. Limestone sources were analyzed by X-ray diffraction to obtain the composition of the limestone. For composition analysis, limestone samples were prepared and analyzed by an outside laboratory with the Malvern/Panalytical Emperian XRD with a Pixcell 1D detector and scanned from 5 °2Θ to 75 °2Θ at 45kV and 40mA. Semiquantitative results were achieved using Rietveld analysis. Composition of the two limestone sources are described in Table 15.

Table 15. Mineral composition of two limestone sources from different mines in the United States

Source	1	2
Composition	(%)	(%)
Calcite, $CaCO_3$	97	100
Dolmite, $CaMg(CO_3)_2$	3	0
Silicia, SiO_2	Trace	Trace

Diets (Tables 16-18) were formulated on a least-cost basis and were composed primarily of corn, soybean meal, corn dried distiller's grains with solubles (DDGS) and porcine MBM. Composite samples of each ingredient were analyzed by an outside laboratory for proximates, calcium, and P, and values were used to formulate all dietary treatments. Birds were fed an isocaloric diet in three dietary phases: starter (d 0-15), grower (d 16-23), and finisher (d 24-35). Diets for each phase were mixed using a 2-ton horizontal double-ribbon Scott mixer, pelleted

using a 1-ton/hr California Pellet Mill equipped with a 4.4-mm diameter die and conditioner, and crumbled using a roller when appropriate. Starter feed was fed as a crumble, and all other phases were fed in a pellet. Feed samples were collected in triplicate and sent to a third-party laboratory for proximate and mineral analysis. During the grow-out, birds started out at total dietary calcium levels of 1.05% or 0.75% and decreased as described above with the Ca:P ratio approximately 2:1. All experimental diets had inclusion of 1500 FTU/kg of phytase with a nutrient credit of 0.16% calcium and 0.15% AvP (Quantum Blue, AB Vista Feed Ingredients, Marlborough, UK).

Table 16. Starter diet formulations and calculated nutrient concentration of different protein sources, calcium levels, and different limestone source on Ross 708 males

	Standard Ca		Low Ca	
Ingredient	Veg	MBM	Veg	MBM
Corn	53.233	54.109	55.200	56.137
Soybean Meal, 48% CP	36.425	34.275	36.225	34.025
DDGS	4.000	4.000	4.000	4.000
MBM	0.000	2.750	0.000	2.750
Soybean Oil	2.020	1.415	1.325	0.710
Limestone	1.340	1.013	0.795	0.465
Monocalcium Phosphate	1.075	0.575	0.550	0.050
Methionine	0.351	0.349	0.347	0.346
L-Lysine	0.244	0.252	0.246	0.255
Sodium Bicarb	0.225	0.215	0.228	0.218
Salt	0.213	0.188	0.210	0.188
Vitamins[1]	0.125	0.125	0.125	0.125
Threonine	0.100	0.098	0.100	0.098
Choline	0.090	0.090	0.090	0.090
Valine	0.073	0.060	0.073	0.058
Trace Mineral[2]	0.050	0.050	0.050	0.050
Phytase[3]	0.030	0.030	0.030	0.030
Xylanase[3]	0.008	0.008	0.008	0.008
Titanium Dioxide	0.400	0.400	0.400	0.400
Total	100.00	100.00	100.00	100.00
Predicted Nutrient Composition				
AME, kcal/kg	3031.58	3031.40	3031.20	3030.92
Protein, %	22.31	22.90	22.38	22.95
Fat, %	4.30	4.00	3.70	3.40
Calcium, %	1.0500	1.0500	0.7500	0.7518
Available P, %	0.4986	0.4999	0.3912	0.3925
Analyzed Nutrient Composition				
Dry Matter, %	89.585	88.99	88.69	89.105
Protein, %	24.00	24.15	23.60	24.75
Fat, %	4.215	3.700	3.755	3.055
Calcium, %	0.850	0.885	0.630	0.640
Total P, %	0.690	0.665	0.575	0.585

[1]Vitamin premix added at this rate yields 11,463 IU vitamin A, 4,012 IU vitamin D3, 47.8 IU vitamin E, 0.02 mg B12, 6.21 mg riboflavin, 47.8 mg niacin, 21.01 mg d-pantothenic acid, 135.8 mg choline, 1.53 mg menadione, 1.82 mg folic acid, 3.06 mg thiamine, 0.57 mg biotin. The carrier is ground rice hulls.

[2]Trace mineral premix added at this rate yields 60.0 mg manganese, 60 mg zinc, 60 mg iron, 7 mg copper, 0.4 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium per kg of diet. The carrier is calcium carbonate and the premix contains less than 1% mineral oil.

[3]AB Vista Feed Ingredients, Marlborough, UK

Table 17. Grower diet formulations and calculated nutrient concentration of variable dietary calcium levels, and different limestone source and particle size on Ross 708 males

	Standard Ca		Low Ca	
Ingredient	Veg	MBM	Veg	MBM
Corn	57.639	58.253	59.384	59.991
Soybean Meal, 48% CP	32.000	30.475	31.800	30.275
DDGS	4.000	4.000	4.000	4.000
MBM	0.000	2.000	0.000	2.000
Soybean Oil	2.420	1.985	1.815	1.385
Limestone	1.228	0.988	0.790	0.550
Monocalcium Phosphate	0.875	0.500	0.375	0.000
Methionine	0.297	0.296	0.295	0.293
L-Lysine	0.207	0.212	0.209	0.214
Sodium Bicarb	0.238	0.230	0.240	0.233
Salt	0.258	0.240	0.255	0.238
Vitamins[1]	0.125	0.125	0.125	0.125
Threonine	0.085	0.080	0.085	0.080
Choline	0.090	0.090	0.090	0.090
Valine	0.053	0.040	0.050	0.040
Trace Mineral[2]	0.050	0.050	0.050	0.050
Phytase[3]	0.030	0.030	0.030	0.030
Xylanase[3]	0.008	0.008	0.008	0.008
Titanium Dioxide	0.400	0.400	0.400	0.400
Total	100.00	100.00	100.00	100.00
Predicted Nutrient Composition				
AME, kcal/kg	3108.23	3108.36	3108.30	3108.76
Protein, %	20.59	21.02	20.64	21.07
Fat, %	4.77	4.55	4.25	4.04
Calcium, %	0.9517	0.9505	0.7002	0.6990
Available P, %	0.4519	0.4506	0.3496	0.2394
Analyzed Nutrient Composition				
Dry Matter, %	87.77	87.515	87.205	87.73
Protein, %	21.35	21.00	21.30	21.50
Fat, %	4.830	4.910	4.535	4.460
Calcium, %	0.810	0.830	0.555	0.575
Total P, %	0.615	0.645	0.555	0.505

[1]Vitamin premix added at this rate yields 11,463 IU vitamin A, 4,012 IU vitamin D3, 47.8 IU vitamin E, 0.02 mg B12, 6.21 mg riboflavin, 47.8 mg niacin, 21.01 mg d-pantothenic acid, 135.8 mg choline, 1.53 mg menadione, 1.82 mg folic acid, 3.06 mg thiamine, 0.57 mg biotin. The carrier is ground rice hulls.

[2]Trace mineral premix added at this rate yields 60.0 mg manganese, 60 mg zinc, 60 mg iron, 7 mg copper, 0.4 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium per kg of diet. The carrier is calcium carbonate and the premix contains less than 1% mineral oil.

[3]AB Vista Feed Ingredients, Marlborough, UK

Table 18. Finisher diet formulations and calculated nutrient concentration of variable dietary calcium levels, and different limestone source and particle size on Ross 708 males

	Standard Ca		Low Ca	
Ingredient	Veg	MBM	Veg	MBM
Corn	62.044	62.502	63.452	63.862
Soybean Meal, 48% CP	28.325	27.175	28.150	27.025
DDGS	4.000	4.000	4.000	4.000
MBM	0.000	1.500	0.000	1.500
Soybean Oil	2.520	2.195	2.040	1.720
Limestone	1.107	0.927	0.755	0.567
Monocalcium Phosphate	0.650	0.375	0.250	0.000
Methionine	0.258	0.257	0.255	0.254
L-Lysine	0.185	0.189	0.187	0.190
Sodium Bicarbonate	0.230	0.225	0.233	0.225
Salt	0.262	0.250	0.263	0.250
Vitamins[1]	0.125	0.125	0.125	0.125
Threonine	0.075	0.072	0.075	0.072
Choline	0.090	0.090	0.090	0.090
Valine	0.040	0.030	0.038	0.030
Trace Mineral[2]	0.050	0.050	0.050	0.050
Phytase[3]	0.030	0.030	0.030	0.030
Xylanase[3]	0.008	0.008	0.008	0.008
Total	100.00	100.00	100.00	100.00
Predicted Nutrient Composition				
AME, kcal/kg	3163.46	3163.44	3163.90	3163.44
Crude Protein, %	19.20	19.52	19.23	19.56
Fat, %	4.9400	4.7800	4.5300	4.3700
Calcium, %	0.8514	0.8515	0.6491	0.6505
Available P, %	0.4012	0.4015	0.3194	0.3248
Analyzed Nutrient Composition				
Dry Matter, %	88.185	89.02	88.58	89.08
Protein, %	20.05	20.85	19.45	19.95
Fat, %	5.285	5.100	4.790	5.090
Calcium, %	0.770	0.800	0.515	0.545
Total P, %	0.515	0.520	0.425	0.440

[1]Vitamin premix added at this rate yields 11,463 IU vitamin A, 4,012 IU vitamin D3, 47.8 IU vitamin E, 0.02 mg B12, 6.21 mg riboflavin, 47.8 mg niacin, 21.01 mg d-pantothenic acid, 135.8 mg choline, 1.53 mg menadione, 1.82 mg folic acid, 3.06 mg thiamine, 0.57 mg biotin. The carrier is ground rice hulls.

[2]Trace mineral premix added at this rate yields 60.0 mg manganese, 60 mg zinc, 60 mg iron, 7 mg copper, 0.4 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium per kg of diet. The carrier is calcium carbonate and the premix contains less than 1% mineral oil.

[3] AB Vista Feed Ingredients, Marlborough, UK

4.2.3. Measured Variables

4.2.3.1. Live Performance

Pen body weight (BW) and feed consumption were recorded on d 15, 23, and 35 to determine average BW and average BW gain (BWG). Feed consumption and daily mortality were used to determine mortality corrected FCR.

4.2.3.2. pH

On d 15 and 23, three birds/pen were randomly selected and used to measure the pH of the gizzard and sections of the small intestine (duodenum, jejunum, and ileum). Following cervical dislocation, pH measurements were obtained directly from the lumen of each section with digesta present. Incisions were made in the distal gizzard, mid-way up the distal duodenum, jejunum, and ileam. The spear-tip electrode was inserted in both the cranial and caudal direction to obtain readings using the digital pH meter (Professional Portable Meat pH Meter, Hanna Instruments, Woonsocket, RI). The two pH readings from each intestinal section of each bird were pooled to get a pen average (Walk et al., 2012).

4.2.3.3. Ileal Digestibility

On d 15 and 23, six birds/pen and four birds/pen, respectively, were euthanized by cervical dislocation for digesta collection. Digesta samples were collected from the entire ileum (defined as Meckel's diverticulum to ~2 cm cranial to ileocecal junction), pooled/pen, and immediately frozen (-20°C) until further analysis. Samples were freeze dried via lypholizer (Labconco FreeZone Freeze Dry Systems, 8811 Prospect Ave, Kansas City, MO 64132) and ground to pass a 1 mm screen. Dried, ground ileal digesta and experimental diets were sampled for amino acids (method 982.30 E(a)), calcium (method 975.03 B(b)), and P (method 968.08)

according to AOAC (2006) and titanium according to Journal of Animal Science (2004) at University of Missouri Agriculture Experiment Station (Columbia, MO).

4.2.3.3.1. Digestibility Calculations

Apparent ileal amino acid digestibility was calculated using the following equation (Paiva et al., 2014):

AID= [(nutrient/TiO2)diet – (nutrient/TiO2)ileum] / (nutrient/TiO2)diet

where: (nutrient/TiO2)diet= the ratio of nutrient to titanium dioxide in the diet

(nutrient/TiO2)ileum= the ratio of nutrient in the ileal digesta.

The following equation was used to calculate digestible nutrient intake in g/d (Walk and Rama Rao, 2018):

Digestible nutrient intake (g/day) = [(diet nutrient, %) x (AID nutrient, % / 100)] / 100 x daily intake (g)

where: Diet nutrient = the analyzed nutrient concentration of the diet

AID nutrient = the calculated apparent ileal nutrient digestibility

4.2.3.4. Intestinal soluble and insoluble elements

On days 15 and 23, digesta from 5 birds/pen was collected from the gizzard, duodenum, and ileum and weighed. Gizzard digesta was suspended in 2x the weight in DI water and centrifuged at 5000 rpm for 5 mins at 18°C. The supernatant was removed and pellet was re-suspended in 2x weight DI water and centrifuged at 5300 g for 5 mins at 18°C. Supernatant from both centrifugations will be combined. Other sections of the intestine (duodenum and ileum) will be centrifuged at 5300 g for 5 min at 18°C. Both the supernatant and pellet were analyzed by inductively coupled plasma (ICP) for elemental content.

4.2.4. Statistical Analysis

A 2 (protein sources) × 2 (calcium dietary levels) × 2 (limestone sources) factorial arrangement of treatments within a RCBD was used. Data were subjected to an ANOVA using the GLM procedure of SAS 9.4 (SAS institute, Inc., 2020), $P \leq 0.05$ and treatment means were further explored with Tukey's HSD test.

4.3. Results

4.3.1. Performance

Due to increased mortality at placement as a result of compromised chick quality, mortality up to d 4 (8%) was removed from statistical analysis as it was not treatment related. After d 4, health of the birds improved, and mortality was decreased which allowed the trial to continue. During the starter phase (d 5-15), there was a 3-way interaction of protein type, calcium level and limestone source on mortality (Table 19). Birds consuming limestone source 2 in the MBM inclusion diet, regardless of calcium level, had increased mortality due to NE as compared to the all-veggie diet at the standard calcium level with source 1 of limestone. There were no differences observed in mortality during the grower or finisher phases. For the periods of d 5-23 and d 5-35, birds fed the veggie diet had reduced mortality compared to the animal protein diets.

At placement of the project, there was no difference observed in BW between treatments (Table 20). On d 15, there was a protein type x calcium level x limestone source interaction on BW. In the veggie protein treatment groups, birds on low dietary calcium of limestone source 1 had reduced BW compared to all other veggie-based treatments. Birds fed standard dietary calcium level of limestone source 1 in the animal protein based-diets had intermediate BW, which was similar to all other treatments, and there were no differences in the remaining

treatments. On d 23, there was a 2-way interaction between protein type and limestone source. Birds on veggie diets with limestone source 1 had reduced BW compared to those on limestone source 2, which were similar in BW to birds on animal protein-based diets with either source of limestone. There was no difference observed in d 35 BW. The BWG results for the starter period of D 0-15 and the period of D0-23 showed the same differences as described for BW (Table 21). There were no other differences observed in BWG during the grower, finisher, or for the cumulative trial period. The only significant differences observed in FCR occurred during the starter period (Table 22). A protein source x calcium level interaction was observed for FCR during the starter phase, in which a reduction in dietary calcium in the all-veggie diets resulted in less efficient FCR compared to the standard calcium level veggie diets, while there were no differences in FCR between calcium levels with animal protein diets.

4.3.2. Nutrient Intake

Similar trends in amino acid nutrient intake digestibility to those reported for Experiment 1 were observed in this experiment. Numerous 2-way interactions were observed at d 14 (Table 23), while at d 23 there was a shift to all 3-way interactions (Table 24). Three-way interaction of protein source, calcium level, and limestone source was observed for threonine and methionine on d 14. In the veggie diet, low dietary calcium with limestone source 1 resulted in decreased digestible intake compared to the standard calcium level. However, this decrease was not evident in the veggie diet with limestone source 2 or the animal protein diets with either limestone source. Results of methionine digestible intake indicated that limestone source 1 was most detrimental to intake at either dietary calcium level in the veggie diets, while results with limestone source 2 were similar to those seen with animal protein diets. At d 14, there were many two-way interactions of protein source and calcium level on AA digestible intake. For

every AA with the exception of valine, methionine, and lysine, standard calcium level in veggie diets increased AA digestible intake compared to low dietary calcium in veggie diets. Conversely, standard calcium level in animal protein diets reduced AA digestible intake compared to these diets with lower calcium level. With valine and lysine, the same difference was observed between dietary calcium levels in veggie diets, but there was no difference in the diets with MBM. Interactions of protein and limestone source influenced digestible intake of glutamine, proline, isoleucine, leucine, and histidine. In the veggie based diets there was no difference in intake between limestone sources. However in the animal protein diets, results were dependent on limestone source, with limestone source 2 resulting in increased intake compared to source 1, other than with glycine. Glycine was also effected by limestone source and calcium level with differences in intake between sources at the standard dietary calcium level, but increased intake with source 1 compared to source 2 at the low dietary calcium level. Both protein source by calcium level and protein source by limestone source interactions resulted in differences in digestible calcium and P intake at d 14. In broilers on animal protein diets, calcium level did not impact calcium or P intake, but with veggie diets intake was reduced with lower dietary calcium. With veggie based protein diets, there was no influence of limestone source, but in animal protein diets, limestone source 2 increased digestible calcium and P intake. Phosphorus digestible intake was also altered by calcium level and limestone source as indicated by increased intake with low dietary calcium of source 2 compared to source 1, but no difference between sources at standard dietary calcium.

With a few exceptions, most of the amino acids had similar interactions of protein source, dietary calcium level, and limestone geographic source. In the diets with veggie protein, and limestone source 1, there were no differences in digestible intake between standard and low

dietary calcium level. In contrast, when diets included animal protein, limestone source 2 didn't result in differences in intake between calcium levels, but with limestone source 1, low dietary calcium level resulted in increased intake compared to standard calcium level. The only AA for which these differences were not consistent included methionine, tyrosine, and lysine. Digestible intake of methionine and tyrosine indicated that the lower levels of calcium resulted in reduced intake with both limestone source 1 and 2 in the veggie-based diet groups. In contrast, in the animal protein groups digestible intake increased with low dietary calcium compared to standard calcium with limestone source 1, but there wasn't a difference in these groups with source 2. In the diets with MBM, there were no differences between standard dietary calcium and lower dietary calcium with source 1 or source 2 of limestone.

Digestible intake of calcium and P at d 23 indicated interactions of diet protein type, calcium level, and limestone geographic source. The calcium data indicated that in veggie diets, standard levels of calcium from limestone source 2 resulted in the lowest intake, but this was not seen with the diets containing MBM. In the MBM diets, low dietary calcium level with source 2 resulted in the highest intake. Similar results were observed with P in the veggie and MBM diets with standard levels of source 2 limestone having lower digestible intake.

4.3.3. pH

There were no differences observed in intestinal pH at d 15 or d 23 in all sections of the small intestine (Table 25).

4.3.4. Intestinal Soluble and Insoluble Elements

The changing environment throughout the intestinal tract could have an impact on how elements are solubilized. Tables 26 through 31 illustrate insoluble and soluble elements throughout the intestinal tract. In the gizzard, two-way and three-way interactions were observed

in insoluble elements with two-way interactions observed in soluble elements. Birds fed standard levels of calcium in the all-veggie diet regardless of limestone source had increased insolubility of aluminum. With birds fed animal protein with low dietary calcium levels, regardless of source, as well as low dietary calcium levels of limestone source 2 in the all-veggie diet had reduced levels of insoluble aluminum in the gizzard. Protein source and dietary calcium level altered the insoluble calcium in the gizzard. Birds fed standard dietary calcium levels regardless of protein source had increased levels of insoluble calcium, compared to birds fed low dietary calcium levels. Soluble calcium in the gizzard (Table 27) followed a similar trend. Birds fed standard levels of calcium regardless of protein source had increased soluble calcium compared with low dietary calcium levels. Soluble manganese in the gizzard was impacted by an interaction of protein source and limestone source. Birds fed all-veggie diets with limestone source 1 had increased soluble manganese, while birds fed animal protein with limestone source 1 had reduced soluble manganese. Intermediate levels of soluble manganese were observed in birds fed limestone source 2 regardless of dietary protein source.

As the digesta moves down the intestinal tract, insoluble and soluble elements are not as impacted by protein source, dietary calcium level, and limestone source in the duodenum. Birds fed standard levels of limestone source 2 had increased insoluble sulfur where as standard levels of limestone source 1 reduced insoluble sulfur. All other insoluble elements were independently affected by dietary factors. All-veggie diets increased insoluble aluminum and manganese. Insoluble duodenal calcium was increased with standard dietary calcium levels, as well as with limestone source 1. Three-way interactions were observed in soluble duodenum calcium. Birds fed standard levels of dietary calcium regardless of protein source or limestone source, had

increased soluble calcium in the duodenum. Reduced levels of soluble calcium were observed in birds fed low dietary calcium of source 2 in the all-veggie diet.

With the absorption of nutrients in the ileum, soluble and insoluble elements were impacted by three-way interactions. Insoluble aluminum in the ileum was increased in birds fed standard dietary calcium levels of source 2 in the all-veggie diets. Birds fed low dietary calcium levels in the animal protein diets regardless of calcium source had reduced insoluble aluminum in the ileum. Insoluble calcium, phosphorus, and zinc followed a similar trend with birds fed standard dietary calcium of limestone source 2 having increased ileal levels of these. Insoluble manganese was similar to other elements with birds fed standard calcium levels of source 2 in the all-veggie diets having increased content, but birds fed low dietary calcium levels of source 1 in the all-veggie diets had increased content in the ileum. Insoluble sodium was increased in birds consuming all-veggie diets with low dietary calcium levels of source 2. Solubility of the elements in the ileum followed a trend much closer than that of the insoluble elements. Birds fed all-veggie diets with standard dietary calcium levels of source 2 had increased solubility of ileal calcium, manganese, and phosphorus. Results indicated that birds fed the all-veggie diets with standard calcium levels of limestone source 2 were similar in levels of soluble zinc to the birds fed low dietary calcium levels of source 1 in the all-veggie diets, which increased solubility.

Table 19. Evaluation of limestone source, particle size, and dietary calcium level on mortality of broilers during naturally occurring necrotic enteritis.

Treatment			Percent Mortality				
			Starter (5-15)*	Grower*	Finisher*	D 5-23*	D 5-35*
Protein	Level	Source					
Veggie	Standard	1	0.926[a]	0.538	1.176	1.296	1.852
Veggie	Low	1	3.519[ab]	0.000	0.000	3.519	3.519
Veggie	Standard	2	3.519[ab]	0.000	0.952	3.519	3.889
Veggie	Low	2	1.667[ab]	0.585	0.379	2.037	2.222
Animal	Standard	1	3.540[ab]	0.278	1.773	3.725	4.465
Animal	Low	1	2.778[ab]	1.229	1.817	3.519	4.259
Animal	Standard	2	4.630[b]	0.908	0.000	5.185	5.185
Animal	Low	2	4.444[b]	0.000	1.258	4.444	5.000
Pooled SEM			0.676	0.273	0.521	0.692	0.754
	Level	Source					
	Standard	1	2.233	0.408	1.474	2.511	3.159
	Standard	2	4.074	0.454	0.476	4.352	4.537
	Low	1	3.148	0.614	0.909	3.519	3.889
	Low	2	3.056	0.292	0.819	3.241	3.611
Pooled SEM			0.522	0.258	0.443	0.531	0.571
Protein							
Veggie			2.407[a]	0.281	0.627	2.593[a]	2.87[a]
Animal			3.848[b]	0.604	1.212	4.218[b]	4.727[b]
Pooled SEM			0.367	0.179	0.311	0.320	0.385
P Value							
Protein			0.0169	0.2101	0.1923	0.0019	0.0011
Level			0.9123	0.9301	0.8027	0.9195	0.8592
Source			0.0174	0.5916	0.2253	0.1279	0.3198
Protein * Level			0.1156	0.9964	0.0903	0.4091	0.8592
Protein * Source			0.1747	0.5297	0.1664	0.4209	0.7443
Level * Source			0.0484	0.4737	0.3110	0.0402	0.1357
Protein * Level * Source			0.0473	0.0045	0.7322	0.123	0.1309

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

* Mortality up to d 4 was not included in summary and analysis due to poor chick quality

n=12

Table 20. Evaluation of limestone source, particle size, and dietary calcium level on body weight of broilers during naturally occurring necrotic enteritis.

Treatment			Body Weight			
			D 0 (g)	D 15 (kg)	D 23 (kg)	D 35 (kg)
Protein	Level	Source				
Veggie	Standard	1	33.41	0.443[a]	0.873	1.876
Veggie	Low	1	33.27	0.408[b]	0.827	1.814
Veggie	Standard	2	33.34	0.448[a]	0.884	1.884
Veggie	Low	2	33.22	0.447[a]	0.887	1.860
Animal	Standard	1	33.42	0.429[ab]	0.891	1.871
Animal	Low	1	33.15	0.442[a]	0.895	1.865
Animal	Standard	2	33.39	0.443[a]	0.882	1.868
Animal	Low	2	33.34	0.448[a]	0.900	1.898
	Pooled SEM		0.117	0.006	0.012	0.021
Protein	Level					
Veggie	Standard		33.38	0.445[a]	0.879	1.880
Veggie	Low		33.24	0.427[b]	0.857	1.837
Animal	Standard		33.41	0.436[ab]	0.886	1.870
Animal	Low		33.24	0.445[a]	0.898	1.882
	Pooled SEM		0.081	0.005	0.009	0.015
Protein		Source				
Veggie		1	33.34	0.425	0.850[b]	1.845
Veggie		2	33.28	0.447	0.885[a]	1.872
Animal		1	33.28	0.436	0.893[a]	1.868
Animal		2	33.36	0.446	0.891[a]	1.883
	Pooled SEM		0.083	0.005	0.009	0.015
	P Value					
	Protein		0.8707	0.3370	0.0096	0.2626
	Level		0.0758	0.3302	0.5761	0.3108
	Source		0.9093	0.0005	0.0720	0.1681
	Protein * Level		0.8378	0.0030	0.0707	0.0755
	Protein * Source		0.4033	0.1774	0.0472	0.6988
	Level * Source		0.4966	0.1586	0.0889	0.2261
	Protein * Level * Source		0.5441	0.0232	0.3416	0.9871

a-b Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).
n=12

Table 21. Evaluation of limestone source, particle size, and dietary calcium level on body weight gain of broilers during naturally occurring necrotic enteritis.

Treatments			Body Weight Gain (kg)				
			Starter	Grower	Finisher	D0-23	D0-35
Protein	Level	Source					
Veggie	Standard	1	0.409[a]	0.431	1.003	0.84	1.843
Veggie	Low	1	0.375[b]	0.419	0.987	0.794	1.781
Veggie	Standard	2	0.415[a]	0.436	1.000	0.851	1.851
Veggie	Low	2	0.413[a]	0.440	0.973	0.853	1.827
Animal	Standard	1	0.396[ab]	0.431	0.981	0.857	1.838
Animal	Low	1	0.409[a]	0.453	0.970	0.862	1.832
Animal	Standard	2	0.410[a]	0.439	0.987	0.848	1.835
Animal	Low	2	0.415[a]	0.452	0.998	0.867	1.865
Pooled SEM			0.006	0.010	0.016	0.012	0.021
Protein	Level						
Veggie	Standard		0.412[a]	0.433	1.001	0.845	1.847
Veggie	Low		0.394[b]	0.429	0.980	0.823	1.804
Animal	Standard		0.403[ab]	0.450	0.984	0.853	1.836
Animal	Low		0.412[a]	0.453	0.984	0.865	1.848
Pooled SEM			0.005	0.008	0.011	0.009	0.015
Protein		Source					
Veggie		1	0.392	0.425	0.995	0.817[b]	1.811
Veggie		2	0.414	0.438	0.987	0.852[a]	1.839
Animal		1	0.402	0.457	0.975	0.860[a]	1.835
Animal		2	0.412	0.446	0.992	0.858[a]	1.850
Pooled SEM			0.005	0.008	0.012	0.009	0.015
P Value							
Protein			0.3382	0.0135	0.5461	0.0097	0.2631
Level			0.3468	0.9186	0.3845	0.5875	0.3157
Source			0.0005	0.9177	0.7106	0.0724	0.1685
Protein * Level			0.0029	0.6815	0.3754	0.0706	0.0754
Protein * Source			0.1722	0.1208	0.2910	0.0466	0.6955
Level * Source			0.1620	0.2354	0.8178	0.0903	0.2278
Protein * Level * Source			0.0224	0.8548	0.4728	0.3392	0.9844

a-b Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).
n=12

Table 22. Evaluation of limestone source, particle size, and dietary calcium level on feed conversion ratio (FCR) of broilers during naturally occurring necrotic enteritis.

Treatment			Feed Conversion Ratio				
			Starter	Grower	Finisher	D0-23	D0-35
Protein	Level						
Veggie	Standard		1.205[a]	1.475	1.566	1.319	1.415
Veggie	Low		1.260[b]	1.486	1.563	1.357	1.437
Animal	Standard		1.242[ab]	1.485	1.579	1.345	1.434
Animal	Low		1.243[ab]	1.491	1.574	1.347	1.430
Pooled SEM			0.012	0.017	0.019	0.010	0.008
Protein		Source					
Veggie		1	1.255	1.483	1.551	1.353	1.431
Veggie		2	1.211	1.478	1.577	1.324	1.421
Animal		1	1.247	1.475	1.588	1.344	1.436
Animal		2	1.238	1.501	1.565	1.348	1.429
Pooled SEM			0.012	0.017	0.019	0.010	0.009
Level		Source					
Standard		1	1.236	1.492	1.578	1.344	1.434
Standard		2	1.212	1.468	1.566	1.321	1.415
Low		1	1.270	1.466	1.561	1.353	1.432
Low		2	1.237	1.511	1.576	1.352	1.435
Pooled SEM			0.012	0.017	0.019	0.010	0.008
P Value							
Protein			0.3978	0.6533	0.5576	0.4251	0.4906
Level			0.0190	0.6257	0.8610	0.0441	0.3138
Source			0.0237	0.5370	0.9543	0.2035	0.3393
Protein * Level			0.0238	0.8908	0.9821	0.0631	0.1640
Protein * Source			0.1366	0.3772	0.2319	0.0973	0.8396
Level * Source			0.8265	0.0538	0.5143	0.2431	0.2241
Protein * Level * Source			0.1374	0.7286	0.8945	0.4875	0.6696

a-b Means within a column lacking a common superscript are significantly different from each other ($P<0.05$).
n=12

Table 23 Evaluation of limestone source, particle size, and dietary calcium level on digestible intake (g/bird) of broilers during naturally occurring necrotic enteritis at d 14

Treatment			Digestible Intake (g/bird)								
			Aspartic	Threonine	Serine	Glutamic	Proline	Glycine	Alanine	Valine	Methionine
Protein	Level	Source									
Veggie	Standard	1	0.579	0.230^{ab}	0.243	1.116	0.327	0.220	0.278	0.295	0.171^{d}
Veggie	Low	1	0.527	0.201^{d}	0.224	1.036	0.301	0.204	0.261	0.263	0.160^{e}
Veggie	Standard	2	0.596	0.227^{b}	0.251	1.143	0.336	0.230	0.285	0.307	0.175^{c}
Veggie	Low	2	0.533	0.222^{bc}	0.235	1.046	0.303	0.201	0.610	0.275	0.183^{ab}
Animal	Standard	1	0.527	0.211^{cd}	0.230	1.053	0.326	0.247	0.279	0.283	0.181^{b}
Animal	Low	1	0.553	0.225^{b}	0.244	1.098	0.344	0.271	0.298	0.278	0.181^{b}
Animal	Standard	2	0.555	0.227^{b}	0.244	1.094	0.323	0.238	0.267	0.285	0.186^{a}
Animal	Low	2	0.575	0.242^{a}	0.253	1.136	0.344	0.253	0.303	0.289	0.174^{cd}
	Pooled SEM		0.006	0.003	0.002	0.007	0.004	0.003	0.003	0.001	0.003
Protein	Level										
Veggie	Standard		0.588^{a}	0.229^{a}	0.247^{a}	1.129^{a}	0.332^{b}	0.225^{c}	0.281^{b}	0.301^{a}	0.173^{c}
Veggie	Low		0.530^{c}	0.211^{c}	0.229^{c}	1.041^{c}	0.302^{c}	0.202^{d}	0.261^{c}	0.269^{c}	0.171^{c}
Animal	Standard		0.541^{c}	0.219^{b}	0.237^{b}	1.073^{b}	0.324^{b}	0.243^{b}	0.283^{b}	0.284^{b}	0.184^{a}
Animal	Low		0.564^{b}	0.233^{a}	0.249^{a}	1.117^{a}	0.344^{a}	0.262^{a}	0.300^{a}	0.284^{b}	0.177^{b}
	Pooled SEM		0.004	0.002	0.002	0.006	0.003	0.002	0.003	0.003	0.001
Protein		Source									
Veggie		1	0.553	0.215	0.233	1.076^{b}	0.314	0.212^{c}	0.269	0.279	0.165^{b}
Veggie		2	0.565	0.224	0.243	1.094^{b}	0.319	0.215^{c}	0.273	0.291	0.179^{a}
Animal		1	0.540	0.218	0.237	1.075^{b}	0.335	0.259^{a}	0.288	0.280	0.181^{a}
Animal		2	0.565	0.235	0.249	1.163^{a}	0.334	0.246^{b}	0.295	0.287	0.180^{a}
	Pooled SEM		0.006	0.003	0.002	0.009	0.004	0.003	0.003	0.003	0.001
	Level	Source									
	Standard	1	0.553	0.221^{b}	0.237	1.085	0.326	0.234^{ab}	0.279	0.289	0.176^{b}
	Standard	2	0.577	0.227^{ab}	0.248	1.120	0.330	0.234^{ab}	0.286	0.296	0.180^{a}
	Low	1	0.540	0.213^{c}	0.234	1.067	0.322	0.237^{a}	0.279	0.270	0.170^{c}
	Low	2	0.554	0.232^{a}	0.244	1.091	0.323	0.227^{b}	0.282	0.282	0.178^{ab}
	Pooled SEM		0.006	0.003	0.002	0.009	0.004	0.005	0.004	0.003	0.002

Table 23. Continued

Treatment	Digestible Intake (g/bird)								
	Aspartic	Threonine	Serine	Glutamic	Proline	Glycine	Alanine	Valine	Methionine
P Value									
Protein	0.1436	0.0022	0.0063	0.0516	<0.0001	<0.0001	<0.0001	0.6303	<0.0001
Level	<0.0001	0.4400	0.0775	<0.0001	0.0509	0.396	0.5111	<0.0001	<0.0001
Source	<0.0001	<0.0001	<0.0001	<0.0001	0.4290	0.0307	0.0396	0.0003	<0.0001
Protein * Level	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002
Level * Source	0.2748	0.0017	0.6544	0.3524	0.6754	0.018	0.4164	0.2955	0.0100
Protein * Source	0.1177	0.0682	0.6603	0.0398	0.2273	0.0004	0.5208	0.3281	<0.0001
Protein * Level * Source	0.7862	0.0032	0.2429	0.4778	0.3468	0.6664	0.7084	0.3588	<0.0001

a-e Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=12

Table 23 Continued.

Treatment			Digestible Intake (g/bird)								
			Ile	Leu	Tyr	Phe	Lys	His	Arg	Ca	P
Protein	Level	Source									
Veggie	Standard	1	0.261	0.503	0.204	0.298	0.388	0.156	0.434	0.168	0.164
Veggie	Low	1	0.237	0.464	0.185	0.272	0.352	0.144	0.396	0.133	0.114
Veggie	Standard	2	0.267	0.515	0.212	0.305	0.406	0.161	0.447	0.167	0.149
Veggie	Low	2	0.240	0.474	0.194	0.280	0.372	0.146	0.409	0.138	0.135
Animal	Standard	1	0.236	0.473	0.192	0.279	0.382	0.146	0.422	0.157	0.150
Animal	Low	1	0.244	0.493	0.201	0.291	0.401	0.149	0.435	0.152	0.151
Animal	Standard	2	0.248	0.496	0.195	0.294	0.403	0.154	0.435	0.185	0.165
Animal	Low	2	0.259	0.523	0.205	0.306	0.402	0.157	0.442	0.196	0.172
	Pooled SEM		0.003	0.005	0.002	0.003	0.003	0.001	0.002	0.006	0.003
Protein	Level										
Veggie	Standard		0.264^{a}	0.509^{a}	0.208^{a}	0.301^{a}	0.397^{a}	0.158^{a}	0.441^{a}	0.167^{a}	0.157^{a}
Veggie	Low		0.238^{c}	0.469^{c}	0.190^{c}	0.276^{c}	0.362^{b}	0.145^{c}	0.402^{c}	0.136^{b}	0.124^{b}
Animal	Standard		0.242^{c}	0.484^{b}	0.193^{c}	0.286^{b}	0.392^{a}	0.150^{b}	0.428^{b}	0.170^{a}	0.157^{a}
Animal	Low		0.251^{b}	0.508^{a}	0.203^{b}	0.299^{a}	0.401^{a}	0.153^{b}	0.439^{a}	0.174^{a}	0.161^{a}
	Pooled SEM		0.002	0.004	0.001	0.002	0.003	0.001	0.002	0.005	0.003
Protein		Source									
Veggie		1	0.249^{a}	0.483^{b}	0.194	0.285	0.370	0.150^{bc}	0.415	0.150^{b}	0.139^{c}
Veggie		2	0.253^{a}	0.495^{b}	0.203	0.292	0.389	0.153^{ab}	0.428	0.152^{b}	0.142^{c}
Animal		1	0.24^{b}	0.483^{b}	0.196	0.285	0.392	0.148^{c}	0.429	0.154^{b}	0.150^{b}
Animal		2	0.253^{a}	0.510^{a}	0.200	0.300	0.503	0.155^{a}	0.439	0.191^{a}	0.169^{a}
	Pooled SEM		0.003	0.005	0.002	0.003	0.004	0.0001	0.003	0.005	0.003
	Level	Source									
	Standard	1	0.248	0.488	0.198	0.289	0.385	0.151	0.428	0.162	0.157^{a}
	Standard	2	0.258	0.506	0.204	0.300	0.405	0.157	0.441	0.176	0.157^{a}
	Low	1	0.240	0.780	0.193	0.282	0.376	0.147	0.416	0.143	0.132^{b}
	Low	2	0.249	0.499	0.200	0.293	0.387	0.151	0.425	0.167	0.154^{a}
	Pooled SEM		0.003	0.005	0.002	0.003	0.004	0.001	0.003	0.005	0.004

Table 23. Continued

Treatment	Digestible Intake (g/bird)								
	Ile	Leu	Tyr	Phe	Lys	His	Arg	Ca	P
P Value									
Protein	0.0372	0.0461	0.6217	0.0542	<0.0001	0.8829	<0.0001	<0.0001	<0.0001
Level	0.0002	0.0224	0.0012	0.0016	<0.0001	<0.0001	<0.0001	0.0013	<0.0001
Source	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Protein * Level	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001
Level * Source	0.9936	0.7357	0.6131	0.8865	0.0631	0.3685	0.2784	0.2517	<0.0001
Protein * Source	0.035	0.0407	0.0553	0.075	0.1279	0.0275	0.3993	0.0002	0.0003
Protein * Level * Source	0.4449	0.5564	0.9333	0.9095	0.0291	0.3381	0.3152	0.5646	0.0007

a-e Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=12

Table 24 Evaluation of limestone source, particle size, and dietary calcium level on digestible intake (g/bird) of broilers during naturally occurring necrotic enteritis at d 23

Treatment			Digestible Intake (g/bird)								
			Aspartic	Threonine	Serine	Glutamic	Proline	Glycine	Alanine	Valine	Met
Protein	Level	Source									
Veggie	Standard	1	1.142^{c}	0.436^{cd}	0.485^{c}	2.167^{d}	0.627^{d}	0.440^{de}	0.565^{de}	0.558^{d}	0.357^{b}
Veggie	Low	1	1.117^{c}	0.421^{d}	0.484^{c}	2.144^{d}	0.617^{d}	0.422^{e}	0.546^{e}	0.547^{d}	0.316^{f}
Veggie	Standard	2	1.145^{c}	0.430^{d}	0.495^{bc}	2.243^{c}	0.672^{c}	0.446^{d}	0.588^{cd}	0.562^{d}	0.373^{a}
Veggie	Low	2	1.224^{b}	0.469^{ab}	0.517^{a}	2.303^{bc}	0.684^{bc}	0.473^{c}	0.601^{bc}	0.591^{c}	0.335^{e}
Animal	Standard	1	1.246^{b}	0.468^{ab}	0.482^{c}	2.306^{b}	0.683^{b}	0.506^{b}	0.613^{bc}	0.623^{b}	0.337^{e}
Animal	Low	1	1.328^{a}	0.477^{a}	0.514^{ab}	2.492^{a}	0.754^{a}	0.542^{a}	0.674^{a}	0.659^{a}	0.351^{bc}
Animal	Standard	2	1.236^{b}	0.457^{ab}	0.488^{c}	2.294^{bc}	0.689^{bc}	0.570^{b}	0.613^{bc}	0.614^{bc}	0.341^{de}
Animal	Low	2	1.238^{b}	0.454^{bc}	0.495^{bc}	2.300^{bc}	0.699^{bc}	0.502^{b}	0.625^{b}	0.604^{bc}	0.347^{cd}
Pooled SEM			0.010	0.005	0.004	0.014	0.006	0.005	0.006	0.006	0.001
P Value											
Protein			<0.0001	<0.0001	0.8963	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.1762
Level			<0.0001	0.0208	<0.0001	<0.0001	<0.0001	0.0052	0.0001	0.0095	<0.0001
Source			0.7539	0.4969	0.0252	0.4422	0.0002	0.1693	0.0866	0.3382	<0.0001
Protein * Level			0.3002	0.1643	0.1602	0.0002	<0.0001	0.099	<0.0001	0.5784	<0.0001
Level * Source			0.3921	0.0015	0.8399	0.0176	0.0124	0.7139	0.2962	0.7322	0.2101
Protein * Source			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Protein * Level * Source			<0.0001	<0.0001	0.0006	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0014

a-f Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=12

Table 24 Continued.

Treatment			Digestible Intake (g/bird)								
			Ile	Leu	Tyr	Phe	Lys	His	Arg	Ca	P
Protein	Level	Source									
Veggie	Standard	1	0.513^{d}	0.999^{c}	0.396^{e}	0.595^{c}	0.769^{e}	0.312^{cd}	0.833^{e}	0.258^{a}	0.316^{a}
Veggie	Low	1	0.500^{d}	0.982^{c}	0.379^{f}	0.587^{c}	0.798^{d}	0.304^{d}	0.822^{e}	0.199^{abc}	0.265^{bcd}
Veggie	Standard	2	0.506^{d}	1.047^{b}	0.406^{de}	0.606^{c}	0.757^{e}	0.318^{c}	0.858^{d}	0.135^{c}	0.222^{e}
Veggie	Low	2	0.542^{c}	1.073^{b}	0.430^{b}	0.648^{b}	0.834^{abc}	0.335^{b}	0.893^{bc}	0.222^{ab}	0.316^{a}
Animal	Standard	1	0.567^{b}	1.067^{b}	0.412^{cde}	0.642^{b}	0.845^{ab}	0.339^{b}	0.906^{bc}	0.169^{bc}	0.289^{ab}
Animal	Low	1	0.602^{a}	1.166^{a}	0.453^{a}	0.686^{a}	0.850^{a}	0.364^{a}	0.959^{a}	0.171^{bc}	0.249^{cde}
Animal	Standard	2	0.562^{bc}	1.078^{b}	0.414^{bcd}	0.638^{b}	0.820^{bcd}	0.338^{b}	0.887^{c}	0.214^{ab}	0.244^{de}
Animal	Low	2	0.559^{bc}	1.084^{b}	0.428^{bc}	0.644^{b}	0.816^{cd}	0.336^{b}	0.910^{b}	0.248^{a}	0.279^{bc}
Pooled SEM			0.005	0.008	0.004	0.005	0.006	0.002	0.005	0.015	0.007
P Value											
Protein			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.8026	0.0071
Level			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.1577	0.0771
Source			0.3418	0.0055	0.0013	0.0609	0.0366	0.1969	0.0386	0.6240	0.0075
Protein * Level			0.5227	0.0001	<0.0001	0.2405	<0.0001	0.0315	0.0004	0.8553	0.0269
Level * Source			0.4869	0.0498	0.2024	0.4104	0.0263	0.9573	0.2861	0.0001	<0.0001
Protein * Source			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.1692
Protein * Level * Source			<0.0001	<0.0001	<0.0001	<0.0001	0.0008	<0.0001	<0.0001	0.0107	0.0016

a-f Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=12

Table 25. Evaluation of limestone source, particle size, and dietary calcium level on intestinal pH of broilers during naturally occurring necrotic enteritis.

Treatments			Intestinal pH							
			Day 15				Day 23			
			Gizzard	Duodenum	Jejunem	Ileum	Gizzard	Duodenum	Jejunem	Ileum
Protein	Level	Source								
Veggie	Standard	1	2.255	6.108	5.889	5.833	2.258	6.114	5.847	5.759
Veggie	Low	1	2.083	5.990	5.781	5.834	2.132	6.024	5.824	5.66
Veggie	Standard	2	2.178	6.073	5.821	5.831	2.414	6.115	5.818	5.51
Veggie	Low	2	2.178	6.079	5.877	5.731	2.435	6.157	5.853	5.681
Animal	Standard	1	2.205	6.090	5.862	5.649	2.465	6.181	5.840	5.718
Animal	Low	1	2.209	6.098	5.831	5.598	2.332	6.176	5.764	5.690
Animal	Standard	2	2.343	6.104	5.852	5.717	2.247	6.203	5.881	5.808
Animal	Low	2	2.141	6.094	5.851	5.665	2.244	6.083	5.814	5.670
Pooled SEM			0.083	0.042	0.039	0.079	0.084	0.040	0.031	0.065
P Value			0.5846	0.5694	0.6587	0.2499	0.0889	0.06	0.3708	0.111

n=12

Table 26 Evaluation of limestone source, particle size, and dietary calcium level on mineral insolubility in gizzard of broilers during naturally occurring necrotic enteritis at d 15

Treatments			Gizzard D 15 Insoluble (ppm)							
			Aluminum	Calcium	Iron	Mn	Sodium	Phosphorus	Sulfur	Zinc
Protein	Level	Source								
Veggie	Standard	1	11.943[a]	543.750	51.042	2.669	79.708	263.333	362.167	7.867
Veggie	Low	1	11.233[ab]	414.750	46.483	3.913	83.650	263.583	376.333	9.016
Veggie	Standard	2	13.322[a]	496.167	58.233	3.872	77.042	274.500	367.750	8.767
Veggie	Low	2	8.037[cde]	334.583	40.650	1.846	75.133	245.583	342.000	6.275
Animal	Standard	1	8.881[bcd]	509.417	33.650	3.833	78.842	294.333	387.500	11.128
Animal	Low	1	6.183[e]	457.083	20.875	2.390	77.508	260.917	398.250	8.809
Animal	Standard	2	9.241[bc]	514.250	35.817	2.971	79.733	290.917	413.750	8.852
Animal	Low	2	6.452[de]	412.667	24.267	2.116	77.600	263.750	371.833	7.790
	Pooled SEM		0.553	24.210	2.930	0.564	2.839	10.472	15.953	1.238
Protein	Level									
Veggie	Standard		12.632	519.958[a]	54.638	3.244	78.375	268.917	364.958	8.317
Veggie	Low		9.635	374.667[b]	43.567	3.086	79.392	254.583	359.167	7.646
Animal	Standard		9.061	511.833[a]	34.733	3.341	79.288	292.625	400.625	9.990
Animal	Low		6.317	434.875[b]	22.871	2.244	77.554	262.333	390.042	8.300
	Pooled SEM		0.421	17.908	2.121	0.426	2.055	7.384	11.448	0.962
Protein		Source								
Veggie		1	11.588	479.250	48.763	3.291	81.679	263.458	369.250	8.441
Veggie		2	10.679	415.375	49.442	3.079	76.088	260.042	354.875	7.521
Animal		1	7.532	483.250	27.263	3.056	78.175	277.625	382.875	9.969
Animal		2	7.846	463.458	30.042	2.544	78.667	277.333	397.792	8.321
	Pooled SEM		0.505	20.940	2.449	0.443	2.007	7.810	11.466	0.960

Table 26 Continued

Treatments		Gizzard D 15 Insoluble (ppm)							
		Aluminum	Calcium	Iron	Mn	Sodium	Phosphorus	Sulfur	Zinc
Level	Source								
Standard	1	10.412[a]	526.583	42.346	3.057	79.275	278.833	374.833	9.498
Standard	2	11.281[a]	505.208	47.025	3.492	78.388	282.708	390.750	8.809
Low	1	8.708[b]	435.917	33.679	3.352	80.579	262.250	387.217	8.913
Low	2	7.244[b]	373.625	32.458	1.981	76.368	254.667	361.917	7.033
Pooled SEM		0.533	17.782	3.110	0.435	2.032	7.561	11.844	0.962
P Value									
Protein		<0.0001	0.1423	<0.0001	0.3684	0.8225	0.0388	0.0050	0.3177
Level		<0.0001	<0.0001	<0.0001	0.2171	0.8620	0.0038	0.4802	0.3107
Source		0.4547	0.0196	0.4567	0.3550	0.2180	0.8052	0.6831	0.2705
Protein * Level		0.7496	0.0553	0.8140	0.2976	0.5052	0.2901	0.8361	0.6608
Protein * Source		0.1257	0.2135	0.6510	0.7616	0.1424	0.8354	0.4059	0.7540
Level * Source		0.0041	0.2480	0.2055	0.0547	0.4207	0.4468	0.0772	0.6082
Protein * Level * Source		0.0057	0.8133	0.1271	0.0331	0.5406	0.2408	0.9527	0.2932

a-e Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=12

Table 27 Evaluation of limestone source, particle size, and dietary calcium level on mineral solubility in gizzard of broilers during naturally occurring necrotic enteritis at d 15

Treatments			Gizzard D 15 Soluble (ppm)							
			Aluminum	Calcium	Iron	Mn	Sodium	Phosphorus	Sulfur	Zinc
Protein	Level	Source								
Veggie	Standard		3.576	160.700[a]	5.551	3.207	91.222	125.265	65.117	0.613
Veggie	Low		3.238	95.092[b]	5.753	3.025	84.425	108.642	51.404	0.730
Animal	Standard		3.086	144.908[a]	5.567	2.848	86.504	123.833	58.958	1.006
Animal	Low		3.005	105.921[b]	4.826	3.011	84.442	115.963	53.459	0.455
	Pooled SEM		0.095	6.487	0.363	0.089	3.880	4.977	3.175	0.192
Protein		Source								
Veggie		1	3.503	137.670	5.486	3.238[a]	91.952	116.522	60.304	0.960
Veggie		2	3.308	117.163	5.815	2.995[ab]	83.725	117.021	56.017	0.469
Animal		1	3.027	118.675	5.403	2.847[b]	82.121	112.788	52.405	0.531
Animal		2	3.064	132.154	4.990	3.012[ab]	88.825	127.008	60.013	0.786
	Pooled SEM		0.098	8.252	0.365	0.088	3.776	5.014	3.247	0.192
	P Value									
	Protein		0.0004	0.7786	0.2921	0.0405	0.5422	0.5321	0.5241	0.9254
	Level		0.0362	<0.0001	0.5298	0.8999	0.2576	0.0162	0.0036	0.5345
	Source		0.4152	0.5398	0.9139	0.6576	0.8382	0.1458	0.6284	0.7940
	Protein * Level		0.1878	0.0376	0.2827	0.0573	0.5394	0.3902	0.2041	0.1545
	Protein * Source		0.2235	0.0063	0.3993	0.0255	0.0600	0.1606	0.0628	0.0555
	Level * Source		0.7616	0.2349	0.0773	0.9952	0.6702	0.2069	0.2813	0.0220
	Protein * Level * Source		0.2029	0.3513	0.7419	0.3067	0.7977	0.5962	0.7748	0.5900

a-b Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=12

Table 28 Evaluation of limestone source, particle size, and dietary Ca level on mineral insolubility in duodenum of broilers during naturally occurring necrotic enteritis at d 15

Treatments			Duodenum D 15 Insoluble (ppm)							
			Aluminum	Calcium	Iron	Mn	Sodium	Phosphorus	Sulfur	Zinc
Protein	Level	Source								
	Standard	1	17.413	1681.460	402.479	19.979	773.000	981.833	840.208[b]	35.733
	Standard	2	17.796	1473.540	340.546	21.096	765.042	1097.330	964.208[a]	32.246
	Low	1	17.007	1061.500	89.582	19.542	783.667	1083.460	905.708[ab]	33.283
	Low	2	11.534	793.913	1426.940	22.870	731.870	1045.170	897.261[ab]	33.561
	Pooled SEM		2.488	108.419	471.166	2.607	20.875	55.972	29.688	2.107
Protein										
Veggie			20.240[a]	1297.710	753.369	23.869[a]	770.479	1064.230	906.104	35.675
Animal			11.637[b]	1216.300	354.057	17.768[b]	756.83	1039.550	897.596	31.698
	Pooled SEM		1.871	92.331	414.619	1.878	14.969	40.299	21.703	1.461
	Level									
	Standard		17.604	1577.500[a]	371.513	20.538	769.021	1039.580	902.208	33.990
	Low		14.329	930.553[b]	744.038	21.170	758.319	1064.720	901.575	33.419
	Pooled SEM		2.103	79.287	419.113	1.983	15.073	39.948	21.719	1.486
		Source								
		1	17.210	1371.480[a]	246.035	19.760	778.333	1032.650	872.958	34.508
		2	14.732	1140.960[b]	872.185	21.964	748.809	1071.810	931.447	32.889
	Pooled SEM		2.081	90.864	403.776	1.972	14.917	40.078	21.327	1.485

Table 28 Continued

Treatments	Duodenum D 15 Insoluble (ppm)							
	Aluminum	Calcium	Iron	Mn	Sodium	Phosphorus	Sulfur	Zinc
P Value								
Protein	0.0105	0.4301	0.5427	0.0439	0.5095	0.6583	0.7739	0.0635
Level	0.3111	<0.0001	0.5906	0.8583	0.5999	0.6793	0.9757	0.7941
Source	0.4375	0.0372	0.3610	0.4872	0.1670	0.5133	0.0602	0.4495
Protein * Level	0.6976	0.1699	0.1647	0.3328	0.8716	0.4764	0.8851	0.1858
Protein * Source	0.3736	0.8322	0.3064	0.4503	0.3401	0.4187	0.5839	0.7197
Level * Source	0.3726	0.8021	0.3145	0.7459	0.3091	0.1789	0.0307	0.3633
Protein * Level * Source	0.2396	0.2726	0.3646	0.7887	0.4815	0.2636	0.4729	0.2534

a-b Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=12

Table 29 Evaluation of limestone source, particle size, and dietary calcium level on mineral solubility in duodenum of broilers during naturally occurring necrotic enteritis at d 15

Treatments			Duodenum D 15 Soluble							
			Aluminum	Calcium	Iron	Mn	Sodium	Phosphorus	Sulfur	Zinc
Protein	Level	Source								
Veggie	Standard	1	5.301	507.000[a]	25.025	10.343	755.000	496.750	635.167	7.847
Veggie	Low	1	6.484	403.833[bc]	35.858	12.261	750.750	570.250	649.000	10.578
Veggie	Standard	2	5.041	520.583[a]	22.900	9.447	721.083	469.083	618.833	7.253
Veggie	Low	2	11.769	299.417[d]	40.600	11.745	751.917	526.083	652.833	9.513
Animal	Standard	1	4.840	513.833[a]	21.492	10.134	750.667	497.833	617.333	8.698
Animal	Low	1	3.608	316.636[cd]	29.755	11.432	751.734	586.181	664.272	10.532
Animal	Standard	2	6.762	483.583[ab]	28.400	9.913	758.917	593.500	706.417	9.371
Animal	Low	2	3.247	362.900[cd]	22.920	11.339	708.600	536.900	641.000	10.215
Pooled SEM			1.424	22.727	3.553	0.466	19.453	53.305	35.682	0.616
P Value										
Protein			0.1781	0.4177	0.1027	0.4991	0.8687	0.3279	0.4868	0.0450
Level			0.6727	<0.0001	0.0198	<0.0001	0.6791	0.2973	0.7800	<0.0001
Source			0.3803	0.2613	0.8497	0.2340	0.2288	0.8697	0.6123	0.4668
Protein * Level			0.0937	0.9225	0.0509	0.3030	0.1733	0.5250	0.5286	0.1975
Protein * Source			0.6439	0.1100	0.8347	0.4476	0.9747	0.4469	0.4569	0.2611
Level * Source			0.6633	0.5323	0.6118	0.7247	0.7748	0.2997	0.3815	0.4140
Protein * Level * Source			0.2975	0.0042	0.1231	0.8611	0.1245	0.4088	0.2095	0.7716

a-d Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=12

Table 30 Evaluation of limestone source, particle size, and dietary calcium level on mineral insolubility in ileum of broilers during naturally occurring necrotic enteritis at d 15

Treatments			Ileum D 15 Insoluble (ppm)							
			Aluminum	Calcium	Iron	Mn	Sodium	Phosphorus	Sulfur	Zinc
Protein	Level	Source								
Veggie	Standard	1	50.242^{b}	2443.170^{ab}	338.417	55.417^{ab}	737.833^{bcd}	991.083^{b}	604.000	55.017^{abc}
Veggie	Low	1	36.900^{c}	1820.830^{c}	239.000	58.617^{a}	779.417^{abc}	908.167^{bc}	599.333	59.575^{ab}
Veggie	Standard	2	65.683^{a}	2812.170^{b}	319.750	63.283^{a}	651.917^{d}	1284.420^{a}	633.417	61.458^{a}
Veggie	Low	2	27.308^{c}	1260.750^{d}	157.167	45.992^{c}	863.917^{a}	652.000^{d}	639.750	46.500^{d}
Animal	Standard	1	32.867^{c}	2603.670^{ab}	191.750	49.658^{bc}	694.750^{cd}	1070.500^{b}	680.833	52.383^{bcd}
Animal	Low	1	15.092^{d}	1460.500^{cd}	104.042	44.750^{c}	804.000^{ab}	616.000^{d}	665.417	50.167^{cd}
Animal	Standard	2	32.617^{c}	2303.250^{b}	177.250	45.108^{c}	694.917^{cd}	933.083^{b}	669.583	47.200^{cd}
Animal	Low	2	16.142^{d}	1673.580^{cd}	132.867	49.533^{bc}	725.167^{bcd}	723.000^{cd}	711.417	54.700^{abc}
Pooled SEM			1.795	91.286	24.572	1.736	23.729	44.243	21.009	1.751
P Value										
Protein			<0.0001	0.2995	0.0001	<0.0001	0.0994	0.0004	<0.0001	0.0007
Level			<0.0001	<0.0001	0.0007	0.0056	<0.0001	<0.0001	0.6486	0.3227
Source			0.2875	0.3289	0.4457	0.3799	0.2462	0.9601	0.0921	0.1604
Protein * Level			0.0061	0.1609	0.2513	0.0094	0.0999	0.7069	0.6879	0.0030
Protein * Source			0.4186	0.7153	0.3102	0.3330	0.2632	0.6166	0.5692	0.2480
Level * Source			0.0002	0.1463	0.8604	0.0322	0.1861	0.0258	0.2694	0.0601
Protein * Level * Source			<0.0001	<0.0001	0.3464	<0.0001	0.0005	<0.0001	0.4533	<0.0001

a-d Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=12

Table 31 Evaluation of limestone source, particle size, and dietary calcium level on mineral solubility in ileum of broilers during naturally occurring necrotic enteritis at d 15

Treatments			Ileum D 15 Soluble (ppm)							
			Aluminum	Calcium	Iron	Mn	Sodium	Phosphorus	Sulfur	Zinc
Protein	Level	Source								
Veggie	Standard	1	12.581	1333.330^{ab}	39.100	31.517^{ab}	894.500	553.333^{ab}	344.583	25.825^{ab}
Veggie	Low	1	11.193	1034.250^{cd}	44.783	34.983^{a}	976.167	533.167^{ab}	336.250	34.092^{a}
Veggie	Standard	2	13.383	1574.750^{a}	38.642	34.308^{a}	866.083	681.083^{a}	351.833	29.392^{ab}
Veggie	Low	2	9.491	469.417^{f}	32.100	25.308^{c}	956.417	354.000^{c}	350.000	20.575^{b}
Animal	Standard	1	9.536	1259.500^{bc}	38.325	25.508^{c}	895.917	568.750^{a}	353.750	25.463^{ab}
Animal	Low	1	4.923	590.250^{ef}	28.283	26.325^{bc}	953.583	358.417^{c}	364.250	25.217^{ab}
Animal	Standard	2	10.364	1333.330^{ab}	37.117	26.667^{bc}	921.167	549.167^{ab}	375.917	25.525^{ab}
Animal	Low	2	4.744	791.830^{de}	31.825	27.842^{bc}	892.500	407.083^{bc}	373.833	28.483^{ab}
	Pooled SEM		0.501	63.652	3.142	1.256	23.964	35.685	11.153	2.020
	P Value									
	Protein		<0.0001	0.0230	0.0440	<0.0001	0.6617	0.0230	0.0101	0.3678
	Level		<0.0001	<0.0001	0.0862	0.3320	0.0042	<0.0001	0.9570	0.7073
	Source		0.7277	0.7998	0.2499	0.2496	0.2223	0.8287	0.1064	0.2517
	Protein * Level		0.0012	0.3076	0.1245	0.0412	0.0393	0.9601	0.5670	0.5712
	Protein * Source		0.4290	0.0021	0.1008	0.0100	0.8572	0.4362	0.7404	0.0230
	Level * Source		0.0441	0.0005	0.4253	0.0013	0.2588	0.0227	0.8512	0.0176
	Protein * Level * Source		0.2560	<0.0001	0.0723	0.0007	0.1680	0.0005	0.5566	0.0007

a-f Means within a column lacking a common superscript are significantly different from each other (P<0.05).
n=12

4.4. Discussion

In Experiment 2, mortality due to NE was not as prevalent as in previous trials, but that could be attributable to complications with early chick quality and high (~8%) 4 day mortality, which were indicative of an *E. coli* infection. As indicated in Experiment 1, the role of dietary calcium in predisposing or contributing to NE is more complex than originally hypothesized. Not only was there an interaction of calcium dietary level and limestone source, as in Experiment 1, but the results were also dependent on if the diet was veggie or animal protein based. Results suggested that the higher level of dietary calcium in diets with animal protein was more contributory to mortality resulting from NE. The possibility of animal protein as a risk factor agrees with other published research showing increased incidence of NE mortality (Williams, 2005; Zanu, et al., 2020). The mortality could possibly be due to increased nutrient availability in the lower intestine. With the current research, increased mortality was observed in birds fed animal protein with standard dietary calcium levels of limestone source 2. Additionally, analysis of soluble nutrients in the ileal indicated soluble calcium was increased in birds on this diet. The increased levels of soluble calcium in the ileum, as well as soluble zinc, could be from the influx of ions from the pore forming toxins of *C. perfringens*. A large percentage of the toxins reported to be similar to the NetB toxin are part of the pore-forming group, which form pores in the phospholipid bilayer of cells, causing an influx of ions (i.e, Na^+, Cl^-, Ca^{2+}) (Keyburn et al., 2010). This research aligns with previous work that has indicated that calcium possibly plays a role in the pathogenesis of NE (Keyburn et al., 2008; Paiva et al., 2014; Paiva et al., 2013; Zanu et al., 2020d).

There are multiple hypotheses for the mode of action of risk factors contributing to natural pathogenesis of NE. One possible scenario is an increase in the intestinal pH due to the

difference in dietary calcium levels and limestone solubility (Selle et al., 2009). Another possibility for the increase in NE-associated mortality resulting from higher dietary calcium levels would be a connection between dietary calcium level and pathogenesis of *C. perfringens* from increased bacterial activation and toxin production. In this experiment, there was not a change in pH, but there was an increase in soluble calcium in the lower GI tract, which increases nutrients in the lower intestine for activation of *C. perfringens*. There has been recent discoveries that intestinal calcium and bile salts facilitate germination of *Clostridium difficile* spores (Kochan et al., 2017). Therefore, the possibility that increased soluble calcium in the ileum could allow for germination of C. perfringens which would allow for pore forming in the intestinal membrane. In previous work in the lab, there was an increase in pH in the ileum that allowed for an environment favorable for the proliferation of *C. perfringens* (Eagleson et al., In progress). With the increase in NE-related mortality in both experiments, soluble calcium in the ileum and ileal pH could have impacted the incidence of NE. Previous work provides evidence that increased pH and changes in calcium digestibility play a role in pathogenesis of NE (Paiva et al., 2014). Lastly, a suggested mode of action for the pathogenesis of NE is that animal proteins are favorable substrates for clostridial growth, and high concentrations in the diet are often associated with NE. There has been reported to be a positive association with crude protein derived from fish meal and ileal and cecal counts of *C. perfringens* (Drew et al., 2004). Wilkie et al. (2005) further evaluated other protein sources and the impact they have on the intestinal *Clostridium* population. Animal-source protein, along with potato meal support increased *C. perfringens* colonization in the intestine. Alternatively, plant-based protein sources contain constituents that inhibit *C. perfringens* colonization (Wilkie et al., 2005). The current research

provides further evidence that animal protein can contribute to an increase in NE-related mortality, as birds fed animal protein had increased mortality throughout the duration of the trial.

The data from this trial also indicated differences in digestible amino acid intake in the lower intestine. In considering the effect of protein source, there was an increase in digestible amino acid intake with birds fed animal protein. In the current trial, increased digestible intake of methionine was observed in birds fed all-veggie diets with standard dietary calcium levels regardless of source. Drew et al. (2004) reported an association of methionine with high numbers of intestinal *C. perfringens*. The methionine present in the distal sections of the small intestine may influence *C. perfringens* populations. Another explanation could be that the change in the solubility of calcium alters intestinal health or environment and more nutrients are available for bacterial overgrowth. Animal protein should have a highly soluble calcium source from the bone that could easily be passing through gizzard and small intestine to be become available for activation of *C. perfringens*. This could be seen in previous work in our lab with the fine particle size or the more soluble calcium source increasing mortality (Eagleson et al., In progress). In the current study, the all-veggie diets increased soluble calcium in the ileum compared to the animal protein diets which is not what was hypothesized that the more soluble calcium from the animal protein increasing calcium content in the lower intestine. The availability of nutrients in the lower intestine for *C. perfringens* could allow for the uptake and proliferation of the bacteria, which would hinder the performance of the birds. With previous work with *C. difficile*, it has been determined that amino acid concentrations were inadequate to support high levels of germination without adequate intestinal calcium content (Kochan et al., 2017). Therefore, with increased soluble calcium in the lower intestine, this could possibly be all that *C. perfringens*

needs to germinate and form pores in the intestinal lining. Leading to the inability of the broiler to absorb nutrients hindering performance or increasing mortality.

During the early growth period, all veggie diets with low calcium levels of limestone source 1 resulted in reduced BW. This contradicts results from other researchers that reported reduction of BWG in birds with the addition of MBM (Zanu et al., 2020). The reduction in performance in early growth with the veggie diets is similar to results reported by Drew et al. (2004). Interestingly, the performance of broilers was impacted by potential differences in source of limestone. Similar results were observed with birds fed animal-based protein having reduced levels of limestone, which would be similar to the current research with different calcium sources altering the body weight (Drew et al., 2004). Amino acid digestible intake in this trial was similar to results in work done previously in our lab with starter period results being affected predominantly by dietary calcium levels and post NE results indicating three-way interactions of protein type, limestone source and dietary calcium level (Eagleson et al., In progress).

In conclusion, these results indicate that not only does dietary calcium level in general, but more specifically even limestone geographic source, have an influence on development of NE and broiler performance. This trial further indicated that the calcium influence on NE and performance may also be influenced by the diet-based protein type, veggie vs animal protein. This experiment suggests the risk for NE development and resulting mortality may be exacerbated with an increase in the use of dietary animal protein. The most substantial effects on performance were in the early phases of the grow out, which could further impair intestinal health and result in risk or predisposition for NE development. Again, the results necessitate further research to understand the contribution of different limestone sources, which influence the solubility characteristics of the calcium in the intestinal tract. These results have direct

application to the poultry industry for consideration of not only level of dietary calcium in formulation, but calcium sources with regard to the characteristics of the limestone and the impact on intestinal health, potential risk for NE, and broiler performance dependent on the protein type of diet.

5. CONCLUSIONS

Poultry is a low-cost protein source, making the industry constantly on the lookout for ways to cut cost. Several methodologies are currently implemented by nutritionist to reduce the cost of the feed, which amounts to approximately 70% of the cost of production. Some methods implemented range from enzyme use to synthetic amino acids. However, the easiest way to reduce the production cost is to reduce the incidences of enteric diseases, such as necrotic enteritis (NE). The methods utilized in the current experiments could be implemented by the poultry industry to reduce the incidence of NE and reduce the cost of production due to disease.

When evaluating the roles calcium dietary level, limestone particle size, and limestone geographic source have on the pathogenesis of NE, birds fed standard dietary calcium levels of the fine particle size (200 microns) had increased NE-related mortality compared to the low levels of the fine particle size during the grower phase. When grower mortality was further investigated to determine if the mortality was similar to typical industry occurring NE mortality curve, the mortality occurrence was similar with the differences occurring from d 14-21. Then, from d 21-28 the mortality declined. Decreased broiler performance, as is commonly observed with NE, was observed with reduced body weight (BW), body weight gain (BWG), and increased feed conversion ratio (FCR). At d 14, coarse particle size (120 microns) of limestone source 1 reduced BW of birds by approximately 10 grams. At d 28, standard levels of limestone source 1 reduced BW by over 100 grams. At d 35, all three dietary factors impacted BW, and birds continued to increase in the spread of BW. Birds fed industry (standard) calcium levels of limestone source 1 in a coarse particle size had reduced BW of approximately 300 grams, which would be a loss of approximately 210 grams of consumable meat or major losses in profit if birds

were consistently coming in with reduced body weights. Similar trends were observed in BWG and FCR with early in the grow-out limestone particle size and source having an impact, but as the bird aged then all three factors impacted the performance of the birds. From the overall performance data, it could be suggested that diets with the fine particle size at industry dietary calcium levels had the most impact on the pathogenesis of NE. Overall, a reduction in the dietary calcium level could reduce the detrimental impact associated with NE due to the reduced BW, BWG, increased FCR, and increased mortality.

Commonly, NE is associated with coccidiosis damage to the intestinal tract, but with these trials there were no differences observed in *Eimeria* counts and macroscopic lesions. Additionally, there were no differences observed in the percent bone ash with varying levels of dietary calcium, which suggests the poultry industry could slightly reduce the dietary calcium levels with no impact on leg health. Necrotic enteritis can develop with a mild coccidiosis reaction and not impact the skeletal structure of the bird, but it can still cause performance issues with the intestinal damage that occurs. The intestinal damage can be observed in the differences in nutrient intake of the bird, but the difference can be accounted for by the lysis of the intestinal epithelial cells by *C. perfringens*. The change in pH in the lower intestine due to the different calcium levels, as well as particle size, could have allowed for an ideal pH for the *C. perfringens* to proliferate in the lower intestine and affect the absorption of the nutrients in the ileum. At d 14, the nutrient intake for most of the amino acids analyzed other than methionine, threonine, aspartic acid, valine, and tyrosine all had reduced intake when fed industry levels of dietary calcium at the fine particle size. For the other amino acids mentioned, other than methionine, there was reduced intake when birds were fed industry levels of fine particle size of limestone

source 1. As for methionine, birds fed industry levels of coarse particle size of source 1 reduced the intake. At d 25, after the NE outbreak, for all amino acids analyzed all dietary factors contributed to the intake of amino acids. Amino acids, other than methionine and leucine, birds fed industry levels of source 1 in the fine particle size had reduced intake. For methionine and leucine, birds fed industry levels of source 1 in the coarse particle size had reduced intake of the amino acids. As for calcium, there was no difference in the intake. Overall, observing the health of the birds, the birds that had the worst performance were not the ones that consumed the reduced amount of amino acids other than methionine. Therefore, dietary calcium level, limestone particle size and limestone source demonstrated to impact the intestinal environment and the absorption of amino acids during the natural occurrence of NE.

In Experiment 1 results for bird performance, those fed industry (Standard) levels of source 1 with the coarse limestone particle size had the most impact. However, NE-related mortality occurred more in groups fed the industry (Standard) levels of the fine limestone particle size. Therefore, for the second experiment, the coarse limestone was used as it is more similar to the particle size commonly used in the poultry industry. Due to the increased mortality at placement because of compromised chick quality, mortality up to d 4 (8%) was removed from statistical analysis as it was not treatment related. During the starter phase, increased mortality was observed with birds fed source 2 at both dietary calcium levels with animal protein in the diets. Protein type, calcium level, and limestone source had effects on BW and BWG up to d 15; then until d 23 protein source and limestone source resulted in significant difference, but there were no differences observed past d 23. At d 15, birds fed low dietary calcium levels of limestone source 1 in an all-veggie diet had reduced BW and BWG. At d 23, differences were

observed when birds were fed an all-veggie diet with limestone source 1 with resulting reduced BW and BWG. For FCR, birds were only impacted during the starter phase with birds fed low dietary calcium levels in an all-veggie diet having less efficient feed conversion. There was no difference observed in mortality after the starter phase, and that could possibly be due to the lower intestine not being an ideal pH for *C. perfringens* as the ileum had no difference in the pH and was below a 6. Though there was no difference observed in the pH and mortality occurring early in the grow-out, dietary calcium level, limestone source, and protein source had an impact on the absorption of nutrients in the ileum. Similar to Experiment 1 at d 14, all amino acids expect for methionine, threonine, lysine, and phosphorus (P) were impacted by protein level and dietary calcium level at d 15. For all amino acids except methionine, threonine and lysine, as well as P, birds fed low dietary calcium in all-veggie diets had reduced intake of all nutrients, which correlates to birds that had reduced BW. For methionine and threonine, birds fed low levels of source 1 limestone in the all veggie diets had reduced intake of the two amino acids. At d 23, all factors impacted the intake of nutrients and there was no clear treatment that impacted intake one way or another. Therefore, results from Experiment 2, suggests that animal protein did not have a major impact on NE pathogenesis like previously reported, but that could be due to the reduced inclusion level of the animal protein to be able to keep some inclusion of the limestone. If the animal protein was increased there could have possibly been a different outcome. Overall, there is still evidence that birds fed diets with reduced calcium levels can perform similarly to those on diets with standard dietary calcium levels with reduced dietary calcium levels.

In conclusion, from this dissertation, there is evidence that dietary calcium levels, limestone source, and limestone particle size have an impact on the pathogenesis of NE, but further research needs to be done to determine if there are specific limestones that contribute as a risk factor to the pathogenesis of NE. There also needs to be work to determine if the differences in solubility change the availability of the calcium. There is evidence the dietary calcium level can be reduced without a negative impact on broiler performance and that reducing dietary calcium can possibly reduce NE-related mortality, which would increase poultry producer's profits. The main conclusion would be that limestone needs to be looked at closer as an ingredient, as well as the particle size impacts performance of the birds.

REFERENCES

Akbari Moghaddam Kakhki, R., T. Heuthorst, A. Mills, M. Neijat, and E. Kiarie. 2019. Interactive effects of calcium and top-dressed 25-hydroxy vitamin D3 on egg production, egg shell quality, and bones attributes in aged Lohmann LSL-lite layers. Poultry Science 98:1254-1262. doi 10.3382/ps/pey446

Al-Baadani, H., A. Abudabos, S. Al-Mufarrej, and M. Alzawqari. 2016. Effects of dietary inclusion of probiotics, prebiotics and synbiotics on intestinal histological changes in challenged broiler chickens. South African Journal of Animal Science 46:157. doi 10.4314/sajas.v46i2.6

Al-Sheikhly, F., and A. Al-Saieg. 1980. Role of Coccidia in the Occurrence of Necrotic Enteritis of Chickens. Avian Diseases 24:324. doi 10.2307/1589700

Amerah, A. M., P. W. Plumstead, L. P. Barnard, and A. Kumar. 2014. Effect of calcium level and phytase addition on ileal phytate degredation and amino acid digestibility of broilers fed corn-based diets. Poultry Science 93:906-915.

Atteh, J. O., and S. Leeson. 1985. Influence of Age, Dietary Cholic Acid, and Calcium Levels on Performance, Utilization of Free Fatty Acids, and Bone Mineralization in Broilers. Poultry Science 64:1959-1971. doi 10.3382/ps.0641959

Brennan, J., R. Bagg, D. Barnum, J. Wilson, and P. Dick. 2001. Efficacy of Narasin in the Prevention of Necrotic Enteritis in Broiler Chickens. Avian Diseases 45:210. doi 10.2307/1593030

Burkholder, K. M., K. L. Thompson, M. E. Einstein, T. J. Applegate, and J. A. Patterson. 2008. Influence of Stressors on Normal Intestinal Microbiota, Intestinal Morphology, and Susceptibility to Salmonella Enteritidis Colonization in Broilers. Poultry Science 87:1734-1741. doi 10.3382/ps.2008-00107

Callaway, T. R., T. S. Edrington, R. C. Anderson, R. B. Harvey, K. J. Genovese, C. N. Kennedy, D. W. Venn, and D. J. Nisbet. 2008. Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. Animal Health Research Reviews 9:217-225. doi 10.1017/s1466252308001540

Conway, D. P., and M. E. McKenzie. 2007. Poultry Coccidiosis Diagnostic and Testing Procedures. Blackwell Publishing.

Cooper, K. K., and J. G. Songer. 2009. Necrotic enteritis in chickens: A paradigm of enteric infection by Clostridium perfringens type A. Anaerobe 15:55-60. doi 10.1016/j.anaerobe.2009.01.006

Crouch, C. F., G. S. K. Withanage, V. De Haas, F. Etoré, and M. J. Francis. 2010. Safety and efficacy of a maternal vaccine for the passive protection of broiler chicks against necrotic enteritis. Avian Pathology 39:489-497. doi 10.1080/03079457.2010.517513

De La Rosa, C., D. E. Hogue, and M. L. Thonney. 1997. Vaccination schedules to raise antibody concentrations against epsilon-toxin of Clostridium perfringens in ewes and their triplet lambs. Journal of Animal Science 75:2328. doi 10.2527/1997.7592328x

Dharne, H. 2008. Maintaining Gut Integrity5m Editor, The Poultry Site.

Drew, M. D., N. A. Syed, B. G. Goldade, B. Laarveld, and A. G. Van Kessel. 2004. Effects of Dietary Protein Source and Level on Intestinal Populations of Clostridium perfringens in Broiler Chickens. Poultry Science 83:414-420. doi 10.1093/ps/83.3.414

Driver, J. P., G. M. Pesti, R. I. Bakalli, and H. M. J. Edwards. 2005. Effects of calcium and nonphytate phosphorus concentrations on phytase efficacy in broiler chicks. Poultry Science 84:1406-1417. doi 10.1093/ps/84

Eagleson, C., A. Bergeron, C. Williams, C. Wyatt, D. Klamforth, and A. McElroy. In progress. The role of dietary calcium level and limestone source and particle size in natural necrotic enteritis pathogenesis and bird performance.

Eagleson, C., T. Clark, B. Hill, B. Daniels, A. Eagleson, H. L. Goodwin, and S. Watkins. 2018. Impact of meat and bone meal nutritional variability on broiler performance. Journal of Applied Poultry Research 27:172-179. doi 10.3382/japr/pfx053

Food and Drug Administration. 2020. Veterinary Feed Directive. F. a. D. Administration ed.

Foreign Agriculture Service. 2020. Livestock and Poultry: World Markets and Trade. U. S. D. o. Agriculture ed. World Agricultural Outlook Board.

Fukata, T., Y. Hadate, E. Baba, T. Uemura, and A. Arakawa. 1988. Influence of Clostridium perfringens and its toxin in germ-free chickens. Research in Veternarian Science 44:68-70.

Hamdi, M., S. López-Vergé, E. G. Manzanilla, A. C. Barroeta, and J. F. Pérez. 2015. Effect of different levels of calcium and phosphorus and their interaction on the performance of young broilers. Poultry Science 94:2144-2151. doi 10.3382/ps/pev177

Hargis, B. M. 2014. Overview of Necrotic Enteritis in Poultry in Merck Manual-Veternarian ManualMerck, Merck Manual.

Helmboldt, C. F., and E. S. Bryant. 1971. The Pathology of Necrotic Enteritis in Domestic Fowl. Avian Diseases 15:775. doi 10.2307/1588866

Hofacre, C., D. Reynolds, G. Mathis, B. Lumpkins, N. Ollis, J. Smith, and V. Demey. 2019. Effect of a Competitive Exclusion Culture in a Necrotic Enteritis Challange Model in Broilers. Journal of Applied Poultry Science 28:350-355. doi 10.3382/japr

Immerseel, F. V., J. D. Buck, F. Pasmans, G. Huyghebaert, F. Haesebrouck, and R. Ducatelle. 2004. Clostridium perfringensin poultry: an emerging threat for animal and public health. Avian Pathology 33:537-549. doi 10.1080/03079450400013162

Johnson, J., and W. M. Reid. 1970. Anticoccidial drugs: Lesion scoring techniques in battery and floor-pen experiments with chickens. Experimental Parasitology 28:30-36. doi 10.1016/0014-4894(70)90063-9

Juneja, V., J. Novak, and R. Labbe. 2010. Clostridium perfringens. Pages 53-70 in Pathogens and Toxins in Foods: Challanges and Interventions. V. K. Juneja, and J. N. Sofos eds. ASM Press.

Kaldhusdal, M., and E. Skjerve. 1996. Association between cereal contents in the diet and incidence of necrotic enteritis in broiler chickens in Norway. 28:1-16. doi 10.1016/0167-5877(96)01021-5

Keyburn, A. L., T. L. Bannam, R. J. Moore, and J. I. Rood. 2010. NetB, a Pore-Forming Toxin from Necrotic Enteritis Strains of Clostridium perfringens. Toxins 2:1913-1927. doi 10.3390/toxins2071913

Keyburn, A. L., J. D. Boyce, P. Vaz, T. L. Bannam, M. E. Ford, D. Parker, A. Di Rubbo, J. I. Rood, and R. J. Moore. 2008. NetB, a New Toxin That Is Associated with Avian Necrotic Enteritis Caused by Clostridium perfringens. PLoS Pathogens 4:e26. doi 10.1371/journal.ppat.0040026

Keyburn, A. L., R. W. Portela, K. Sproat, M. E. Ford, T. L. Bannam, X. Yan, J. I. Rood, and R. J. Moore. 2013. Vaccination with recombinant NetB toxin partially protects broiler chickens from necrotic enteritis. Veterinary Research 44:54. doi 10.1186/1297-9716-44-54

Keyburn, A. L., S. A. Sheedy, M. E. Ford, M. M. Williamson, M. M. Awad, J. I. Rood, and R. J. Moore. 2006. Alpha-Toxin of Clostridium perfringens Is Not an Essential Virulence Factor in Necrotic Enteritis in Chickens. 74:6496-6500. doi 10.1128/iai.00806-06

Khalique, A., D. Zeng, M. Shoaib, H. Wang, X. Qing, D. S. Rajput, K. Pan, and X. Ni. 2020. Probiotics mitigating subclinical necrotic enteritis (SNE) as potential alternatives to antibiotics in poultry. AMB Express 10. doi 10.1186/s13568-020-00989-6

Kim, S. W., W. Li, R. Angel, and M. Proszkowiec-Weglarz. 2018. Effects of limestone particle size and dietary Ca concentration on apparent P and Ca digestibility in the presence or absence of phytase. Poultry Science 97:4306-4314. doi 10.3382/ps/pey304

Kochan, T. J., M. J. Somers, A. M. Kaiser, M. S. Shoshiev, A. K. Hagan, J. L. Hastie, N. P. Giordano, A. D. Smith, A. M. Schubert, P. E. Carlson, and P. C. Hanna. 2017. Intestinal calcium and bile salts facilitate germination of Clostridium difficile spores. PLOS Pathogens 13:e1006443. doi 10.1371/journal.ppat.1006443

Kozyreva, T. V., L. S. Eliseeva, and G. M. Khramova. 2009. Modulating Effects of Calcium on Immune Response of Homoiothermal Animal under Thermoneutral Conditions and during Deep Cooling. Bulletin of Experimental Biology and Medicine 148:360-362. doi 10.1007/s10517-010-0712-y

Kratzer, F. H., and P. N. Davis. 1959. The Feeding Value of Meat and Bone Meal Protein. Poultry Science 38:1389-1393. doi 10.3382/ps.0381389

Lee, K. W., H. S. Lillehoj, W. Jeong, H. Y. Jeoung, and D. J. An. 2011. Avian necrotic enteritis: Experimental models, host immunity, pathogenesis, risk factors, and vaccine development. Poultry Science 90:1381-1390. doi 10.3382/ps.2010-01319

Li, W., R. Angel, S.-W. Kim, K. Brady, S. Yu, and P. W. Plumstead. 2016. Impacts of dietary calcium, phytate, and nonphytate phosphorus concentrations in the presence or absence of phytase on inositol hexakisphosphate (IP6) degredation in different segments of broilers digestive tract. Poultry Science 95:581-589.

Liu, N., Y. J. Ru, A. J. Cowieson, F. D. Li, and X. C. Cheng. 2008. Effects fo Phytate and Phytase on the performance and Immune Function of Broilers Fed Nutritionally Marginal Diets. Poultry Science 87:1105-1111.

Long, J., and R. B. Truscott. 1976. Necrotic enteritis in broiler chickens. III. Reproduction of the disease. Canadian Journal of Comparative of Medicine 40:53-59.

Lovland, A., M. Kaldhusdal, K. Redhead, E. Skjerve, and A. Lillehaug. 2004. Maternal vaccination against subclinical necrotic enteritis in broilers. Avian Pathology 33:81-90. doi 10.1080/0379450310001636255

Maga, J. A. 1982. Phytate: its chemistry, occurrence, food interactions, nutritional significance, and methods of analysis. Journal of Agricultural and Food Chemistry 30:1-9. doi 10.1021/jf00109a001

Majeed, S., R. Qudsieh, F. W. Edens, and J. Brake. 2020. Limestone particle size, calcium and phosphorus levels, and phytase effects on live performance and nutrients digestibility of broilers. Poultry Science 99:1502-1514. doi 10.1016/j.psj.2019.11.009

Martin, T., and J. A. Smyth. 2008. Prevalence of netB among some clinical isolates of Clostridium perfringens from animals in the United States. Veterinary Microbiology 136:202-205.

McNaughton, J. L. 1981. Effect of Calcium Carbonate Particle Size on the Available Phosphorus Requirement of Broiler Chicks. Poultry Science 60:197-203. doi 10.3382/ps.0600197

McReynolds, J. L., J. A. Byrd, K. J. Genovese, T. L. Poole, S. E. Duke, M. B. Farnell, and D. J. Nisbet. 2007. Dietary Lactose and its Effect on the Disease Condition of Necrotic Enteritis. Poultry Science 86:1656-1661. doi 10.1093/ps/86.8.1656

Moore, R. J. 2016. Necrotic enteritis predisposing factors in broiler chickens. Avian Pathology 45:275-281. doi 10.1080/03079457.2016.1150587

National Research Council. 1994. Nutrient Requirements of Poultry: Ninth Revised Edition. The National Academic Press, Washington, DC.

Nelson, T. S. 1967. The Utilization of Phytate Phosphorus by Poultry- A Review. Poultry Science 46:862-871.

Paiva, D., C. Walk, and A. McElroy. 2014. Dietary calcium, phosphorus, and phytase effects on bird performance, intestinal morphology, mineral digestibility, and bone ash during a natural necrotic enteritis episode. 93:2752-2762. doi 10.3382/ps.2014-04148

Paiva, D. M., C. L. Walk, and A. P. McElroy. 2013. Influence of dietary calcium level, calcium source, and phytase on bird performance and mineral digestibility during a natural necrotic enteritis episode. Poult Sci 92:3125-3133. doi 10.3382/ps.2013-03298

Petit, L., M. Gibert, and M. R. Popoff. 1999. Clostridium perfringens: toxinotype and genotype. Trends in Microbiology 7:104-110. doi 10.1016/s0966-842x(98)01430-9

Pilvi, T. K., M. Storvik, M. Louhelainen, S. Merasto, R. Korpela, and E. M. Mervaala. 2008. Effect of Dietary Calcium and Dairy Proteins on the Adipose Tissue Gene Expression Profile in Diet-Induced Obesity. Journal of Nutrigenetics and Nutrigenomics 1:240-251. doi 10.1159/000151238

Prescott, J. F. 1979. The prevention of experimentally induced necrotic enteritis in chickens by avoparcin. American Association of Avian Pathologists 23:1072-1074.

Ravindran, V. 1995. Phytases in Poultry Nutrition-An Overview. Australian Poultry Science.

Reid, B. L., and C. W. Weber. 1976. Calcium Availability and Trace Mineral Composition of Feed Grade Calcium Supplements. Poultry Science 55:600-605. doi 10.3382/ps.0550600

Rood, J. I., V. Adams, J. Lacey, D. Lyras, B. A. McClane, S. B. Melville, R. J. Moore, M. R. Popoff, M. R. Sarker, J. G. Songer, F. A. Uzal, and F. Van Immerseel. 2018. Expansion of the Clostridium perfringens toxin-based typing scheme. Anaerobe 53:5-10. doi 10.1016/j.anaerobe.2018.04.011

Ross. 2018. Broiler Management Handbook. Aviagen ed.

Schoepe, H., C. Pache, A. Neubauer, H. Potschka, T. Schlapp, L. H. Wieler, and G. Baljer. 2001. Naturally Occurring Clostridium perfringens Nontoxic Alpha-Toxin Variant as a Potential Vaccine Candidate against Alpha-Toxin-Associated Diseases. 69:7194-7196. doi 10.1128/iai.69.11.7194-7196.2001

Schrezenmeir, J., and M. De Vrese. 2001. Probiotics, prebiotics, and synbiotics—approaching a definition. The American Journal of Clinical Nutrition 73:361s-364s. doi 10.1093/ajcn/73.2.361s

Selle, P. H., A. J. Cowieson, and V. Ravindran. 2009. Consequences of calcium interactions with phytate and phytase for poultry and pigs. Livestock Science 124:126-141. doi 10.1016/j.livsci.2009.01.006

Selle, P. H., V. Ravindran, A. Caldwell, and W. L. Bryden. 2000. Phytate and phytase: consequences for protein utilisation. Nutrition Research Reviews 13:255-278. doi 10.1079/095442200108729098

Shafey, T. M., M. W. McDonald, and J. G. Dingle. 1991. Effects of dietary calcium and available phosphorus concentration on digesta pH and on the availability of calcium, iron, magnesium and zinc from the intestinal contents of meat chickens. British Poultry Science 32:185-194. doi 10.1080/00071669108417339

Singh, N., S. Kuhar, K. Priya, R. Jaryal, and R. Yadav. 2018. Phytase: The Feed Enzyme, an Overview. Pages 269-327Springer Singapore.

Soares, J. H. 1984. Calcium Metabolism and Its Control - A Review. Poultry Science 63:2075-2083.

Songer, J. G. 1996. Clostridial Enteric Diseases of Domestic Animals. Clinical Microbiology Reviews 9:216-234.

Springer, S., and H. J. Selbitz. 1999. The control of necrotic enteritis in sucking piglets by means of aClostridium perfringenstoxoid vaccine. FEMS Immunology & Medical Microbiology 24:333-336. doi 10.1111/j.1574-695x.1999.tb01302.x

Stanford, M. 2006. Calcium Metabolism in Clinical Avian Medicine.

Stringfellow, K., J. McReynolds, J. Lee, J. Byrd, D. Nisbet, and M. Farnell. 2009. Effect of bismuth citrate, lactose, and organic acid on necrotic enteritis in broilers. Poultry Science 88:2280-2284. doi 10.3382/ps.2008-00456

Titball, R. W., C. E. Naylor, and A. K. Basak. 1999a. The Clostridium perfringensα-toxin. 5:51-64. doi 10.1006/anae.1999.0191

Titball, R. W., C. E. Naylor, and A. K. Basak. 1999b. The Clostridium perfringensα-toxin. Anaerobe 5:51-64. doi 10.1006/anae.1999.0191

Toyoda, T., K. Isobe, T. Tsujino, Y. Koyata, F. Ohyagi, T. Watanabe, M. Nakamura, Y. Kitamura, H. Okudera, K. Nakata, and T. Kawase. 2018. Direct activation of platelets by addition of CaCl2 leads coagulation of platelet-rich plasma. International Journal of Implant Dentistry 4. doi 10.1186/s40729-018-0134-6

Troxel, T. R., G. L. Burke, W. T. Wallace, L. W. Keaton, S. R. McPeake, D. Smith, and I. Nicholson. 1997. Clostridial vaccination efficacy on stimulating and maintaining an immune response in beef cows and calves. Journal of Animal Science 75:19. doi 10.2527/1997.75119x

Tsiouris, V., I. Georgopoulou, C. Batzios, N. Pappaioannou, R. Ducatelle, and P. Fortomaris. 2015. The effect of cold stress on the pathogenesis of necrotic enteritis in broiler chicks. Avian Pathology 44:430-435. doi 10.1080/03079457.2015.1083094

Tsiouris, V., I. Georgopoulou, C. Batzios, N. Pappaioannou, R. Ducatelle, and P. Fortomaris. 2018. Heat stress as a predisposing factor for necrotic enteritis in broiler chicks. Avian Pathology 47:616-624. doi 10.1080/03079457.2018.1524574

Uzal, F. A., J. C. Freedman, A. Shrestha, J. R. Theoret, J. Garcia, M. M. Awad, V. Adams, R. J. Moore, J. I. Rood, and B. A. McClane. 2014. Towards an understanding of the role ofClostridium perfringenstoxins in human and animal disease. Future Microbiology 9:361-377. doi 10.2217/fmb.13.168

Vasin, A. L., D. V. Samigullin, and E. A. Bukharaeva. 2010. The role of calcium in modulation of the kinetics of synchronous and asynchronous quantal release at the neuromuscular junction. Biochemistry (Moscow) Supplement Series A: Membrane and Cell Biology 4:77-84. doi 10.1134/s1990747810010125

Wade, B., and A. Keyburn. 2016. The true cost of necrotic enteritis in Poultry WorldPoultry World, https://www.poultryworld.net/Meat/Articles/2015/10/The-true-cost-of-necrotic-enteritis-2699819W/.

Walk, C. L., M. R. Bedford, and A. P. McElroy. 2012. Influence of limestone and phytase on broiler performance, gastrointestinal pH, and apparent ileal nutrient digestibility. Poultry Science 91:1371-1378. doi 10.3382/ps.2011-01928

Walk, C. L., and S. V. Rama Rao. 2018. High doses of phytase on growth performance and apparent ileal amino acid digestibility of broilers fed diets with graded concentrations of digestible sulfur amino acids. Poultry Science 97:3610-3621. doi 10.3382/ps/pey218

Watkins, K. L., T. R. Shryock, R. N. Dearth, and Y. M. Saif. 1997. In-vitro antimicrobial susceptibility of Clostridium perfringens from commercial turkey and broiler chicken origin. Veterinary Microbiology 54:195-200. doi 10.1016/s0378-1135(96)01276-x

Watkins, R. M., B. C. Dilworth, and E. J. Day. 1977. Effect of Calcium Supplement Particle Size and Source on the Performance of Laying Chickens. Poultry Science 56:1641-1647. doi 10.3382/ps.0561641

Wilkie, D. C., A. G. V. Kessel, L. J. White, B. Laarveld, and M. D. Drew. 2005. Dietary amino acids affect intestinal Clostridium perfringens populations in broiler chickens. Canadian Journal of Animal Science 85:185-193.

Williams, R. B. 2005. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathology 34:159-180. doi 10.1080/03079450500112195

Xing, R., H. Yang, X. Wang, H. Yu, S. Liu, and P. Li. 2020. Effects of Calcium Source and Calcium Level on Growth Performance, Immune Organ Indexes, Serum Components, Intestinal Microbiota, and Intestinal Morphology of Broiler Chickens. Journal of Applied Poultry Research 29:106-120.

Zanu, H. K., C. Keerqin, S. K. Kheravii, N. Morgan, S. B. Wu, M. R. Bedford, and R. A. Swick. 2020a. Influence of meat and bone meal, phytase, and antibiotics on broiler chickens challenged with subclinical necrotic enteritis: 2. intestinal permeability, organ weights,

hematology, intestinal morphology, and jejunal gene expression. Poultry Science. doi 10.1016/j.psj.2019.12.049

Zanu, H. K., C. Keerqin, S. K. Kheravii, N. K. Morgan, S. B. Wu, M. R. Bedford, and R. A. Swick. 2020b. Influence of meat and bone meal, phytase, and antibiotics on broiler chickens challenged with subclinical necrotic enteritis: 1. growth performance, intestinal pH, apparent ileal digestibility, cecal microbiota, and tibial mineralization. Poultry Science 99:1540-1550. doi 10.1016/j.psj.2019.11.021

Zanu, H. K., S. K. Kheravii, M. R. Bedford, and R. A. Swick. 2020c. Dietary calcium and meat and bone meal as potential precursors for the onset of necrotic enteritis. World's Poultry Science Journal:1-14. doi 10.1080/00439339.2020.1831419

Zanu, H. K., S. K. Kheravii, N. K. Morgan, M. R. Bedford, and R. A. Swick. 2020d. Interactive effect of two dietary calcium and phytase levels on broilers challenged with subclinical necrotic enteritis: Part 1. broiler performance, gut lesions and pH, bacterial counts and apparent ileal digestibility. Poultry Science. doi 10.1016/j.psj.2020.05.033