DETERMINATION OF CALCIUM DIGESTIBILITY AND BIOAVAILABILITY ON FIVE LIMESTONE SOURCES

BY

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THESIS

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ABSTRACT

Four experiments were conducted to evaluate the effect of 5 limestones (L1-L5) varying in calcium (Ca) solubility on Ca utilization in laying hens, broiler chickens, and crossbred chickens using different methods. The 5 commercial limestones varied in solubility (88-97%), mean particle size (500-700 microns), and geographical origin and were evaluated for digestibility and bioavailability of Ca. The first experiment was conducted on Lohman LSL Lite hens which were used to determine if the different limestones can impact laying hen performance and egg quality when fed in diets containing different dietary Ca levels. Hens were randomly allotted to and fed 1 of 10 corn soybean meal-based diets containing 1 of 5 limestones at 2 different dietary Ca levels of 3.8% and 2.65%. Results from the laying hen trial indicated there was a significant ($P < 0.05$) main effect for dietary Ca level for the hen-day egg production, egg mass, feed efficiency, egg specific gravity, and eggshell breaking strength, with the values being decreased for hens fed the lower 2.65% Ca level. No effect of limestone source was observed for feed consumption, egg weight, egg specific gravity and eggshell breaking strength, except the hens fed L2 has significantly lower ($P < 0.05$) egg specific gravity and eggshell breaking strength, than hens fed L3 at the higher dietary Ca level. The results of this study indicated that the L2 limestone source had the lowest solubility (88%) and was generally inferior to the other 4 commercial limestones, primarily based on responses for egg production, egg mass, and feed efficiency. In Experiment 2, both crossbred and commercial chicks were used to determine the effect of dietary Ca levels on tibia bone ash to develop a slope-ratio Ca bioavailability assay. The chicks were fed diets containing increasing levels from 0.2 to 0.95% Ca from 8 to 22 d of age. Regressions of bone ash in mg/tibia and % on supplemental Ca intakes yielded linear and quadratic responses for supplemental Ca intake in both types of chicks. Experiment 3 was performed to determine bioavailability of Ca in the 5 limestones using bone ash as the primary

response criterion. Commercial Ross 308 broiler males were fed 1 of 13 diets which consisted of a Ca deficient diet (0.3% Ca) or that diet supplemented with 0.15% or 0.30% Ca from either reagent grade calcium carbonate ($CaCO_3$) or 1 of the 5 commercial limestones from 8 to 22 d of age. Multiple linear regression of bone ash (mg/tibia and %) on supplemental Ca intake yielded slope-ratio relative Ca bioavailability values ranging from 90 to 106%. In Experiment 4, a broiler chicken assay was conducted to determine apparent ileal Ca digestibility and total tract Ca retention for the 5 limestones using corn-based diets. The ileal Ca digestibility (%) and total tract Ca retention (%) values at 21 d of age were low and variable with a range of 20 to 34% for ileal Ca digestibility and 12 to 31% for total tract Ca retention among limestones. The results of these studies indicate a slope-ratio bone ash assay can be used to measure relative bioavailability of Ca in limestones, and relative Ca bioavailability, ileal Ca digestibility, and total tract Ca retention generally did not differ significantly among the 5 commercial limestones.

This thesis is dedicated to my grandparents, John and Pat Kovac. I know they have been watching over me this entire journey.

“What lies behind us, and what lies before us are small matters compared to what lies within us.”

- Ralph Waldo Emerson

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TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

The biggest contributor of calcium (Ca) in poultry feed is limestone (Kim et al., 2019). Limestone is known to be the least expensive and possibly the most readily available source of Ca (Walk, 2016). Even though limestone is not an expensive ingredient, it is a source of Ca which is very important to the body. Calcium and phosphorus (P) are the most abundant macro minerals in the body. Both of these minerals are largely found in the skeleton (Walk et al., 2021a). Calcium has several different roles in the growth and development of poultry. Other than skeletal growth, it aids in muscle contraction, nerve impulse transmission, blood clotting, enzyme activation, metabolic reactions, protein synthesis, maintenance of osmotic acid-base balances in the membranes, eggshell formation and egg quality (David et al., 2021a). When there are deficiencies with Ca or P, they can cause an imbalance with the Ca to P ratio which can lead to issues with bone mineralization, feed efficiency, reduced growth performance, and in some cases death. Having too much Ca can also affect the chickens by reducing feed efficiency (Walk et al., 2021b). There are many properties that may affect the digestibility of Ca, including particle size distribution, origin of the limestone, geological origin, chemical composition, and physical properties of the rock (Kim et al., 2019). Another factor that is often observed is the solubilization of Ca within the digestive system. Studies have shown that birds fed a coarse limestone have higher gizzard Ca concentrations because the coarser particles are retained longer in the gizzard, which increases digesta Ca solubilization. Thus, Ca utilization in chickens is affected by its degree of solubilization in the digestive tract. Data have shown that laying hens are more efficient in absorbing Ca from limestone than broilers (David et al., 2021a). Calcium plays an important role is eggshell quality in laying hens. Hens deposit approximately 2 grams of

Ca into an eggshell daily. That eggshell is composed of 95% Ca carbonate, with 60 to 70% of Ca needed to produce an egg coming from the diet, and the rest comes from the medullary bones (Hervo et al., 2022). Another problem Ca deficiencies can cause in laying hens is a reduction in egg production (Scott et al., 1971). Thus, Ca is very important in poultry diets because it can greatly influence many aspects of poultry production.

Dietary Sources of Calcium

In poultry nutrition, there are several dietary sources of Ca. In the past, there has not been a lot of attention on Ca digestibility because of the low cost and amount of limestone available. Some studies have evaluated Ca from sources other than limestone that could provide Ca and be as useful as limestone, including meat and bone meal, monocalcium phosphate, and dicalcium phosphate (David et al., 2019). Other earlier studies also evaluated oyster shells and seashells as a source of Ca (Guinotte and Nys, 1991). It is well known that there is a relatively low Ca concentration in plant sources, so inorganic sources such as limestone and dicalcium phosphate are commonly used in poultry diets (Adeola and Zahng, 2018). The limestone may vary in composition because of the location where it was mined and presence of other nutrients that can affect the utilization of the Ca in the limestone and dicalcium phosphate source (Reid and Weber, 1976). Sources can also vary in particle size and solubility which may affect the Ca digestibility (Walk et al., 2021a). Cost of the product can also affect how much the source is used. The cost of oyster shells is about twice the price of limestone, and not all feed mills will carry it (Miller and Sunde, 1975). When considering the bioavailability of different Ca sources, they are usually compared to Ca carbonate which is assumed to have a Ca bioavailability of 100% (Anwar et al., 2016). All Ca sources may be variable in their Ca content and bioavailability but limestone is one of the least expensive and most consistent when compared to other sources.

Limestone as a Dietary Calcium Source

Usually, the primary source of Ca for diet supplementation is ground limestone, and that is due to the fact that it is so abundant and inexpensive. More than 80% of the Ca in the earth's crust exists as limestone (McDowell, 2003). Calcium from limestone is considered a highly bioavailable Ca source and variations of limestone Ca availability have not generally been taken into account. Limestone can contribute more than 50% of total analyzable Ca in a broiler diet depending on the growth stage and ingredients used. The average Ca concentration was recently surveyed from 47 U.S. limestones and found to be 36.58% with a geometric diameter of 0.318 mm. There were also different concentrations of various minerals such as manganese, zinc, and iron, along with others, but they did not seem to correlate with the concentration of Ca. It has been suggested that the most important characteristics affecting limestone solubility are the physical properties of the rock and not so much its particle size or particle size distribution. The average true Ca digestibility coefficient of limestone was found to be 0.51 (Kim et al., 2019). The geological age of limestone can be determined by the color of the limestone as well. The darker the limestone, the geologically older is it, and associated with that are more impurities and typically lower solubility and Ca availability (Hy-line, 2016). In a different study, there were 2 basal diet types, corn based, and corn starch based purified diets, with 4 Ca sources as the sole source of Ca, and it was found that the analyzed Ca concentration of limestone was determined to be 400 g/kg. When the Ca digestibility in limestone was compared with meat and bone meal, monocalcium phosphate, and dicalcium phosphate, the limestone Ca digestibility was higher than the other Ca sources (David et al., 2019). Limestone can be variable but when good sources are found, it can be an excellent source of Ca, especially when compared with other sources like oyster shells and meat and bone meal.

Calcium and Phosphorus Interrelationship

The absorption of Ca and P from the digestive tract can be affected by several factors, including the dietary concentrations of Ca and P and Ca:total P ratio. These values can range for broilers, turkeys, and swine from about 1:1 to 2:1 (NRC, 1994). Diets for egg laying birds can have a Ca:P ratio of 4:1 or greater to allow for eggshell production. About 80% of the body's P and 98% of Ca are present in the skeleton as hydroaxyapatite, $Ca_5(PO_4)_3(OH)$. Skeletal development is what P is mostly needed for, but it is also needed for energy metabolism (Hurwtiz and Bar, 1965). The rest of the Ca is in the extracellular fluid and plasma and in the cell and is used for several other functions. The skeleton provides support for the vital internal organs and aids in locomotion and contains about 98% of the body Ca as Ca phosphate. Calcium and P availability are very important during the laying period. Laying hens need a large amount of Ca to deposit in the eggshell (De Vries et al., 2010).

The efficiency with which Ca and P are absorbed from the intestine depends on the quality and form of the elements in the feed. The absorption of Ca and P occurs predominantly in the duodenum and jejunum of the small intestine (Partridge, 1978). Calcium absorption occurs by passive and active transport, and each these processes accounts for about half of the Ca absorption in the small intestine (Hurwitz, 1996; Pond et al., 2005). Plasma Ca^{2+} and P concentrations are controlled by a feedback mechanism with parathyroid hormone, which is activated by vitamin D_3 and the receptors in the small intestine, bone, and kidney. When plasma Ca^{2+} and P are too low, parathyroid hormone and vitamin D_3 are released to increase plasma Ca^{2+} and P by intestinal absorption and bone resorption and to reduce Ca^{2+} and P excretion by the kidney. As the plasma Ca^{2+} concentration increases past the physiological limit, cells in the thyroid gland release calcitonin, which is a peptide hormone that lowers Ca^{2+} in the plasma.

When plasma Ca^{2+} and P are high, calcitonin reduces intestinal and bone absorption of Ca^{2+} and P and increases excretion by the kidney. The main conclusion that can be drawn is Ca and P homeostasis is maintained by feedback mechanisms regulated by plasma Ca^{2+} and P concentrations, which trigger the release of hormones that affect intestinal absorption, bone resorption and kidney excretion of Ca^{2+} and P (Veum, 2010). Excess levels of Ca and P can interact with other elements to cause deficiencies in nutrients, including zinc and magnesium (Maynard et al., 1979).

Calcium and Vitamin D

Choeclaciferol, otherwise known as vitamin D_3, is a fat-soluble vitamin. Since vitamin D_3 is essential for absorption of Ca and P, it is important to know how it can be obtained by the body. It can be taken directly from the diet, or it can be synthesized from 7-dehydroxycholesterol which is formed in the liver. When it is originates from the liver, it is transported to the skin and then is converted to vitamin D_3 when it is exposed to ultraviolet light and skin temperature. Vitamin D_3 can be transformed in the liver, and to some extent the kidney and intestine, to become 25-hydroxyvitamin D_3 (25-OHD_3). It is then converted in the intestine, bone, and skin into 1, 25$(OH)_2D_3$ (Soares, 1984). Vitamin D_3, is not metabolically active until it is converted into 1, 25$(OH)_2D_3$ which is called calcitriol. Calcitriol is a metabolite that is classified as a secosteroid hormone because of its functions in absorption of Ca and P in the intestine, resorption of Ca^{2+} and P in the kidney, and mobilization or accumulation of Ca^{2+} and P in bone (Henry and Norman, 1984; Lee et al., 1994; Bouillon et al., 1995). Vitamin D, thus, has to be converted to calcitriol to enhance Ca and P absorption and metabolism (DeLuca, 2008). Calcitriol seems to have a direct effect on bone tissue growth by regulating the differentiation and proliferation of the cellular elements in bone as well as other issues and systems (Pols et al.,

1990). The absorption efficiency of vitamin D_3 in poultry feed is about 60 to 70 % (Bar et al., 1980).

Calcium in Laying Hen Metabolism and Nutrition

The dietary requirement of Ca for laying hens ranges from 3.91 to 4.09 % of the diet (Hy-line, 2016). The Ca requirement can depend on their growth and production stage (David et al., 2021b). In addition, the utilization of dietary Ca primarily depends on how well it is solubilized in the digestive tract in poultry. Thus, digestibility of Ca in limestone can affect the dietary Ca requirement for growth and development of poultry which can include the eggshell formation and egg quality. About 99% of all Ca in the egg is in the shell and most of the P is in the yolk. Eggshell formation starts about 4 hours after ovulation. The metabolic need for Ca may vary depending on the stage of the eggshell formation process during the day. The longer stage of eggshell formation takes place during the scotophase especially when ovulation occurs later in the day. During the dark hours of the light cycle, the Ca from dietary Ca intake is low because there is almost no feed left in the gut. Thus, late at night, Ca has to be mobilized from the medullary bone reserves to fulfill the requirements for eggshell formation (De Vries et al., 2010). About 20 to 40% of the Ca needed to produce an eggshell comes from the bone (Bar, 2009). Later, when the photophase begins again, eggshell formation is done, so less Ca is needed, and dietary Ca intake is also increased. Thus, Ca can be deposited back into the bones to replenish the medullary bone Ca (De Vries et al., 2010).

The skeleton of domestic birds is composed of 3 different types of bone tissues. The compact cortical bone is found in the diaphysis of the long bones, cancellous bone is found in the vertebrae and epiphyses of the long bone, and specialized medullary bone is in the marrow cavities of certain bones (Hodges, 1974). All of these bones are fully developed during the

rearing period. The main bones that contribute to the skeletal structure are the cortical and cancellous bones that are both forms of lamellar bone. Once the bones are formed, they are maintained by a continuous process of bone deposition by osteoblasts and bone resorption by osteoclasts (Whitehead and Fleming, 2000). Osteoclast cells are responsible for bone resorption and the subsequent release of Ca and P from the skeleton to supply to the other organ systems or for excretion. Osteoblasts are used for the deposition of organic matrix, which eventually becomes mineralized bone tissue (Hurwitz, 1992). When there is an imbalance in the processes, it can lead to issues such as a net loss in the cortical bone which can cause osteoporosis (Whitehead and Fleming, 2000). When the hens become sexually mature, there are increased levels of estrogen which trigger the osteoblasts to completely switch to woven soft formation of bone tissues called the medullary bone. After this, the formation of structural bone stops (Whitehead, 2004; Hudson et al., 1993). Osteoclastic activity is slowed by estrogen but then increases during eggshell formation. Because of this process over time, the quantity of structural bones decreases (Dacke et al., 1993). It has been stated that in long term cases of Ca deficiency, medullary bone reserves are restored from structural bone which can lead to osteopenia and osteoporosis (Whitehead, 2004; Fleming, 2008). Calcium and P deficiencies in fast-growing broiler chickens and turkeys can cause skeletal abnormalities such as tibial dyschondroplasia, and Ca turnover rate in bone can vary greatly depending on the type of bone. Cortical bone can have a life of several months; where as, medullary bone has a half-life of only 1 to 2 days (Hurwitz, 1996). When a diet is deficient in Ca and P ions, the bones have a very large reservoir that can be used when needed (Aurbach et al., 1985). Calcium plays an important role in the growth and development of bones and can affect subsequent eggshell quality, which is why it is necessary to have the correct inclusions of Ca, P and vitamin D during the rearing period.

Calcium and Eggshell Quality

The Ca requirements for egg production can change depending on the circadian changes and the stage of ovulatory sequence. Usually, a hen will have 3 to 9 ovulatory cycles and then a day of rest when no oviposition occurs. Each cycle starts with ovulation and ends with oviposition. Each oviposition is followed by ovulation for the next cycle within 1 hour. The first oviposition in a sequence occurs within 3 hours of the start of the photophase. Each consecutive oviposition occurs slightly later in the day. The day of rest occurs after oviposition starting at about 7 hours from start of the photoperiod (Etches, 1987). An average egg weighs 60 grams and contains 2 grams of Ca (Taylor, 1963). The length of the laying cycle is about 24 to 25 hours with the shell being added during the last 21 hours (Johnson, 2015). The Ca for the eggshell comes from blood Ca that was absorbed in the intestine or from resorption from the medullary bone. While the shell is going through the calcification process, the Ca that is being absorbed from the intestine is not enough to provide the high Ca requirement, so about 40% of the Ca in the eggshell comes from Ca stored in the bone. The Ca in the bone is then refilled with Ca from intestinal absorption when the eggshell gland is inactive (Nys and Guyot, 2011). In a study (David et al., 2021a), some layers that received a Ca-free diet consumed their eggshells in attempt to satisfy their Ca needs for eggshell formation. The conclusion was based on the amount of broken eggs found in cages and the presence of eggshell in ileal digesta (David et al., 2021a). Other studies found that Ca-deprived layers would rather choose Ca enriched diets when given the choice between 2 diets with and without Ca carbonate (Wood-Gush and Kare, 1966). This was thought to explain the behavior of hens eating their eggs when being fed a diet deficient in Ca (David et al., 2021a).

Another paper explained that hens have to export 2 grams of Ca daily into the eggshell that is mainly composed of Ca carbonate (Nys, 2017). It was concluded that 60 to 70% of required Ca for eggshells is provided by the diet and the remainder comes from the medullary bone. The medullary bone is produced when the hens reach sexual maturity (Hadley et al., 2019). Over time, the osteoclasts will resorb the medullary bone, and the cortical bone is gradually degraded, which will cause bone weakness with aging. Along with bone weakness, it was reported that the eggshell quality was reduced from 49 weeks and continued to decline (Wistedt et al., 2019). When eggshell quality decreases, it can cause an increase in cracked eggs, which causes an economic loss for producers (Whitehead and Flemming, 2000). It was also observed that the capacity of intestinal absorption of Ca decreased with increasing age (Beck and Hasen, 2004). Fleming et al. (2006) found that coarse limestone particles are retained longer in the gizzard, which can provide a greater amount of soluble Ca for a longer amount of time during the dark period when compared with fine limestone particles. According to Fleming et al. (2006), hens fed coarse limestone particles resulted in a continuous soluble Ca diffusion from the stored particles in the crop and gizzard during the dark period and reduced bone Ca mobilization in laying hens. In the study done by Hervo et al. (2022), however, it was found that there was no overall significant interaction between limestone particle size and age on egg specific gravity, eggshell breaking strength, or eggshell thickness. Other statistical models showed that specific gravity, eggshell thickness, and eggshell breaking strength linearly increased with limestone particle size. The age of birds did show a significant linear effect on egg specific gravity but not on eggshell thickness or eggshell breaking strength. It was concluded that increased limestone particle size did not affect egg production performance but did improve eggshell quality (Hervo et al., 2022).

During peak egg production, the requirement for Ca is very high in laying hens. The Ca metabolism is greatly affected by the later stages of egg formation (Al-Batshan et al., 1994). Researchers have hypothesized that the larger particle size limestone stays in the crop and gizzard longer than finely ground Ca sources which will allow it to be available to hen for a longer period of time (Scott et al., 1971). It was thought that a large particle would be more beneficial during the dark period when there is no feed being consumed in order to provide some the Ca needed to make the eggshell (Etches, 1987). Other experiments have shown that larger particle size limestone does not affect shell quality when the Ca levels in the diets are optimal (Roland, 1986).

The composition of different Ca sources can vary (Reid and Weber, 1976) and is believed to be in relation to the location in which it is mined (Saunders-Blades et al., 2009). For example, limestone sources can vary in the amount of Ca and other nutrients which could affect the utilization of the Ca source by the laying birds (Reid and Weber, 1976). Another study was done using 3 different local limestones as dietary Ca sources for laying hens and also evaluated the limestones at 2 particle sizes. Saunder-Blades et al. (2009) used a commercial ground limestone and oyster shells with large particle size as 2 controls. The 3 other limestones were local ones that had different physical properties, such as color, but the limestones all contained similar amounts of Ca (38.03-38.86%). The limestones were fed at 2 particle sizes consisting of 100% ground, or a mixture of 67% ground and 33% large particle Ca. In the study, Saunder-Blades et al. (2009) found that the Ca sources that were being tested were similar in the concentration of Ca and impurities of other minerals. They found, however, that other mineral components were different with regard to their concentration within the Ca sources and that could affect how beneficial the Ca source is for the laying hen. In the same study, it was found that egg weight

increased throughout the trial and varied among the different Ca sources. Hens fed the control and Ca source C, which was a local limestone, had similar egg weights during phases 2 and 3 but egg weights were increased for 100% ground Ca source A and B, which were both local limestones, and as the control mixed particle which consisted of a commercial ground limestone for the ground Ca source and oyster shells of the large particle Ca source treatment groups. The egg specific gravity did not change with Ca source and particle size during any phase of laying cycle. Specific gravity did decrease during 1 phase of the experiment, but the researchers concluded that it was due to higher environmental temperatures. It was noted that the specific gravity it went back up during the next phase when the temperature went back down. During the last phase of the laying cycle, the specific gravity decreased again, but it was concluded that the effect was due to the hen's increased age (Saunders-Blades et al., 2009).

Calcium and Bone Integrity

Bone fractures are not always directly affected by nutrition but when there are insufficiencies in the diet, they can increase the rate and severity of bone fractures, particularly leg fractures (Rennie et al., 1997). As discussed earlier, particle size of Ca sources and the amount of Ca in the diet are important because the larger particle size Ca stays in the gizzard longer and can provide a more sustained source of Ca during the night when the eggshell is formed (Bar et al., 2002). Since eggshell quality and bone health are closely related and they both require a large amount of Ca, it is important to know when the physical attributes of different Ca sources affect the birds. Some researchers have noted that when increasing the dietary Ca concentrations, bone strength was also increased in older layers of 80 weeks of age (Nascimento et al., 2014). Other researchers showed that tibia bone breaking strength was higher in hens fed larger particle limestone than in those fed finely ground limestone (Cheng and Coon,

1990). It was stated in the latter study that other researchers also showed that increased dietary Ca concentrations and increased limestone particle size significantly increased tibia breaking strength in laying hens at 72 weeks of age.

The study mentioned above performed by Saudners-Blades et al., 2009, used 3 local limestones at 2 different particle sizes as dietary Ca sources for laying hens. The study was done in phases to allow for diet changes to accommodate the different Ca requirements of the aging laying hens. When this study concluded, it was found that there were no differences due to dietary Ca source on total and cortical bone mineral density as well as total and cortical bone mineral content. Dietary Ca source and particle size did have an effect on the trabecular bone mineral density of tibias from the hens, in that Ca source A yielded greater trabecular bone mineral density of hens than Ca source B or the control treatments. Since this measurement included medullary bone, it might mean that there is a larger pool of readily available skeletal Ca for those hens fed Ca source A. It was also found that the tibiae of birds fed Ca sources A and B were longer than those from hens fed Ca source C. There was a pattern of increased bone quality of tibiae from hens fed the mixed particle size limestones. The study concluded that even though both particle size treatments showed similar results for egg production and eggshell quality, the hens in the mixed particle size treatment groups were able to maintain egg production and eggshell quality with less support from structural bone reserves (Saunder-Blades et al., 2009).

Digestibility and Bioavailability Calcium in Broiler Chicken and Laying Hen Nutrition

Broiler chicks 0 to 3 weeks of age require approximately 1% of Ca per kilogram of diet but the amount decreases as the birds age, and this recommendation was based on maximizing bone ash in broilers (NRC, 1994). Broilers are usually selected for rapid growth and because of that, they require higher levels of Ca and P for skeletal development and energy metabolism

during the early part of their lives. If there are deficiencies in minerals such as Ca, it can cause issues, especially in the fast-growing lines. Deficiencies in Ca can cause skeletal problems such as tibial dyschondroplasia which causes bone deformities, lameness and mortality in some cases (Fleming, 2008). There are published values of available P for most ingredients but with Ca, the values are restricted to total Ca. The latter also confounds the Ca to P ratio since it is usually defined as total Ca to available P in the diet. What is really important is the amount of Ca and P that are actually digested, absorbed, and potentially used for body tissues or metabolic needs. It is also necessary to obtain a better understanding of the other factors that can influence the ability of birds to digest or absorb Ca in different sources. As discussed earlier, some of those factors include the physical from of concentrated Ca-containing ingredients like limestone, and their solubility, particle size, and where they originated (Li et al., 2021). Based on previous and current research, there are 2 primary methods used to measure the bioavailability and digestibility of Ca in limestone and other Ca sources. These will be discussed below.

Calcium Digestibility

When formulating diets for broiler chickens, inorganic Ca sources such as limestone and dicalcium phosphate (DCP) are often used because there are low Ca concentrations in most plant sources. Calcium digestibility of ingredients can be expressed as apparent, standardized, or true digestibility (Adeola and Zhang, 2018). One experiment in the latter study was done to test the difference in true ileal digestibility of Ca in limestone and DCP and to see if the values are additive in mixed diets. In this experiment, they used limestone, DCP, and a mixture of the 2 at a ratio of 1:1 and with 3 dietary Ca concentrations. This research found that intake of Ca, ileal Ca digested, and Ca retained linearly increased as the Ca concentration in the diet increased. It was also stated that no Ca concentration effect was observed on apparent ileal digestibility of Ca. It

was found that the regression method used to determine the true ileal digestibility of Ca for limestone yielded a value of 63.7%, and values for limestone and DCP were additive in mixed semi-purified diets for broilers (Adeola and Zhang, 2018).

Depending on the age and growth stage and the other ingredients used, limestone can contribute more than 50% of total analyzable Ca in a broiler diet. Limestones are not always similar in source, mineral content, or physical characteristics and can vary in particle size and Ca concentrations (Adeola and Zhang, 2018). An experiment was conducted to determine the effect of basal diet composition on the true Ca digestibility of limestone, meat and bone meal, monocalcium phosphate, and DCP (David et al., 2019). One of the experiments in this study used 8 experimental diets that were developed based on 2 basal diets, one being corn-based and the other one being corn starch-based and each basal diet was used for each of the 4 Ca sources. Titanium dioxide was added in all the diets as an indigestible indicator. This study found that analyzed Ca concentrations in limestone, meat and bone meal, monocalcium phosphate and DCP were determined to be 400, 90, 174, and 260 g/kg, respectively, and analyzed Ca concentrations of experimental diets ranged between 8.9 and 10.8 g/kg. The study found limestone had the highest Ca digestibility when compared with the other 3 sources, and DCP was the lowest. It was also noted that digestibility of Ca in meat and bone meal and monocalcium phosphate was similar. There was an effect of diet type where the Ca digestibility determined in birds fed the corn-based diet was higher than those fed the corn starch-based purified diet (David et al., 2019). The researchers thought this could be explained by the coarser particle size of the corn-based diet, since it could decrease the rate of digesta passage, allowing for more contact time of Ca source with digestive secretions, resulting in greater Ca solubility and digestibility (O'Dell et al.,

1959; Rochell et al., 2012). The David et al. 2019 study showed that the measurement of Ca digestibility is influenced by the composition of the type of basal diet.

Walk et al. (2021b) stated that the challenge with a digestible Ca formulation system in broilers is not the absence of proper values for raw materials but rather highly variable results due to the difference in methods employed, inherent ingredient variability and particle size, differences in dietary Ca to P ratios, the presence or absence of phytate, and the age of the birds. There is a growing awareness that over-supply of Ca and variability in the nutritional quality of common Ca sources such as limestone can greatly impact the digestibility of alternative nutrients such as amino acids and P (Walk et al., 2021b). Hens solubilize comparatively less limestone in their digestive systems as the level of dietary limestones increase (Rao and Roland, 1990). The avoidance of excess and non-digested dietary Ca through observing and understanding the Ca concentration and contamination in feed ingredients is likely to improve uniformity and digestibility of P and amino acids, reduce intestinal pH, and allow for space in the diet and reduce the need for some added fat or oil (Walk, 2016). After reviewing serval studies, researchers found that regardless of the dietary calcium:non-phytate P(Ca:NPP) ratio or the use of a diet adaptation period, the ileal Ca digestibility coefficients for several limestones determined at different ratios or adaption period were not different to the from an overall ileal Ca digestibility coefficient average over all Ca:NPP ratio and adaptation periods. Thus, an average Ca digestibility coefficient for limestone may be suitable to use in feed formulations until a standardized method is developed and employed (Walk et al., 2021b).

Li et al. (2021) performed a study to determine the effects of limestone geometric mean diameter, phytate, Ca source, and phytase on standardized ileal digestibility of Ca and P in broilers. They used a corn and corn germ-based basal diet for treatment 1that had no added Ca or

inorganic P. The 3 Ca sources evaluated were a commercial limestone, a subsample of the commercial limestone ground into smaller particle size, or bone from swine meat meal that was separated manually and then ground to the same particle size as the smaller limestone. These ingredients were added into the diet to achieve 0.71% Ca in order to determine the impacts of limestone particle size or Ca source on Ca digestibility. The authors concluded that the larger particle size limestone had higher values for standardized ileal digestibility of Ca than the finer ground limestone. It was also stated that reducing the limestone particle size had a negative impact on the standardized ileal digestibility of P (Li et al., 2021).

Calcium Bioavailability

Bone status is mainly utilized as an indicator of mineral adequacy in poultry diets. The most important minerals that help produce the matrix of the bone are Ca and P. Bone mineralization can affect the bone strength (Reichmann and Connor, 1977). Weak legs often result in reduced feed intake which can then affect weight gain and lower other production aspects (Rowland et al., 1967). There are 2 ways to evaluate bone mineralization, which are the noninvasive and invasive methods. Invasive methods include bone ash, bone breaking strength, bone weight, and bone volume. One method that could be performed as either invasive or noninvasive is bone densitometry. The most noninvasive method would be to use ultrasound (Rao et al., 1993). While noninvasive methods are not as frequently used, they can be valuable measurements because they allow for an in vivo measurement of bone status. These can be a useful research tool because they permit measurements of bone mineralization process over a longer time period using fewer birds. Bone densitometry, also known as photon absorptiometry, includes moving a low energy, gathered monoenergetic gamma-photon beam across a stationary bone. A focused light detector moves together with the photon source on the opposite side of the

bone and measures the photon flux. As the photon beam passes through the bone, a curve is generated. The area of the curve is equal to the amount of bone mineral content present (Meyer et al., 1968). In a study performed by Onyango et al. (2003), they used the relative sensitivity of bone mineral content, bone mineral density, shear force, and bone ash as indicators of dietary Ca and P adequacy in broiler chicks. In this study, they had 3 different diets with different P levels, but all of the diets had the same Ca:P ratio. This research found weight gain, feed intake, and gain/feed increased linearly as levels of dietary Ca and P increased. The bone mineral density, bone mineral content, and percent ash also increased linearly as the levels of dietary Ca and P increased. It was also noted that the bone mineral density and bone mineral content showed a quadratic increase with an increase in the levels of dietary Ca and P. This paper concluded that bone ash, bone mineral density, and bone mineral content are more sensitive than shear force as indicators of dietary Ca and P bioavailability in broiler chicks (Onyango et al., 2003).

The NRC (1994) suggests a Ca:NPP ratio of 2.22 for broilers from 1 to 21 days of age. Díaz-Alonso et al (2019) conducted a study to evaluate the growth performance and tibia measurements of broiler chicks fed increasing levels of Ca. The researchers evaluated 6 dietary levels of available P (aP) and 2 series of Ca:aP ratios, adjusted or variable. For the adjusted Ca:P ratio, it was kept at 2:1 and for the variable Ca:aP ratio, it varied from 7.69 to 1.59. The increasing Ca diets yielded a quadratic response on the bone ash percentage, rising from 35% with 0.26% Ca up to 48% with 1.26% Ca. The researchers did note a plateau at approximately 0.86% Ca for the tibia ash. The tibia ash weight for this trial was between 1.05 and 1.47 grams and it was concluded that maximum weight of ash was obtained in broilers fed the diets with the Ca level of 1.06% (Díaz-Alonso et al., 2019).

CONLUSION

Limestone is often the primary source of Ca in poultry diets but can be variable depending on its origin, particle size, and solubility. Some studies have shown limestone source can have large impacts on eggshell quality, bone quality, and Ca digestibility in poultry. Limestone is one the best options to provide Ca in the diet because it is inexpensive and contains a high amount of Ca. More research needs to be conducted to evaluate the effects of limestone origin on Ca digestibility and bioavailability in poultry at different ages and in different types of birds, including laying hens and broilers. Therefore, the overall objective of this thesis was to evaluate the effect of several limestones with varying in vitro Ca solubility on egg production performance and eggshell quality in laying hens and to determine Ca digestibility and relative Ca bioavailability for the limestone in broiler chickens using different methods. In the first experiment, Lohman LSL Lite hens were used to determine if the different limestones can impact laying hen performance and egg quality when fed in diets varying in dietary Ca. In the second experiment, cross bred and commercial chicks were used to develop a Ca bioavailability assay based on response in bone ash from feeding increasing dietary Ca levels from limestone in the diets. In the third experiment, commercial broilers were then used to evaluate the relative bioavailability of Ca in the different limestones using bone ash as the primary response. In the fourth experiment, commercial broilers were used to determine the digestibility of Ca in the different limestones based on ileal digestibility or total tract retention.

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CHAPTER 2:

EVALUATION OF LIMESTONES VARYING IN CALCIUM SOLUBILITY ON EGG PRODUCTION PERFOMRANCE AND TOTAL TRACT CALCIUM RETENTION IN LOHMAN LSL LITE LAYING HENS

ABSTRACT

The objective of this study was to determine the effect of feeding 5 different commercial limestones varying in solubility (88-97%) on laying hen performance, eggshell quality, and total tract Ca retention when the limestones were fed at 2 different dietary Ca levels. Lohmann LS Lite laying hens were fed 1 of 10 diets from 50 to 66 weeks of age. All diets were corn-soybean meal based and contained 16% protein. The 10 dietary treatments were arranged in a 5 x 2 factorial design with 5 limestones (L1-L5) and 2 dietary Ca levels which were 3.80 and 2.65%, respectively. Each of the 10 diets were fed to 6 replicate groups of 14 caged hens. The solubilities for L1-L5 were 97, 88, 95, 97, and 93% respectively. Egg production, egg weight, daily egg mass, feed consumption, feed efficiency, egg specific gravity, eggshell breaking strength, tibia bone ash, and total tract Ca retention were measured for each of the 6 replicate groups of hens at 2 or 4 week intervals. Data were analyzed using a two-way ANOVA for a 5 x 2 factorial design. There was a significant ($P < 0.05$) main effect for dietary Ca level for hen-day egg production, egg mass, feed efficiency, egg specific gravity, and eggshell breaking strength, with the values being decreased for hens fed the lower 2.65% Ca level. When comparing the 5 limestones, L1 generally yielded the highest performance values and was selected as the primary reference limestone for comparison with the other limestones. Hen-day egg production was significantly lower ($P < 0.05$) for hens fed diets containing L2, L3, or L5 compared with hens fed L1 in the lower Ca diets. Egg mass for hens fed L2 was decreased compared with hens fed L1 in

the lower Ca diet. Feed efficiency for hens fed L2 was lower ($P < 0.05$) than for hens fed L1 at both dietary Ca levels. No effect of limestone source was observed for feed consumption, egg weight, egg specific gravity, and eggshell breaking strength, except the hens fed L2 had significantly lower ($P < 0.05$) egg specific gravity and eggshell breaking strength than hens fed L3 at the higher Ca level. There was no significant effect of dietary treatments on bone ash expressed as g/tibia or as %. There was also no consistent effect of limestone source on total tract Ca retention and values were higher ($P < 0.05$) at the lower dietary Ca level. The results of this study indicated that the L2 limestone source had the lowest solubility (88%) and was generally inferior to the other 4 commercial limestones primarily based on responses for egg production, egg mass, and feed efficiency.

INTRODUCTION

There is current interest in the inclusion of limestone in poultry diets due to the fact that limestone is an inexpensive ingredient and is usually the most readily available source of calcium (Ca) (Walk, 2016). Limestone is a naturally occurring mineral ingredient composed of Ca carbonate ($CaCO_3$); however, it has been found to vary in Ca solubility and Ca concentration even within the same country or region (Shih et al., 2000; Sa and Boyd, 2017). This ingredient has been routinely added to poultry diets because Ca plays an important role in skeletal health, egg production, eggshell formation, and eggshell quality (David et al., 2021). While particle size and solubility have been some of the main properties of limestone that have been researched, there has been limited research done on other properties of limestone such as the origin of the mineral. More research needs to be conducted to evaluate the effect of limestone origin on Ca digestibility and bioavailability in poultry, including laying hens.

There are several methods that can be used to measure bioavailability and digestibility of Ca in limestone and other Ca sources. Invasive methods include bone ash, bone breaking strength, bone weight, bone volume, and bone densitometry (Rao et al., 1993). Some of the noninvasive methods include excreta collection, feed measurements, and egg production measurements. For example, total tract retention of Ca can be measured by subtracting the total Ca voided in the excreta from the total Ca intake (Khanal et al., 2020). Other ways to measure the efficacy of limestone in laying hens are egg production, egg weight, egg mass, feed efficiency, eggshell breaking strength, and eggshell thickness (Härtel, 1990). In a study performed by Härtel (1990), it was noted that egg breaking strength and shell thickness were influenced by increasing dietary Ca levels. Eggshell quality can be measured by specific gravity as well (Bland et al., 2014). An eggshell is composed of 95% Ca carbonate, with 60 to 70% of Ca needed to produce an egg coming from the diet and the rest coming from the medullary bones (Hervo et al., 2022). In a study, some layers that received a Ca-free diet consumed their eggshells in attempt to satisfy their Ca needs for eggshell formation (David, et., 2021). Thus, when hens are not consuming the proper amount of Ca, it can negatively affect their egg production, and also their bone health.

It has been shown that variabilities in limestone, including particle size and rate of solubility, can affect the solubilization of Ca within the digestive system (David et al., 2021). Limestones with a larger particle size and higher solubility (%) may be particularly beneficial in diets of laying hens in production (David et al., 2021). Therefore, the objective of this study was to determine the effect of feeding 5 different commercial limestones varying in solubility (88-97%) on laying hen performance, eggshell quality, and total tract Ca retention when the limestones were fed at 2 different dietary Ca levels.

MATERIALS AND METHODS

The protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois. (Animal Use Protocol 21034)

Ingredients and Analyses

The 5 different limestone sources (L1-L5) were obtained from Danisco Animal Nutrition & Health/ IFF (1000 41st Ave Dr SW Cedar Rapids, IA, 52404). The limestones varied in particle size and were from different geographical origins within the U.S. Analyses were conducted to determine particle size (method S319.4; ANSI/ASAE, 2008), and solubility (Kim et al., 2019). Titanium concentrations in experimental diets, ileal digesta, and excreta were measured using UV spectroscopy (Myers et al., 2004). The Ca and P were analyzed via inductively coupled plasma-mass spectrometry (method 985.01 A, B, and C; AOAC International, 2007). All analyses except particle size and solubility were conducted at the University of Missouri-Columbia Experiment Station Chemical Laboratory, Columbia, MO. Particle size and solubility were determined by Danisco Animal Nutrition and Health.

Diets and Design

This experiment was conducted to determine the effect of feeding 5 different commercial limestones varying in solubility (88-97%) on laying hen performance, eggshell quality, and total tract Ca retention when the limestones are fed at 2 different dietary Ca levels. There were 10 dietary treatments with 6 replicates of 14 hens per replicate (2 adjacent cages containing 7 hens per cage; cages were 17 inches high x 23 inches wide and 22 inches deep). The Lohmann LSL Lite laying hens were housed in a caged layer house with water and feed provided ad libitum consumption. Hens were fed twice daily in order to minimize feed wastage. Water was provided

by 2 nipple waterers per cage. The hens were fed a corn soybean meal-based diet containing 18% protein and 4% Ca from 18 to 50 wks of age. Starting at 50 weeks of age, 840 hens were randomly allotted to the 10 dietary treatments and fed a corn-soybean meal diet containing 3.8% Ca or 2.65% Ca from the 5 different limestone sources from 50 to 66 weeks of age (Table 2.1). The trial started on March 15th and ended July 15th of 2022.

Body weight was measured at the beginning of the trial and hens were allotted to treatments so that mean body weight was similar among treatments. Eggs produced from all hens were counted and collected each day. Hen-day egg production was calculated every 2 weeks and corrected for mortality. Feed consumption was calculated every 2 weeks (g/hen/day). Mortality was recorded daily. Daily egg mass (g of egg produced per hen per day) was calculated by multiplying hen-day egg production times mean egg weight (g/egg). Feed efficiency (g egg/g feed consumed) was determined every 2 weeks by dividing daily egg mass by amount of feed intake (g/feed/day). All eggs laid in a 24 hour period were collected from each replicate group and measured for specific gravity every 4 weeks using the flotation method with NaCl solutions varying in specific gravity from 1.056 to 1.100 g/cm^3 in 0.004 increments. The eggshell breaking strength was performed every 4 weeks on 6 eggs from each replicate and were measured in Newton's using the Orka Egg Force Reader (Rockford, IL). Total tract Ca retention of diets was determined by collecting excreta at 58 and 66 weeks of age and using titanium dioxide (0.5% of the diet) as a digesta marker. The excreta samples were freeze-dried and analyzed for Ca and Ti at the University of Missouri Experiment Station Chemical Laboratory, Colombia, MO. Diets and freeze-dried ileal digesta and excreta that were collected were analyzed for Ca and titanium. The apparent ileal digestibility and total tract retention values were calculated as shown below. Ileal digestibility (%) = [(Ca diet – Ca ileal digesta) / Ca diet] × 100 where Ca diet = Ca in the

diet (%); Ca ileal digesta = Ca in ileal digesta (%) × titanium in diet (%) / titanium in ileal digesta (%). Total tract retention (%) = [(Ca diet – Ca excreta) / Ca diet] × 100 where Ca diet = Ca in the diet (%); Ca excreta = Ca in excreta (%) × titanium in diet (%) / titanium in excreta (%). At the end of the trial, 2 hens were euthanized from each replicate using CO_2 gas and the tibia were autoclaved, cleaned of any adhering tissue, oven dried at 100 degrees Celsius for 24 hours, and ashed at 600 degrees Celsius in a muffle furnace for 24 hours for a tibia ash analysis.

Statistical Analysis

The SAS software (SAS Institute INC, 2010) was used to analyze the data initially using one-way ANOVA for a completely randomized design. Data were then further analyzed as two-way ANOVA for a 5x2 factorial arrangement of treatments (5 limestone sources and 2 dietary Ca levels). Significant differences among treatments were determined using Fisher's least significant difference test. Differences in values for main effects, interactions and among treatments were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

The analyzed Ca, mean particle size and solubility of the 5 limestones are presented in Table 2.1. The Ca level of the limestones was similar to that reported by the NRC (1994) of 38% where the 5 different limestones, L1-L5, contained 39.3, 36.7, 38.2, 38.7, and 37.9% Ca, respectively. The mean particle sizes of L1-L5 were 516, 590, 652, 507, and 714 microns, respectively. The limestone solubility was also determined at 5, 10, and 30 minutes and it was found that after 30 minutes, the solubility of L1-L5 was 97, 88, 95, 97, and 93%, respectively.

Hens fed the diets with the higher dietary Ca level had generally higher hen day egg production (%) than the hens fed the lower dietary Ca diet (Table 2.3) (significant Ca main effect

for all wk). The higher dietary Ca level diet yielded a high hen day egg production of about 88% for the entire 16-wk experiment. According to the management guide for Lohman LSL-Lite layers, the egg production should be around 90% at 50 wk of age and then continuously decline as age increases. By 66 wks of age, egg production is expected to be approximately 85% (Lohmann Tierzucht, GmbH, 2019). Thus, egg production of the hens in the current study slightly exceeded egg production in the breeder manual. When comparing the effects of limestone sources, at the higher dietary Ca level, there generally were no consistent differences for hen-day egg production. For the lower Ca diets (2.65%), hen-day egg production was significantly lower for hens fed L2 than L1 for several 2-wk periods and was lower for L2, L3, and L5 than L1 for the entire 52-66 wk period. There were similar results for the hen-housed egg production as for the hen-day egg production with there being higher values for hens fed the higher dietary Ca level diets (Table 2.4). For most wk, L2 again yielded lower hen-housed egg production than L1 at the lower dietary Ca level. These results for egg production are somewhat in contrast to those of a previous study where it was found that there were no differences in egg production among Ca sources and their particle sizes (Saunders-Blades, 2009). Solubility of the Ca sources, however, was not determined in the Saunder-Blades (2009) study. For the egg weight, there were no significant differences among treatments (Table 2.5). As expected, egg weight increased with increasing wk and hen age. Table 2.6 shows the results for the daily egg mass. There was a significant limestone source main effect at 64, 66, and 52-66 wk which was primary due to the higher values for L1 versus L2, L3, and L4. There was a significant main effect of dietary Ca level at all wk with egg mass being higher for hens fed diets containing the higher Ca level. Research that has been conducted previously found that there were no differences in egg production and egg weight in hens from 38 to 62 weeks of age when the Ca

content of the diet was increased from 3.5 to 4.0% (Kesharvarz et al., 1993). In the current experiment, the low dietary Ca level of 2.65% was below the Ca levels in the experiment conducted by Keshavarz et al. (1993). The 2.65% Ca level was used in attempt to be more sensitive to any effects of limestone source on egg production, feed efficiency, and eggshell quality.

All the hens consumed approximately 111 g to 120 g of feed per day during this experiment (Table 2.7). The hens slightly increased their feed consumption as the experiment progressed. There was generally no effect of limestone source or Ca level on feed intake. Laying hens are known to possess a specific appetite for Ca and may prefer a diet with a higher amount of Ca to attain the needed nutrients (Wood-Gush and Kare, 1966). This study did not, however, observe a significant difference in feed consumption between the 2 dietary Ca levels. The data in Table 2.8 show the influence of the dietary treatments on feed efficiency. There was a significant difference between the dietary Ca level treatments (main effect of Ca level) with the values being increased at the higher dietary Ca level. There was a significant limestone source effect during the last 6 wk. The latter effect primarily resulted because L1 at the 3.8% dietary Ca level yielded higher values for several wk than L4, and L2 often yielded lower values than L1 at the 2.65% dietary Ca level. In general agreement with our findings, it was reported that feed efficiency was not consistently significantly affected either by the origin or the particle sizes of limestone source (Guinotte and Nys, 1991). The same study also reported, however, a significant limestone source by particle size interaction for feed consumption (Guinotte and Nys, 1991).

Egg specific gravity results are reported in Table 2.9. There were significant differences between the higher and lower dietary Ca level treatments at all time periods (significant main effect). There were only a few, and no consistent, significant limestone source effects. In a

previous study, increasing the dietary Ca level from 3.0 to 3.5% caused an increase in egg specific gravity from 1.0835 to 1.0843 in hens from 38 to 62 weeks of age (Keshavarz et al., 1993). These results agree with the data from the current study wherein there was an increase in specific gravity as dietary Ca increased from 2.65 to 3.80%.

The eggshell breaking strength showed similar results to the egg specific gravity (Table 2.10). There was a significant dietary Ca level main effect with values being higher at the higher dietary Ca level. There was a significant interaction between limestone source and dietary Ca level at 54, 62, and 54-66 wk. This interaction was primarily due to the higher value for L3 versus L1, L2, and L5 at the higher dietary Ca level, but not at the lower Ca level. Thus, as observed for egg specific gravity, there was no consistent effect of limestone source on eggshell breaking strength. At the end of the trial, there were a large number of cracked eggs which prevented egg specific gravity and eggshell breaking strength measurements to be conducted on eggs that were already cracked. The latter could have played a factor in there being little to no effect of limestone source on egg specific gravity and eggshell breaking strength because many of the cracked eggs probably had low specific gravity and breaking strength. As mentioned previously, when hens are lacking Ca in their diets, they will attempt to self-regulate and increase their Ca consumption, and that feeding behavior could have resulted in more pecking and attempted consumption of eggs, thereby resulting in more cracked eggs for hens fed limestone that have lower digestible or bioavailable Ca (Wood-Gush and Kare, 1966). The decrease in egg production from feeding the lower Ca diets also resulted in fewer eggs being measured for eggshell breaking strength and specific gravity, which may have increased the variability and reduced the precision and accuracy of those measurements. Finally, the reduced

egg production resulted in fewer eggs being produced, possibly resulting in thicker shells of those fewer eggs.

For the ending body weight and tibia bone ash, there were no significant differences among dietary treatments (Table 2.11). Keshavarz et al. (1993) reported similar results where they observed no effect on tibia dry weight or ash content in hens 62 weeks of age when the Ca level was increased from 3.5 to 4.0% of the diet. Guinotte and Nys (1991) did find a limestone particle size effect; when fine ground limestone was substituted with coarse limestone, the tibia characteristics were improved. It is well-known that Ca provided in blood following absorption of diet Ca from the intestine or from resorption from the medullary bone in laying hens. During the calcification of eggshell, intestinal absorption of diet Ca is insufficient to satisfy the Ca requirement and as much as 40% of the shell Ca may be derived from bone (David et al., 2021). Since Ca is used from the medullary bone to help with egg calcification, particularly when diets are Ca deficient, it was expected that the lower dietary Ca level of 2.65% would have had a larger effect on bone ash than that observed in the current study. However, the lower dietary Ca level used in this experiment may not have been low enough to see a large effect on the bones.

Table 2.12 shows the effect of the dietary treatments on total tract Ca retention when the birds were 58 and 66 weeks of age. Higher values were observed for the lower dietary Ca level diets (Ca main effect). There was a significant main effect of limestone source but the results were not consistent, except that L3 did yield the lowest Ca retention within both dietary Ca levels. The dietary Ca level effect is assumed to be due to hens being more effective at retaining Ca, because there was not as much in their diet and also because 3.80% Ca may have exceeded their Ca requirement and resulted in more Ca being excreted in the urine. Clunies et al. (1992) performed an experiment with hens fed different dietary Ca levels and reported that dietary Ca

content had no significant effect on absolute Ca retention on non-shell forming days, but on shell forming days, as the dietary Ca level increased, percentage of dietary Ca retained decreased. The latter study also showed that hens fed the lowest Ca diet of 2.5% retained a significantly greater proportion of dietary Ca compared with hens fed 3.5 and 4.5% dietary Ca (Clunies et al., 1992). Hurwitz and Bar (1965) found similar results and stated that as dietary Ca concentrations decreased, a greater proportion was retained. Thus, the results of the Clunies et al. (1992) and Hurwitz and Bar (1965) studies are in agreement with the results of the current study. In a different study using on laying hens fed limestones containing different particle sizes, total tract Ca retention was higher for the coarser limestones than finer ground limestone, which was believed to be explained by lower solubility of the coarse limestone (Khanal et al., 2020). For limestones that were used in the current study, solubility percentage after 30 minutes did not correlate well with total tract Ca retention or limestone particle size. For example, L5, which had the largest particle size, did generally have one of the highest values for total tract Ca retention within dietary Ca level but its solubility at 30 min was not the lowest of the 5 limestones.

In summary, there was a significant effect of the dietary Ca level on several measured production parameters, with the values being increased at the higher dietary Ca level. In contrast, total tract Ca retention was higher at the lower dietary Ca level. There was not a consistent significant effect among the 5 different limestone samples, although L2, which had the lowest solubility (88%), was often inferior to the other 4 higher solubility limestones based on responses for egg production, egg mass and feed efficiency. These results suggest that 30-minute limestone solubility should be in excess of 88%.

TABLES

Table 2.1. Ingredient's composition of diets.

	Dietary treatments									
Ingredients, %	1	2	3	4	5	6	7	8	9	10
Corn	62.69	62.69	62.69	62.69	62.69	68.92	68.92	68.92	68.92	68.92
Soybean meal	22.20	22.20	22.20	22.20	22.20	21.20	21.20	21.20	21.20	21.20
Soybean oil	3.70	3.70	3.70	3.70	3.70	1.50	1.50	1.50	1.50	1.50
Limestone 1	9.03	-	-	-	-	6.01	-	-	-	-
Limestone 2	-	9.03	-	-	-	-	6.01	-	-	-
Limestone 3	-	-	9.03	-	-	-	-	6.01	-	-
Limestone 4	-	-	-	9.03	-	-	-	-	6.01	-
Limestone 5	-	-	-	-	9.03	-	-	-	-	6.01
Dicalcium phosphate	1.36	1.36	1.36	1.36	1.36	1.35	1.35	1.35	1.35	1.35
Salt	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42
Vitamin mix[1]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Mineral mix[2]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
DL-Met	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Choline chloride	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Calculated values As-is:										
MEn	2970	2970	2970	2970	2970	2970	2970	2970	2970	2970
CP, %	15.9	15.9	15.9	15.9	15.9	15.9	15.9	15.9	15.9	15.9
Ca, %	3.8	3.8	3.8	3.8	3.8	2.65	2.65	2.65	2.65	2.65
Non-phytate P, %	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35

[1] Provided per kilogram of diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 µg; DL-α-tocopheryl, 11 IU; vitamin B_{12}, 0.01 mg; riboflavin; 4.41 mg; D-pantothenic, 10 mg; niacin, 22 mg; menadione sodium bisulfate, 2.33 mg.

[2] Provided as milligrams per kilogram of diet: manganese, 75 from $MnSO_4 \cdot H_2O$; iron, 75 from $FeSO_4 \cdot H_2O$; 75 mg from ZnO; copper, 5 mg from $CuSO_4 \cdot 5H_2O$; iodine, 75 from ethylene diamine dihydroidide; selenium, 0.1 from $NaSeO_3$.

Table 2.2. Analyzed calcium, particle size, and solubility of five limestone sources (as fed basis).

Limestone sources	Analyzed Ca (%)	Mean particle size (μm)[1]	Solubility (%)[2]		
			5 min	15 min	30 min
1	39.3	516	71	93	97
2	36.7	590	68	83	88
3	38.2	652	67	89	95
4	38.7	507	84	97	97
5	38.0	714	57	85	93

[1] The particle size was measured using the procedure of method S319.4; ANSI/ASAE, 2008 using different sized sieves.

[2] The solubility was measured using the procedure of Kim et al., 2019, using pH 3 HCl solution buffered with 3 M glycine.

Table 2.3. Influence of dietary treatments on hen-day egg production (%).

Dietary treatment	Age (wk)[1]								
	52	54	56	58	60	62	64	66	Mean
1. 3.80% Ca, L1	87.9^{a}	87.7^{a}	88.8^{ab}	86.7^{a}	88.5^{a}	90.8^{a}	89.1^{a}	90.4^{a}	88.7^{a}
2. 3.80% Ca, L2	86.8^{ab}	87.6^{a}	87.5^{ab}	86.7^{ab}	89.0^{a}	88.3^{ab}	87.0^{ab}	86.3^{abc}	87.4^{ab}
3. 3.80% Ca, L3	86.3^{abc}	88.7^{a}	88.4^{ab}	86.9^{ab}	88.8^{a}	89.1^{ab}	89.5^{a}	88.8^{ab}	88.3^{ab}
4. 3.80% Ca, L4	84.2^{abcd}	86.7^{ab}	86.7^{abc}	83.2^{bc}	87.6^{ab}	88.6^{ab}	86.3^{ab}	84.7^{bcd}	86.0^{bc}
5. 3.80% Ca, L5	86.0^{abc}	89.4^{a}	90.3^{a}	89.1^{a}	88.5^{a}	88.9^{ab}	88.0^{ab}	87.9^{abc}	88.5^{ab}
6. 2.65% Ca, L1	82.4^{cd}	83.4^{bc}	85.3^{bcd}	81.4^{cd}	84.4^{bc}	85.4^{bc}	84.8^{b}	83.4^{cde}	83.8^{cd}
7. 2.65% Ca, L2	83.3^{bcd}	80.8^{cd}	80.1^{e}	78.4^{d}	80.0^{de}	81.7^{cd}	78.4^{c}	79.2^{e}	80.2^{e}
8. 2.65% Ca, L3	86.5^{abc}	80.5^{cd}	82.9^{cde}	79.4^{cd}	76.9^{e}	81.0^{d}	78.9^{c}	80.8^{de}	80.8^{e}
9. 2.65% Ca, L4	84.6^{abc}	82.4^{cd}	82.5^{de}	80.5^{cd}	82.1^{cd}	82.1^{cd}	80.3^{c}	78.8^{e}	81.7^{de}
10. 2.65% Ca, L5	80.2^{d}	79.1^{d}	80.3^{e}	78.7^{d}	81.9^{cd}	84.3^{cd}	80.4^{c}	81.3^{de}	80.8^{e}
Pooled SEM	1.49	1.21	1.37	1.44	1.41	1.35	1.28	1.66	1.0

[a-e] Means within a column with no common superscript differ ($P < 0.05$).

[1] Significant limestone (L) source main effect at 64 and 66 and significant Ca level main effect at all wk ($P < 0.05$). The interaction between limestone source and Ca level was not significant.

Table 2.4. Influence of dietary treatments on hen-housed egg production (%).

Dietary treatment	Age (wk)[1]								
	52	54	56	58	60	62	64	66	Mean
1. 3.80% Ca, L1	87.9^{a}	87.7^{a}	88.8^{ab}	86.7^{ab}	88.5^{a}	90.8^{a}	89.1^{a}	90.4^{a}	88.7^{a}
2. 3.80% Ca, L2	86.8^{ab}	87.6^{a}	87.5^{ab}	86.7^{ab}	88.4^{a}	86.9^{abc}	84.9^{b}	84.3^{bcd}	86.6^{ab}
3. 3.80% Ca, L3	86.3^{abc}	87.7^{a}	87.3^{ab}	85.5^{abc}	86.7^{a}	87.0^{abc}	87.3^{ab}	86.7^{ab}	86.8^{ab}
4. 3.80% Ca, L4	84.2^{abcd}	86.7^{ab}	86.7^{abc}	83.2^{bc}	86.7^{a}	87.5^{ab}	85.2^{ab}	83.7^{bcd}	85.5^{bc}
5. 3.80% Ca, L5	86.0^{abc}	89.4^{a}	90.3^{a}	89.1^{a}	87.8^{a}	87.7^{ab}	85.5^{ab}	84.7^{bc}	87.6^{ab}
6. 2.65% Ca, L1	82.4^{cd}	83.4^{bc}	85.3^{bcd}	81.4^{cd}	84.4^{ab}	74.8^{bcd}	83.8^{bc}	82.3^{bcd}	83.5^{cd}
7. 2.65% Ca, L2	83.3^{bcd}	80.8^{cd}	80.1^{e}	77.8^{e}	78.0^{cd}	78.0^{e}	73.6^{e}	73.9^{e}	78.2^{f}
8. 2.65% Ca, L3	86.5^{abc}	80.5^{cd}	82.9^{cde}	79.4^{cd}	76.9^{d}	81.0^{de}	78.0^{d}	79.8^{cd}	80.6^{def}
9. 2.65% Ca, L4	84.6^{abc}	82.4^{cd}	82.5^{de}	80.5^{cd}	82.1^{cd}	82.1^{de}	80.3^{cd}	78.8^{de}	81.7^{de}
10. 2.65% Ca, L5	80.2^{d}	79.1^{d}	80.3^{e}	78.7^{d}	81.1^{bcd}	82.6^{cde}	78.4^{cd}	79.3^{cde}	80.0^{ef}
Pooled SEM	1.49	1.21	1.37	1.44	1.41	1.35	1.28	1.66	1.06

[a-f] Means within a column with no common superscript differ ($P < 0.05$).

[1] Significant limestone (L) source main effect at 62, 64, and 66 wk and overall and significant Ca level main effect at all wk ($P < 0.05$). The interaction between limestone source and Ca level was not significant.

Table 2.5. Influence of dietary treatments on egg weight (g/ egg).

Dietary treatment	Age (wk)[1]								
	52	54	56	58	60	62	64	66	Mean
1. 3.80% Ca, L1	63.8	65.7	65.5	65.6	65.7	65.7	66.6	66.6	65.6
2. 3.80% Ca, L2	63.0	65.3	65.2	64.6	64.8	64.5	65.4	65.4	64.8
3. 3.80% Ca, L3	63.3	64.6	65.6	64.6	65.2	64.9	66.2	66.9	65.2
4. 3.80% Ca, L4	63.5	64.7	64.0	63.9	64.4	64.7	64.6	64.3	64.3
5. 3.80% Ca, L5	63.4	64.0	64.6	64.1	64.4	64.2	65.0	65.8	64.4
6. 2.65% Ca, L1	63.0	64.7	64.6	64.5	64.8	64.5	65.5	66.2	64.7
7. 2.65% Ca, L2	63.7	65.3	66.0	64.8	64.6	64.9	64.9	66.1	65.0
8. 2.65% Ca, L3	63.8	64.9	66.2	65.0	65.3	64.8	65.4	65.7	65.1
9. 2.65% Ca, L4	63.8	65.0	65.8	64.2	64.7	64.6	65.4	66.5	65.0
10. 2.65% Ca, L5	63.4	65.3	65.5	64.9	65.0	65.5	66.5	66.9	65.4
Pooled SEM	0.48	0.50	0.50	0.55	0.50	0.50	0.52	0.40	0.40

[1] No significant main effect of limestone (L) source or Ca level at any wk.

Table 2.6. Influence of dietary treatment on egg mass (g egg produced per hen day). [1]

Dietary treatment	Age (wk)[2]								
	52	54	56	58	60	62	64	66	Mean
1. 3.80% Ca, L1	56.1^{a}	57.6^{a}	58.1^{a}	56.9^{a}	58.2^{a}	59.6^{a}	59.3^{a}	60.2^{a}	58.2^{a}
2. 3.80% Ca, L2	54.8^{abc}	57.2^{a}	57.0^{abc}	56.1^{a}	57.7^{a}	56.9^{bc}	56.9^{ab}	56.5^{bcd}	56.6^{ab}
3. 3.80% Ca, L3	54.6^{abc}	57.3^{a}	58.0^{ab}	56.1^{a}	57.9^{a}	57.9^{ab}	59.2^{a}	59.5^{ab}	57.6^{a}
4. 3.80% Ca, L4	53.5^{abcd}	56.1^{ab}	55.5^{bcd}	53.1^{b}	56.3^{ab}	57.4^{abc}	55.8^{bc}	54.4^{de}	55.3^{bc}
5. 3.80% Ca, L5	54.5^{abc}	57.2^{a}	58.3^{a}	57.1^{a}	57.0^{ab}	57.1^{abc}	57.2^{ab}	57.9^{abc}	57.0^{a}
6. 2.65% Ca, L1	52.0^{cd}	53.9^{bc}	55.1^{cde}	52.5^{b}	54.6^{bc}	55.0^{cd}	55.5^{bc}	55.2^{cde}	54.2^{cd}
7. 2.65% Ca, L2	53.0^{bcd}	52.7^{c}	52.8^{e}	50.8^{b}	51.7^{de}	53.0^{de}	50.8^{d}	52.3^{e}	52.1^{e}
8. 2.65% Ca, L3	55.2^{ab}	52.2^{c}	54.9^{cde}	51.6^{b}	50.1^{e}	52.4^{e}	51.6^{d}	53.0^{e}	52.6^{de}
9. 2.65% Ca, L4	54.0^{abc}	53.6^{c}	54.2^{de}	51.7^{b}	53.1^{cd}	53.1^{de}	52.5^{d}	52.3^{e}	53.1^{de}
10. 2.65% Ca, L5	50.9^{d}	51.7^{c}	52.5^{e}	51.1^{b}	53.2^{cd}	55.3^{cd}	53.5^{cd}	54.4^{de}	52.8^{de}
Pooled SEM	1.03	0.81	0.90	0.95	0.85	0.90	0.95	1.20	0.61

[a-e] Means within a column with no common superscript differ ($P < 0.05$).

[1] Calculated as hen-day egg production (%) x egg weight (g).

[2] Significant limestone (L)source main effect at 64 and 66 wk and overall and significant Ca level main effect at all wk ($P < 0.05$). The interaction between limestone source and Ca level was not significant.

Table 2.7. Influence of dietary treatment on feed consumption (g/hen/day).

Dietary treatment	Age (wk)[1]								
	52	54	56	58	60	62	64	66	Mean
1. 3.80% Ca, L1	111.5[abc]	113.5	113.0	112.6	112.6	111.5	113.0	113.0	112.6
2. 3.80% Ca, L2	111.9[a]	113.7	113.4	113.7	113.3	112.9	115.3	115.0	113.6
3. 3.80% Ca, L3	111.3[bc]	115.5	114.7	115.4	116.3	114.3	115.8	115.6	114.8
4. 3.80% Ca, L4	111.1[c]	113.4	113.9	114.1	114.6	114.4	115.0	114.2	113.8
5. 3.80% Ca, L5	111.9[ab]	114.0	114.1	113.7	114.8	112.5	115.3	115.1	113.9
6. 2.65% Ca, L1	112.0[a]	114.2	114.2	114.2	114.2	114.9	115.7	115.6	114.3
7. 2.65% Ca, L2	111.0[c]	113.2	112.9	114.0	116.4	116.4	119.7	120.0	115.3
8. 2.65% Ca, L3	111.9[ab]	113.6	113.5	113.8	114.2	112.8	115.1	115.0	113.7
9. 2.65% Ca, L4	112.1[a]	113.8	113.4	114.0	113.8	113.0	113.9	113.8	113.5
10. 2.65% Ca, L5	111.2[c]	113.4	113.7	114.0	114.9	116.2	116.9	116.4	114.5
Pooled SEM	0.2	0.5	0.5	0.5	0.9	1.1	1.3	1.4	0.6

[a-c] Means within a column with no common superscript differ ($P < 0.05$).

[1] Significant limestone source by Ca level interation at 52 wk ($P < 0.05$). No significant limestone source and Ca main effects and no other significant interactions between limestone source and Ca level.

Table 2.8. Influence of dietary treatments on feed efficiency (g egg/ g feed).

Dietary treatment	Age (wk)[1]								
	52	54	56	58	60	62	64	66	Mean
1. 3.80% Ca, L1	0.503^{a}	0.508^{a}	0.514^{a}	0.505^{a}	0.527^{a}	0.535^{a}	0.525^{a}	0.531^{a}	0.518^{bc}
2. 3.80% Ca, L2	0.489^{ab}	0.503^{a}	0.503^{abc}	0.493^{a}	0.502^{b}	0.504^{b}	0.493^{bc}	0.491^{bcd}	0.497^{bc}
3. 3.80% Ca, L3	0.491^{a}	0.497^{a}	0.505^{ab}	0.486^{ab}	0.509^{ab}	0.507^{b}	0.511^{ab}	0.515^{ab}	0.503^{ab}
4. 3.80% Ca, L4	0.481^{abc}	0.495^{a}	0.487^{bcd}	0.466^{bc}	0.486^{bc}	0.501^{bc}	0.485^{c}	0.476^{cde}	0.485^{cd}
5. 3.80% Ca, L5	0.487^{ab}	0.501^{a}	0.511^{a}	0.502^{a}	0.498^{b}	0.504^{b}	0.496^{bc}	0.504^{abc}	0.501^{b}
6. 2.65% Ca, L1	0.464^{bc}	0.472^{b}	0.482^{cde}	0.457^{c}	0.486^{bc}	0.479^{cd}	0.480^{cd}	0.477^{cde}	0.475^{de}
7. 2.65% Ca, L2	0.478^{abc}	0.466^{b}	0.467^{de}	0.445^{c}	0.436^{e}	0.455^{d}	0.424^{f}	0.436^{f}	0.451^{f}
8. 2.65% Ca, L3	0.493^{a}	0.459^{b}	0.483^{bcde}	0.453^{c}	0.452^{de}	0.465^{d}	0.448^{e}	0.461^{ef}	0.464^{ef}
9. 2.65% Ca, L4	0.482^{abc}	0.471^{b}	0.478^{de}	0.453^{c}	0.461^{d}	0.470^{d}	0.461^{de}	0.460^{ef}	0.467^{ef}
10. 2.65% Ca, L5	0.457^{c}	0.456^{b}	0.462^{e}	0.448^{c}	0.465^{cd}	0.476^{d}	0.457^{e}	0.467^{de}	0.461^{ef}
Pooled SEM	0.0093	0.0073	0.0080	0.0085	0.0085	0.0086	0.0076	0.0104	0.0056

[a-f] Means within a column with no common superscript differ ($P < 0.05$).

[1] Significant limestone (L) source main effect for 60, 62, 64 and 66 wk and overall and significant Ca level main effect at all wk ($P < 0.05$). The interaction between limestone source and Ca level was not significant.

Table 2.9. Influence of dietary treatments on egg specific gravity.

Dietary treatment	Age (wk)[1]				
	54	58	62	66	Mean
1. 3.80% Ca, L1	1.0856[a]	1.0859[a]	1.0836[ab]	1.0851[ab]	1.0850[ab]
2. 3.80% Ca, L2	1.0868[a]	1.0830[b]	1.0820[b]	1.0842[abc]	1.0840[b]
3. 3.80% Ca, L3	1.0872[a]	1.0864[a]	1.0841[ab]	1.0860[a]	1.0860[a]
4. 3.80% Ca, L4	1.0859[a]	1.0855[a]	1.0848[a]	1.0843[abc]	1.0851[ab]
5. 3.80% Ca, L5	1.0869[a]	1.0855[a]	1.0830[ab]	1.0852[ab]	1.0851[ab]
6. 2.65% Ca, L1	1.0806[b]	1.0817[bc]	1.0794[c]	1.0814[d]	1.0808[c]
7. 2.65% Ca, L2	1.0808[b]	1.0792[d]	1.0786[c]	1.0815[d]	1.0800[c]
8. 2.65% Ca, L3	1.0822[b]	1.0808[bcd]	1.0789[c]	1.0832[bcd]	1.0813[c]
9. 2.65% Ca, L4	1.0811[b]	1.0797[cd]	1.0785[c]	1.0811[d]	1.0801[c]
10. 2.65% Ca, L5	1.0821[b]	1.0811[bcd]	1.0793[c]	1.0822[cd]	1.0812[c]
Pooled SEM	0.0006	0.0008	0.0008	0.0008	0.0005

[a-d] Means within a column with no common superscript differ ($P < 0.05$).

[1] Significant limestone (L) main effect for 58, 62, and 66 wk and significant Ca level main effect at all wks ($P < 0.05$). The interaction between limestone source and Ca level was not significant.

Table 2.10. Influence of dietary treatment on eggshell breaking strength (Newtons).

	Age (wk)[1]				
Dietary treatment	54	58	62	66	Mean
1. 3.80% Ca, L1	47.0^{ab}	45.2^{abc}	43.0^{b}	40.8	44.0^{bc}
2. 3.80% Ca, L2	44.4^{bc}	43.5^{bcd}	42.3^{b}	42.4	43.2^{bcd}
3. 3.80% Ca, L3	47.5^{a}	46.5^{a}	48.1^{a}	41.9	46.0^{a}
4. 3.80% Ca, L4	47.2^{ab}	46.0^{ab}	44.2^{b}	42.5	45.0^{ab}
5. 3.80% Ca, L5	44.7^{abc}	43.3^{bcd}	44.3^{b}	44.0	44.1^{bc}
6. 2.65% Ca, L1	42.0^{cd}	40.8^{de}	45.0^{ab}	39.2	41.7^{de}
7. 2.65% Ca, L2	42.2^{cd}	39.3^{e}	41.5^{b}	41.1	41.0^{e}
8. 2.65% Ca, L3	39.8^{d}	41.2^{de}	41.8^{b}	40.5	40.8^{e}
9. 2.65% Ca, L4	43.5^{c}	41.6^{de}	42.9^{b}	39.2	41.8^{de}
10. 2.65% Ca, L5	43.8^{c}	42.6^{cd}	43.9^{b}	40.3	42.6^{cde}
Pooled SEM	1.02	1.03	1.28	1.33	0.006

[a-e] Means within a column with no common superscript differ ($P < 0.05$).

[1] Significant Ca level main effect at 54 and 58 wk ($P < 0.05$). Significant interaction between limestone (L) source and Ca level at 54 and 62 wk and overall ($P < 0.05$).

Table 2.11. Effect of dietary treatments on body weight and tibia ash[1].

Dietary treatment	Body weight at 66 wk (g)	Tibia ash at 66 wk	
		(g/tibia)	(%)
1. 3.80% Ca, L1	1,875	2.82	51.7
2. 3.80% Ca, L2	1,849	2.80	53.6
3. 3.80% Ca, L3	1,912	2.93	51.9
4. 3.80% Ca, L4	1,893	2.98	52.4
5. 3.80% Ca, L5	1,918	2.82	51.6
6. 2.65% Ca, L1	1,812	3.12	55.4
7. 2.65% Ca, L2	1,760	2.73	50.4
8. 2.65% Ca, L3	1,780	2.71	53.4
9. 2.65% Ca, L4	1,827	2.47	53.2
10. 2.65% Ca, L5	1,762	2.83	51.9
Pooled SEM	23.1	0.16	1.22

[1] No significant main effect of limestone (L) source or Ca level at any wk.

Table 2.12. Effect of dietary treatments on total tract Ca retention.

Dietary treatment	Total tract Ca (%) retention	
	58 wks[1]	66 wks[1]
1. 3.80% Ca, L1	50[c]	43[de]
2. 3.80% Ca, L2	57[abc]	48[cde]
3. 3.80% Ca, L3	40[d]	40[e]
4. 3.80% Ca, L4	52[bc]	52[bcd]
5. 3.80% Ca, L5	61[abc]	49[cde]
6. 2.65% Ca, L1	60[abc]	59[ab]
7. 2.65% Ca, L2	66[a]	58[abc]
8. 2.65% Ca, L3	58[abc]	45[de]
9. 2.65% Ca, L4	61[ab]	51[bcd]
10. 2.65% Ca, L5	63[a]	66[a]
Pooled SEM	3.4	3.7

[a-e] Means within a column with no common superscript differ ($P < 0.05$).

[1] Significant limestone source and Ca level main effects ($P < 0.05$). No significant interaction between limestone (L) source and Ca level.

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CHAPTER 3:

DETERMINATION OF CALCIUM DIGESTIBILITY AND BIOAVAILABILITY IN FIVE LIMESTONE SOURCES USING COMMERCIAL BROILERS AND CROSSBRED CHICKENS

ABSTRACT

Three experiments were conducted to determine the effect of feeding 5 commercial limestones varying in solubility (88-97%), mean particle size (500-700 microns), and geographical origin on the digestibility and bioavailability of Ca in commercial broilers and crossbred chickens. In Experiment 1, both crossbred and commercial chicks were used to determine the effect of dietary Ca levels on tibia bone ash in order to develop a slope-ratio Ca bioavailability assay. The chicks were fed diets containing increasing levels of 0.2 up to 0.95% Ca from 8 to 22 d of age. Regressions of bone ash in mg/tibia and % on supplemental Ca intakes yielded linear and quadratic regression responses for supplemental Ca intake in both types of chicks. Experiment 2 was performed to determine bioavailability of Ca in 5 limestones using bone ash as the primary response criterion. Commercial Ross 308 broiler males were fed 1 of 13 diets which were either a Ca deficient diet (0.3% Ca) or that diet supplemented 0.15% or 0.30% Ca from either reagent grade calcium carbonate ($CaCO_3$) or one of the 5 commercial limestones from 8 to 22 d of age. To determine Ca bioavailability of the test limestones relative to reagent grade $CaCO_3$, multiple linear regression of bone ash (mg/tibia and %) on supplemental Ca intake yielded slope-ratio relative Ca bioavailability values ranging from 90 to 106%. In Experiment 3, a broiler chicken assay was conducted to determine apparent ileal Ca digestibility and total tract Ca retention for the 5 limestones using corn-based diets. The ileal Ca digestibility (%) and total tract Ca retention (%) values at 21 d of age were low and variable with a range of 20 to 34% for

ileal Ca digestibility and 12 to 31% for total tract Ca retention. The results of these studies indicate that a slope-ratio bone ash assay can be used to measure relative bioavailability of Ca in limestones and that relative Ca bioavailability, ileal Ca digestibility, and total tract Ca retention generally did not differ significantly among the 5 commercial limestones.

INTRODUCTION

Calcium is one of the most abundant macro minerals in the body and is largely found in the skeleton (Walk et al., 2021a). Calcium has several different roles in the growth and development of poultry, which include maintenance of osmotic acid-base balances in membranes, muscle contraction, and enzyme activation (David et al., 2021). When there is a deficiency of Ca, it can cause an imbalance in the body that affects other minerals and leads to issues with bone mineralization, feed efficiency, and reduced growth performance. It is also possible to have too much Ca, which can negatively affect feed efficiency and cause other problems (Walk et al., 2021b). Limestone is usually the biggest contributor of Ca in poultry diets but some of its properties can affect the digestibility and bioavailability, including the particle size, chemical composition, geological origin of the limestone, and solubility (Kim et al., 2019). Based on the NRC (1994), to maximize bone ash, broiler diets should contain approximately 1% Ca from 0 to 3 weeks of age, which will decrease with age. Since broilers are usually selected for rapid growth, they require higher Ca levels for skeletal development and energy metabolism during the early part of their lives (Fleming, 2008). It is important to obtain a better understanding of the other factors that can influence the ability of the birds to digest or absorb Ca in different sources.

There is increased interest in formulation of poultry diets based on digestible Ca rather than the current total Ca. Walk et al. (2021b) stated that the primary challenge with a digestible

Ca formulation system in broilers is not the absence of proper values for raw materials but rather highly variable results due to the differences in methods employed, inherent ingredient variability and particle size, differences in dietary Ca to P ratios, the presence or absence of phytate, and the age of the birds. Over supplying Ca from limestone or other Ca sources can have a serious impact on the digestibility of other nutrients such as P and amino acids (Walk et al., 2021b). Calcium digestibility can be expressed as apparent, standardized, or true digestibility. Since limestones can contribute more than 50% of total analyzable Ca in a broiler diet (Adeola and Zhang, 2018), the digestibility or bioavailability of the Ca in limestone is important. To measure bioavailability and digestibility of Ca in limestone and other Ca sources, there are invasive and noninvasive methods. Invasive methods include bone ash, bone weight, bone volume, bone breaking strength, and bone densitometry (Rao et al., 1993) and ileal digestibility (Walk et al., 2021b). Bone status is mainly utilized as an indicator of mineral adequacy in poultry diets because it is very important in the production of the matrix of the bone and maintaining the bone strength (Reichmann and Connor, 1977). When birds have weak legs, it can also result in reduced feed intake, which can then affect weight gain and lower other production aspects (Rowland et al., 1967). The noninvasive methods include excreta collection for total tract retention and feed intake and feed efficiency (Khanal et al., 2020).

It has been shown that variabilities in limestone, including particle size, rate of solubility, and geological origin can affect Ca digestibility and bioavailability (David et al., 2021). Thus, larger particle size limestone and higher solubility may be beneficial in diets of broiler chickens. Therefore, the objective of this study was to determine the effect of feeding 5 different commercial limestones varying in solubility (88-97%), particle size, and geographical origin to broiler chickens on digestibility and bioavailability of Ca in the limestones.

MATERIALS AND METHODS

The protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois. (Animal Use Protocol Number 19090).

Ingredients and Analyses

The 5 different limestone sources varying in origin were obtained from Danisco Animal Nutrition & Health/ IFF (1000 41st Ave Dr SW, Cedar Rapids, IA, 52404), and were analyzed to determine particle size (ANSI/ASAE method S319.4, 2008) and solubility (Kim et al., 2019). Titanium concentrations in experimental diets, ileal digesta, and excreta were measured using UV spectroscopy (Myers et al., 2004). The Ca and P analyses were performed via inductively coupled plasma-mass spectrometry (method 985.01 A, B, and C; AOAC International, 2007). All analyses except particle size and solubility were conducted at the University of Missouri-Columbia Experimental Station Chemical Laboratory, Columbia, MO. Particle size and solubility were determined by Danisco Animal Nutrition and Health.

Diets and Design

Experiment 1 was conducted to evaluate the response in bone ash from the different dietary Ca levels in both male and female crossbred and male commercial broiler chicks in an attempt to develop a slope-ratio bioassay for determining relative Ca bioavailability in limestone. The latter was done to determine if the Ca response would be similar in both types of chicks, particularly since the crossbred chicks have primarily been used in the current lab previously for this type of bioassay (Boling-Frankenbach et al., 2001). The crossbred (New Hampshire x Columbian) chicks used in this experiment were hatched at the University of Illinois at Urbana-Champaign poultry research field laboratory and the commercial Ross 308 chicks were obtained

from a commercial hatchery. The crossbred and commercial broiler chicks were provided a nutritionally complete corn-soybean meal starter diet until 8 and 9 days of age, respectively. All chicks were housed in Petersime batteries with raised wire floors in an environmentally controlled room. At 8 (crossbred chicks) and 9 (commercial chicks) days of age the chicks were weighed, wing banded, and allotted to one of 6 diets, ensuring consistency in average body weight across treatments with a completely randomized design to constitute 12 total dietary treatments. There were 4 replicate pens per treatment with 5 chicks per replicate, for a total of 240 chicks (120 crossbred, 120 commercial broilers). The initial average weight of the broilers was 121.6 g, and the initial average weight for the crossbred chicks was 94.3 g. The 6 diets were corn-soybean meal based (Table 3.1). Diet 1 was a Ca deficient diet containing 0.2% Ca, diets 2-5 contained increased increments of 0.15% Ca from limestone. Thus, diet 2 contained 0.35% Ca diet 3 contained 0.50% Ca, diet 4 contained 0.65% Ca. Diet 5 contained 0.80% Ca. diet 6 contained 0.95% Ca. Limestone was added in place of Solka Floc. All of the diets contained 0.45% non-phytate phosphorus and a CP level of 22%. At the end of the experiment, weight gain, feed consumption, and feed efficiency were calculated for each replicate. When the crossbred chicks were 20 days of age and the commercial broilers were 21 days of age, they were euthanized using CO_2 gas and the right leg was collected from each chick for subsequent tibia ash analysis. The right tibia was autoclaved, cleaned of any adhering tissues, oven dried at 100 degrees Celsius for 24 hours, and ashed at 600 degrees Celsius in a muffle furnace for 24 hours.

Experiment 2 was conducted to determine bioavailability of Ca in the 5 limestones relative to the Ca in reagent grade $CaCO_3$ using bone ash as the primary response criterion. Commercial Ross 308 males were housed in Petersime batteries with raised wire floors in an environmentally controlled room. The chicks were fed a standard, nutrionally complete corn-

soybean meal diet for 8 days and had ad libitum access to water and feed. On day 9, the chicks were weighed, wing banded, and allotted to one of 13 dietary treatments, ensuring consistency in average body weight across treatments in a completely randomized design. The average starting weight was 139.4 g and there were 6 replicates per treatment with 5 chicks per replicate for a total of 390 birds. The experimental diets were provided for ad libitum consumption from day 9 to day 23. Diet 1 was a Ca deficient diet containing 0.30% Ca (Table 3.2). Diets 2 and 3 contained 0.15 and 0.30% supplemental Ca from reagent grade $CaCO_3$. Diets 4 and 5, 6 and 7, 8 and 9, 10 and 11, and 12 and 13 contained 0.15 or 0.30% supplemental Ca, respectively, from the 5 commercial limestones (L1-L5) (Table 2.2). All of the diets contained 0.45% non-phytate phosphorus and had a 22% CP level, and limestones were added in place of Solka Floc. Chicks were euthanized on the last day of the experiment using CO_2 gas and the right leg was collected from each chick for subsequent tibia ash analysis. The right tibia was autoclaved, cleaned of any adhering tissues, oven dried at 100 degrees Celsius for 24 hours, and ashed at 600 degrees Celsius in a muffle furnace for 24 hours.

Experiment 3 was conducted to determine apparent ileal Ca digestibility and total tract Ca retention of the 5 test limestones. Commercial Ross 308 males were placed in Petersime batteries with raised wire floors in an environmentally controlled room. The chicks were provided a nutritionally complete corn-soybean meal starter diet for 17 days and had ad libitum access to water and feed. On day 17, the chicks were fasted overnight. One day 18, the chicks were weighed and allotted to one of 5 dietary treatments, maintaining a consistent body weight across all replicates in a complete randomized design. The average initial starting weight for the chicks was 536.6 g and there were 8 replicates per treatment 5 chicks per replicate, resulting in a total of 200 chicks. The experimental diets were provided for ad libitum consumption from day

18 to day 21. All of the diets were corn based (93.4% corn) with each diet containing 2.05% of 1 of the 5 test limestones (Table 3.3). Each diet had a calculated CP level of 7.9% and calculated non-phytate phosphorus of 0.45%. Titanium dioxide was added at 0.5% of the diet as an indigestible marker. Chicks were euthanized on the last day of the experiment (21 days) using CO_2 gas and ileal digesta were collected from Meckel's diverticulum to the ileoceceal junction and excreta were also collected for each replicate. The ileal digesta and excreta were freeze dried and analyzed for Ca and titanium (Myers et al., 2004) at the University of Missouri. Diets and freeze-dried ileal digesta and excreta that were collected at 21 d of age were analyzed for Ca and titanium. The apparent ileal digestibility and total tract retention values were calculated using the following equation. Ileal digestibility (%) = [(Ca diet – Ca ileal digesta) / Ca diet] × 100, where Ca diet = Ca in the diet (%); Ca ileal digesta = Ca in ileal digesta (%) × titanium in diet (%) / titanium in ileal digesta (%). Total tract retention (%) = [(Ca diet – Ca excreta) / Ca diet] × 100, where Ca diet = Ca in the diet (%); Ca excreta = Ca in excreta (%) × titanium in diet (%) / titanium in excreta (%).

Statistical Analysis

SAS software (SAS Institute INC, 2010) was used to initially statistically analyze the data using a one-way ANOVA procedure for completely randomized designs. Significant differences among dietary treatments were determined using Fisher's least significant difference test. Difference in values among treatments were considered to be significant at $P< 0.05$. A linear regression (GLM procedure of SAS) was then computed by regressing either tibia ash (mg/tibia) or tibia ash percent (%) on supplemental Ca intake (g/chick) from reagent grade $CaCO_3$ to assess linear and quadratic effects. In Experiment 2, a multiple regression of tibia ash on supplemental Ca from reagent grade $CaCO_3$ or L1-L5 was computed and then the slope ratio method was used

to calculate the bioavailability of Ca in the test limestones relative to the reagent grade $CaCO_3$ (Finney, 1964). The Ca bioavailability value for reagent grade $CaCO_3$ was set at 100% and the regression coefficient (or slope) for each of the limestones was divided by the regression coefficient (or slope) for the reagent grade $CaCO_3$.

RESULTS AND DISCUSSION

Nutrient Composition

The analyzed Ca level of tested limestones was similar to the value reported by the NRC (1994) of 38%. The 5 limestones (L1-L5) had Ca concentrations, of 39.3, 36.7, 38.2, 38.7, and 37.9%, respectively. The mean particle size was 516, 590, 652, 507, and 714 microns, and the limestone solubility was 97, 88, 95, 97, and 93 % for L1-L5, respectively.

Experiment 1

The growth performance and tibia ash results in Experiment 1 are presented in Table 3.4. As expected, weight gain, feed intake, and feed efficiency were significantly higher for the commercial broiler chicks than for the crossbred chicks (significant main effect). There was a significant quadratic response to increasing dietary Ca level, where there was a significant increase as dietary Ca increased at the lower Ca supplemental levels of 0.15 and 0.30%, but there was no further increase at higher supplemental Ca levels. There was no significant interaction between dietary Ca level and chick type. For bone ash expressed as mg/tibia and %, there was a linear increase with the increasing dietary Ca level for both crossbred and commercial broiler chicks (no significant interaction between dietary Ca and chick type). These results indicated that large linear increases in bone ash can be obtained in both crossbred and commercial chicks by supplementation of a corn-soybean meal diet with increased levels of Ca. Thus, this dietary

regimen or type of experiment can potentially be used for determining relative bioavailability of different dietary Ca sources. The current Ca recommendations for broilers are 1.00% for the starter phase (1 to 21 days), 0.90% for the grower phase (22 to 42 days), and 0.80% for the finisher phase (43 to 56 days) (NRC, 1994). In agreement with the current study, Bai (2022) found that dietary Ca level did affect body weight and average daily gain on 21 d of age. The highest average daily gain was at a dietary Ca level of 0.59%. It was also stated that dietary Ca level did not affect the tibia ash Ca or P contents but did affect the tibia ash percentage. As the dietary Ca level increased, tibia ash percentage increased quadratically and reached a plateau at 0.88% Ca. Thus, the authors estimated that the optimal dietary Ca level was 0.80% or higher for broilers fed a conventional corn-soybean meal diet from 1 to 21 days of age. To obtain the optimal growth rate the dietary Ca level can be only 0.59%, but to meet the Ca requirement for metabolism and bone development of broilers, a higher level of approximately 1.00% Ca may be needed (Bai, 2022). Our results are in partial agreement with those of Bai (2022), wherein a large increase in growth and bone ash were obtained when dietary Ca level increased from 0.20 to 0.95%; however, there was no definite plateau in tibia ash even at the highest Ca level of 0.95% in the current study.

Experiment 2

The growth performance and tibia ash for chicks in Experiment 2 are shown in Table 3.5. Weight gain, feed intake, gain to feed ratio, bone ash (mg/tibia), and bone ash (%) were significantly increased with increasing inclusion of reagent grade $CaCO_3$ and the different test limestones compared with the Ca deficient diet. There was generally no significant difference among individual limestones within dietary Ca level except for higher weight gain for chicks fed 0.3% supplemental Ca from reagent grade $CaCO_3$ when compared with chicks fed the same

supplemental Ca level from L1, L2, or L4. In addition, the gain to feed ratio for chicks fed 0.3% supplemental Ca from reagent limestone was significantly higher than for chicks fed diets containing the other limestones. There was no consistent individual limestone effect within supplemental Ca level on the bone ash expressed as mg/tibia or as a %. The multiple regression equations for bone ash regressed on supplemental Ca intake from the limestone are shown in the footnotes of Table 3.5. There was a highly significant linear effect for all limestones (R^2 values = 0.78-0.79).

The bioavailability values of Ca in the test limestones relative to reagent grade $CaCO_3$ from the multiple linear regression analysis for bone tibia ash content (mg/tibia) and tibia ash concentration (%) on supplemental Ca intake are presented in Table 3.6. The slope-ratio values ranged from 89.8 to 106.2% for tibia ash (mg/tibia) and 88.2 to 98.7 for tibia ash (%). Based on the regression equations and the standard errors of the regression coefficients (Table 3.5 footnote), there were no significant differences among limestone relative bioavailability values for Ca. Bioavailable Ca concentrations for the 5 limestones are also shown in Table 3.6; these were similar to the analyzed Ca level since the relative bioavailability values for the limestones were high.

Experiment 3

Growth performance, ileal Ca digestibility, and total tract Ca retention values for Experiment 3 are presented in Table 3.7. Weight gain and gain to feed ratio did not show any significant differences among the 5 different limestones. The ileal Ca digestibility (%) ranged from 21.5 to 34.3, but there were no significant differences among limestones. There were significant differences for the total tract Ca retention among the limestones with total tract Ca retention (%) ranging from 15.6 to 38.6. Limestone 3 and 4 had the highest Ca retention and

were not significantly different from each other but they were significantly higher than L1, L2, and L5, which were not significantly different from each other.

Kim et al. (2019) found that there were large differences in the apparent ileal digestibility of Ca in diets containing different limestones. They also noted that limestones with smaller particle sizes had lower apparent ileal Ca digestibility than limestones with larger particle sizes, but no significant differences in ileal Ca digestibility among 5 limestones varying in particle size was observed in the current study. Kim et al. (2019) also reported that limestone particle size could only explain less than 40% of the variation in Ca digestibility, and they concluded that differences in apparent ileal Ca digestibility among limestone samples may also be due to several other factors such as mine of origin, type of rock, and physical and chemical characteristics of the rock, which may play an important role in how much Ca will be digested.

The ileal Ca digestibility (%) was generally higher than total tract Ca retention (%) in the current study. David et al. (2021) observed similar results with the values being higher for ileal digestibility than the amount of Ca that was retained. They suggested that it may be due to urinary excretion of Ca and that a higher plasma Ca concentration from absorbed Ca may have induced excretion in the urine. The ileal digestibility and total tract retention for Ca in the limestones in the current study were lower than values reported in previous studies (Walk et al., 2021b). The reason for the difference is unknown. Much of the difference may be due to the very high analyzed Ca in several samples of ileal digesta and excreta in the current study. For example, some ileal and excreta samples had analyzed Ca levels well in excess of 4%, whereas other samples contained 2% or less. The high level of Ca in some samples resulted in very low Ca digestibility and retention values for some replicate pens of chicks, with values being

negative in some cases. The reason for the high Ca level in some ileal digesta and excreta samples in unknown.

In summary, a slope-ratio bioassay based on bone ash response was developed to determine relative bioavailability of Ca in feed ingredients such as limestone. The bioavailability of Ca in 5 commercial limestones was high relative to the Ca in reagent grade limestone, whereas apparent ileal Ca digestibility and total tract retention of Ca in the 5 limestones were much lower and highly variable.

TABLES

Table 3.1. Ingredient composition of diets in Experiment 1.

	Diet					
Ingredient, %	1	2	3	4	5	6
Corn	57.44	57.44	57.44	57.44	57.44	57.44
Soybean meal	36.00	36.00	36.00	36.00	36.00	36.00
Soybean oil	1.60	1.60	1.60	1.60	1.60	1.60
Limestone	-	0.39	0.79	1.18	1.58	1.97
Dicalcium phosphate	0.42	0.42	0.42	0.42	0.42	0.42
Monosodium phosphate	1.11	1.11	1.11	1.11	1.11	1.11
Potassium chloride	0.25	0.25	0.25	0.25	0.25	0.25
Solka Floc[1]	1.97	1.58	1.18	0.79	0.39	-
L-Lys HCl	0.28	0.28	0.28	0.28	0.28	0.28
Vitamin mix[2]	0.20	0.20	0.20	0.20	0.20	0.20
Mineral mix[3]	0.15	0.15	0.15	0.15	0.15	0.15
DL-Met	0.37	0.37	0.37	0.37	0.37	0.37
Choline chloride	0.08	0.08	0.08	0.08	0.08	0.08
L-Thr	0.13	0.13	0.13	0.13	0.13	0.13
Calculated values:						
CP, %	22.0	22.0	22.0	22.0	22.0	22.0
Ca, %	0.20	0.35	0.50	0.65	0.80	0.95
Non-phytate P, %	0.45	0.45	0.45	0.45	0.45	0.45

[1]Fiber Corporation, Urbana, OH 43078. Limestone was added in place of Solka Floc.
[2]Provided per kilogram of diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 µg; DL-α-tocopheryl, 11 IU; vitamin B_{12}, 0.01 mg; riboflavin; 4.41 mg; D-pantothenic, 10 mg; niacin, 22 mg; menadione sodium bisulfate, 2.33 mg.
[3]Provided as milligrams per kilogram of diet: manganese, 75 from $MnSO_4 \cdot H_2O$; iron, 75 from $FeSO_4 \cdot H_2O$; 75 mg from ZnO; copper, 5 mg from $CuSO_4 \cdot 5H_2O$; iodine, 75 from ethylene diamine dihydroidide; selenium, 0.1 from $NaSeO_3$.

Table 3.2. Ingredient composition of diets in Experiment 2.

	Dietary treatments												
Ingredient, %[1]	1	2	3	4	5	6	7	8	9	10	11	12	13
Corn	58.72	58.72	58.72	57.44	57.44	57.44	57.44	57.44	57.44	57.44	57.44	57.44	57.44
Soybean meal	36.00	36.00	36.00	36.00	36.00	36.00	36.00	36.00	36.00	36.00	36.00	36.00	36.00
Soybean oil	1.60	1.60	1.60	1.60	1.60	1.60	1.60	1.60	1.60	1.60	1.60	1.60	1.60
Reagent grade $CaCO_3$	-	0.40	0.80	-	-	-	-	-	-	-	-	-	-
Limestone 1	-	-	-	0.40	0.80	-	-	-	-	-	-	-	-
Limestone 2	-	-	-	-	-	0.40	0.80	-	-	-	-	-	-
Limestone 3	-	-	-	-	-	-	-	0.40	0.80	-	-	-	-
Limestone 4	-	-	-	-	-	-	-	-	-	0.40	0.80	-	-
Limestone 5	-	-	-	-	-	-	-	-	-	-	-	0.40	0.80
Dicalcium phosphate	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87
Monosodium phosphate	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63
Potassium chloride	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Salt	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Solka Floc[2]	0.80	0.40	-	0.40	-	0.40	-	0.40	-	0.40	-	0.40	-
L-Lys HCl	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28
Vitamin mix[3]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Mineral mix[4]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
DL-Met	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37
Choline chloride	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
L-Thr	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Calculated values:													
CP, %	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0	22.0
Ca, %	0.30	0.45	0.60	0.45	0.60	0.45	0.60	0.45	0.60	0.45	0.60	0.45	0.60
Non-phytate P, %	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45

[1] Limestone in diets was added in place of Solka Floc.

[2] Powdered Cellulose; International Fiber Corporation, Urbana, OH 43078.

[3] Provided per kilogram of diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 µg; DL-α-tocopheryl, 11 IU; vitamin B_{12}, 0.01 mg; riboflavin; 4.41 mg; D-pantothenic, 10 mg; niacin, 22 mg; menadione sodium bisulfate, 2.33 mg.

[4] Provided as milligrams per kilogram of diet: manganese, 75 from $MnSO_4 \cdot H_2O$; iron, 75 from $FeSO_4 \cdot H_2O$; 75 mg from ZnO; copper, 5 mg from $CuSO_4 \cdot 5H_2O$; iodine, 75 from ethylene diamine dihydroidide; selenium, 0.1 from NaSeO.

Table 3.3. Ingredient composition of diets in Experiment 3.

	Dietary treatments				
Ingredient, %	1	2	3	4	5
Corn	93.40	93.40	93.40	93.40	93.40
Soybean oil	2.00	2.00	2.00	2.00	2.00
Limestone 1	2.05	-	-	-	-
Limestone 2	-	2.05	-	-	-
Limestone 3	-	-	2.05	-	-
Limestone 4	-	-	-	2.05	-
Limestone 5	-	-	-	-	2.05
Monosodium phosphate	1.50	1.50	1.50	1.50	1.50
Vitamin mix[1]	0.20	0.20	0.20	0.20	0.20
Mineral mix[2]	0.15	0.15	0.15	0.15	0.15
Sodium chloride	0.20	0.20	0.20	0.20	0.20
Titanium dioxide	0.50	0.50	0.50	0.50	0.50
Composition values:					
CP, % (calculated)	7.9	7.9	7.9	7.9	7.9
Ca, % (analyzed)	0.94	0.88	0.91	0.92	0.91
Non-phytate P, % (calculated)	0.56	0.56	0.56	0.56	0.56

[1] Provided per kilogram of diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 µg; DL-α-tocopheryl, 11 IU; vitamin B_{12}, 0.01 mg; riboflavin; 4.41 mg; D-pantothenic, 10 mg; niacin, 22 mg; menadione sodium bisulfate, 2.33 mg.

[2] Provided as milligrams per kilogram of diet: manganese, 75 from $MnSO_4 \cdot H_2O$; iron, 75 from $FeSO_4 \cdot H_2O$; 75 mg from ZnO; copper, 5 mg from $CuSO_4 \cdot 5H_2O$; iodine, 75 from ethylene diamine dihydroidide; selenium, 0.1 from $NaSeO_3$.

Table 3.4. Growth performance and tibia ash for crossbred and commercial broiler chicks, Experiment[1].

Diet	Weight gain[2,3] (g/chick)	Feed intake[2,3] (g/chick)	Gain: feed[2,3] (g/kg)	Bone ash[2,4] (mg/tibia)	Bone ash[2,4] (%)
Crossbred chicks					
1. Ca deficient diet -0.20% Ca	203.9[d]	370.1[c]	551[c]	271.8[d]	32.3[f]
2. As 1 + 0.15% Ca from limestone	234.6[c]	417.4[b]	563[c]	278.5[c]	34.8[e]
3. As 1 + 0.30% Ca from limestone	294.5[a]	455.7[a]	647[a]	365.5[b]	37.3[d]
4. As 1 + 0.45% Ca from limestone	270[b]	446.3[ab]	606[b]	391.5[b]	39.2[c]
5. As 1 + 0.60% Ca from limestone	283.3[ab]	465.8[a]	608[b]	442.5[a]	41.3[b]
6. As 1 + 0.75% Ca from limestone	286.5[ab]	467.7[a]	613[b]	477.5[a]	42.5[a]
Pooled SEM	6.0	9.9	9.3	12.2	0.35
Commercial broiler chicks					
1. Ca deficient diet -0.20%	425.3[c]	604.3[c]	702[c]	382.8[f]	35.8[e]
2. As 1 + 0.15% Ca from limestone	503.2[b]	695.6[b]	721[bc]	481.3[e]	39.0[d]
3. As 1 + 0.30% Ca from limestone	611.8[a]	794.9[ab]	770[ab]	654.5[d]	42.3[c]
4. As 1 + 0.45% Ca from limestone	628.4[a]	803.5[a]	783[a]	732.3[c]	44.6[b]
5. As 1 + 0.60% Ca from limestone	639.8[a]	822.4[a]	778[a]	795.3[b]	46.6[a]
6. As 1 + 0.75% Ca from limestone	645.2[a]	811.0[a]	796[a]	849.5[a]	47.5[a]
Pooled SEM	20.5	16.3	18.0	23.0	0.64

[a-f] Means within a column with no common superscript are significantly different ($P < 0.05$).

[1] Values are means of 4 pens of 5 chicks; average initial body weight was 94.3 g for crossbred chicks and 121.6 g for commercial broilers. Diets were fed from 8 to 20 days of age for crossbred chicks and from 9 to 21 days of age for commercial chicks.

[2] Significant main effect of chick type (crossbred versus commercial) and dietary Ca level ($P < 0.05$). There was no significant interaction between chick type and dietary Ca level.

[3] Significant quadratic effects for dietary Ca level.

[4] Significant linear effects of dietary Ca level.

Table 3.5. Growth performance and tibia ash for commercial broiler chicks in Experiment 2[1].

Dietary treatment	Weight gain (g/chick)	Feed intake (g/chick)	Gain: feed (g/kg)	Bone ash[2] (mg/tibia)	Bone ash[3] (%)
1. Ca deficient diet -0.30% Ca	565.9^{e}	783.5^{d}	723^{c}	521.4^{c}	37.2^{f}
2. As 1 + 0.15% Ca from reagent grade $CaCO_3$	685.5^{bc}	895.5^{ab}	766^{b}	715.1^{b}	41.7^{cd}
3. As 1 + 0.30% Ca from reagent grade $CaCO_3$	758.1^{a}	910.0^{ab}	834^{a}	836.3^{a}	44.3^{ab}
4. As 1 + 0.15% Ca from limestone 1	675.5^{bcd}	876.5^{abc}	771^{b}	689.9^{b}	41.2^{d}
5. As 1 + 0.30% Ca from limestone 1	684.7^{bc}	867.7^{abc}	790^{b}	807.4^{a}	43.6^{ab}
6. As 1 + 0.15% Ca from limestone 2	661.1^{cd}	865.7^{abc}	763^{b}	704.1^{b}	40.8^{de}
7. As 1 + 0.30% Ca from limestone 2	686.6^{bc}	875.0^{abc}	785^{b}	788.1^{a}	43.0^{bc}
8. As 1 + 0.15% Ca from limestone 3	653.7^{cd}	857.9^{bc}	762^{b}	652.0^{b}	39.8^{e}
9. As 1 + 0.30% Ca from limestone 3	714.9^{ab}	905.4^{ab}	791^{b}	846.3^{a}	44.8^{a}
10. As 1 + 0.15% Ca from limestone 4	633.8^{d}	828.9^{dc}	763^{b}	667.1^{b}	41.0^{de}
11. As 1 + 0.30% Ca from limestone 4	692.4^{bc}	884.3^{abc}	783^{b}	802.1^{a}	43.5^{ab}
12. As 1 + 0.15% Ca from limestone 5	685.5^{bc}	884.9^{abc}	776^{b}	707.2^{b}	41.4^{d}
13. As 1 + 0.30% Ca from limestone 5	724.0^{ab}	919.1^{a}	788^{b}	855.6^{a}	44.0^{ab}
Pooled SEM	19.4	20.8	16.6	23.0	0.46

[a-f] Means within a column with no common superscript are significantly different ($P < 0.05$).

[1] Values are means of 6 pens of 5 chicks; average initial body weight was 139.4 g. Diets were fed 9 to 21 days of age.

[2] Multiple regression of tibia ash (Y;mg) on supplemental Ca intake (g) from reagent grade $CaCO_3$ (X_1), limestone 1 (X_2), limestone 2 (X_3), limestone 3 (X_4), limestone 4 (X_5), limestone 5 (X_6) yielded the equation: = $Y = 542.0 + 107.3 \pm 8.61\ X_1 + 100.1 \pm 8.98\ X_2 + 103.3 \pm 9.57\ X_3 + 106.0 \pm 8.95\ X_4 + 96.4 \pm 9.04\ X_5 + 114.0 \pm 9.25\ X_6$ ($R^2 = 0.79$). The ($\pm$) values are standard errors of the regression coefficients.

[3] Multiple regression of tibia ash (%) on supplemental Ca intake (g) from reagent grade $CaCO_3$ (X_1), limestone 1 (X_2), limestone 2 (X_3), limestone 3 (X_4), limestone 4 (X_5), limestone 5 (X_6) yielded the equation: = $Y = 37.8 + 2.38 \pm 0.193X_1 + 2.20 \pm 0.201\ X_2 + 2.10 \pm 0.214\ X_3 + 2.35 \pm 0.200\ X_4 + 2.13 \pm 0.202\ X_5 + 2.30 \pm 0.207\ X_6$ ($R^2 = 0.78$). The ($\pm$) values are standard errors of the regression coefficient.

Table 3.6. Relative Ca bioavailability in the test limestone sources in Experiment 2[1].

Limestone source	Total Ca%	Bioavailability values[2] (%)		Bioavailable content[3] (%)	
		Tibia ash (mg/tibia)	Tibia ash (%)	Tibia ash (mg/tibia)	Tibia ash (%)
1	39.3	93.3	92.4	36.7	36.3
2	36.7	96.3	88.2	35.3	35.3
3	38.2	98.8	98.7	37.7	37.7
4	38.7	89.8	89.5	34.8	34.6
5	38.0	106.2	96.6	40.4	36.7

[1] No significant differences among values within columns.

[2] Calculated by the slope-ratio method using the regression equation in footnotes 2 and 3 in Table 2.5. Bioavailability values are relative to the Ca in reagent grade $CaCO_3$ which was set at 100%.

[3] Bioavailable content = (Total Ca x bioavailability value)/100. Values are presented on as-fed basis.

Table 3.7. Growth performance, ileal Ca digestibility, and total tract Ca retention values for ad libitum-fed chicks in Experiment 3[1].

Limestone source[2]	Weight gain (g/chick)	Feed intake (g/chick)	Gain: feed (g/kg)	Ileal Ca digestibility (%)	Total tract Ca retention (%)
1	67.8[a]	243.9[ab]	278.0[a]	22.4	19.2[b]
2	67.9[a]	258.3[ab]	262.8[a]	21.5	15.6[b]
3	67.1[a]	240.5[b]	279.4[a]	29.3	38.6[a]
4	70.3[a]	261.0[a]	270.0[a]	33.5	30.9[a]
5	67.8[a]	249.0[ab]	272.6[a]	34.3	20.4[b]
Pooled SEM	2.2	4.3	6.7	14.2	8.7

[a-b] Means within a column with no common superscript are significantly different ($P < 0.05$).

[1] Values are means of 8 pens of 5 chicks at 21 days of age.

[2] Corn-based diets containing approximately 0.90% Ca from the limestone sources (Table 3.3).

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