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Research Article

Apparent calcium retention and digestibility coefficients of limestone with different particle sizes in laying hens

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ABSTRACT: Apparent calcium (Ca) retention and digestibility coefficients are affected by limestone particle size in the diet of laying hens. This study aimed to determine the apparent retention and digestibility coefficients of Ca in limestone of different particle sizes in laying hens. The study comprised 288 Lohmann Brown laying hens (50 weeks of age; 1,964 ± 98 g) distributed in a completely randomized experimental design with a 3 × 2 factorial arrangement [three Ca concentrations (10, 20, and 30 g kg^{-1}) × two limestone particle sizes (480 and 1,978 μm)] with eight repetitions per treatment and six birds per experimental unit. The experiment included five days for adaptation and five days for total excreta collection. All birds were slaughtered at the end of the ten days to collect the ileal contents. The total or ileal Ca content was plotted against the Ca of diets concentration using linear regression analysis. The regression line slope represented the apparent retention (CaR) and digestibility coefficients of Ca (CaD) in limestone. There was interaction between Ca concentration in the diet and limestone granulometry on CaD (p = 0.001) and CaR (p < 0.001). The CaD and CaR of fine- and coarse-grained limestone increased linearly with increasing Ca concentrations in the diet. The apparent digestibility coefficients estimated for laying hens fed with fine-grained and coarse-grained limestone were 0.72 and 0.35, respectively. The apparent retention coefficients estimated for laying hens fed fine-grained and coarse-grained limestone were 0.96 and 0.47, respectively.

Keywords: birds, gizzard, minerals, regression

Introduction

The reliable determination of retention and digestibility of calcium (Ca) sources and an adequate protocol for this process are required to measure accurately Ca utilization in laying hens. Ca is the main mineral responsible for the internal and external quality of the egg in laying hens (Sahin et al., 2018). Eggshell formation directly affects Ca retention and ileal digestibility (Bar, 2009); thus, understanding their underlying mechanisms is essential. In addition, limestone is the main ingredient used as a Ca source in diets of laying hens. However, Ca digestibility can vary according to limestone particle sizes (limestone with fine *vs.* coarse grains) (Diana et al., 2021). Studies show that fine-grained limestone has a higher *in vitro* solubility than coarser-grained limestone and that as *in vitro* solubility increases, *in vivo* solubility decreases (Cheng and Coon, 1990a; Zhang and Coon, 1997). Anwar et al. (2016; 2017) studied the true digestibility of Ca from limestone with different particle sizes in broiler chicken and observed a lower coefficient than in fine-grained limestone. Nevertheless, it is difficult to affirm whether coarser limestone is the most indicated, as it requires evaluating the particle size limit of limestone (ideal particle size) used as Ca source for laying hens. Moreover, limestone concentration in the diet of laying hens is higher than that of broiler chicken (Adedokun et al., 2018), which may hamper the digestibility of coarse-grained limestone compared to fine-grained limestone.

The collection and analysis of ileal digesta and total excreta are the most widely used methods to determine nutrient digestibility in birds; however, the results of these methods vary (An et al., 2020). On the other hand, total excreta collection is less costly and does not require sacrificing the animals (Ravindran and Bryden, 1999). Nevertheless, the elimination of Ca through urine must be taken into account to measure Ca digestibility using the total collection method, which is unnecessary for the ileal collection method. Ca elimination in urine depends on hormonal control in renal excretion, where the plasmatic amount of phosphorus (P) and Ca participate in this control. The parathyroid hormone is activated at low Ca levels in the plasma through Ca-sensitive receptors, Ca excretion in urine, and increased Ca absorption by the intestine. Calcitonin is activated at high Ca levels in the plasma, acting inversely to the parathyroid hormone (Leeson and Summers, 2001). Therefore, it is essential to adopt the most appropriate method to determine Ca digestibility in laying hens.

This study aimed to determine the apparent digestibility coefficient of Ca in calcitic limestone of different particle sizes using different sampling methods in laying hens.

Materials and Methods

This research was conducted according to the institutional committee on animal use (06/2020). The experiment was conducted in Viçosa (20°45′14″ S, 42°52′53″ W, altitude 648.74 m), in the state of Minas Gerais, Brazil.

Birds and experimental design

The stud comprised 288 Lohmann Brown laying hens (1,964 ± 98 g). Before the experiment, the birds were reared in cages with free access to water and fed *ad libitum* with a corn and soybean meal-based standard mash diet to meet their nutritional requirements (including Ca and P levels), according to Rostagno et al. (2017).

At 50 weeks of age, the birds were distributed, based on their body weight and egg production, in a 3 × 2 [three concentrations of total Ca in the diet × two limestone granulometries (fine and coarse)] completely randomized factorial design, with eight replicates per treatment and six birds per experimental unit.

The birds were housed in cages equipped with a trough feeder and two nipple-type drinking troughs, in metabolic cages [25 cm wide, 35 cm long, and 40 cm high; two birds per cage (three cages comprised the experimental unit)] for 10 days and fed the experimental diets. The shed was kept at average maximum and minimum temperatures of 28.30 °C and 19.33 °C, respectively, under a 16 h photoperiod.

Limestone

The fine- and coarse-grained limestone used in this study were purchased from a local commercial source, from the same limestone source, to compare the particle size effects. A representative sample was used to evaluate the granulometry and *in vitro* solubility of each limestone (Table 1). The mean geometric diameter (MGD) and geometric standard deviation (GSD) of limestones were performed at the municipality of Concórdia, Santa Catarina State, Brazil, using conventional standard methodology (Zanotto and Bellaver, 1996; Embrapa, 2013). We also determined the contents of calcium (Ca), phosphorus (P), magnesium (Mg), fluor (Fl), sulfur (S), and iron (Fe) of each limestone type.

Table 1 – Particle size analysis, mean geometric diameter, geometric standard deviation, *in vitro* solubility and contents of calcium (Ca), phosphorus (P), magnesium (Mg), fluor (Fl), sulfur (S), and iron (Fe) of fine and coarse limestone.

Particle size (μm)	Fine	Coarse
	%	
2,000	0.1	77.1
1,190	1.4	21.8
595	45.6	0.6
297	35.1	0.1
210	4.8	0.1
149	4.9	0.1
125	1.1	0.0
37	7.0	0.2
Mean geometric diameter (μm)	480	1,978
Geometric standard deviation (μm)	2.06	1.31
In vitro solubility (%)	24.49	15.52
Mineral Composition (g kg^{-1})		
Ca	375.5	375.1
CaO	527.0	526.5
P	–	–
Mg	2.6	2.6
MgO	4.6	4.6
S	0.12	0.12
Fl	0.15	0.15
Fe	0.09	0.09

Diets

Six isoproteic and isoenergetic diets were formulated using fine and coarse limestone as the main Ca source (Table 2). The diets were prepared based on corn and soybean meal supplemented with amino acids, energy, vitamins, and minerals as recommended by Rostagno et al. (2017), except for the level of available P (aP) and total Ca.

Table 2 – Ingredients and nutritional composition of experimental diets.

Ingredients (g kg^{-1})	Level 1	Level 2	Level 3
Corn	569.3	569.3	569.3
Soybean meal	252.0	252.0	252.0
Cornstarch	75.0	37.5	0.0
Corn cob	42.0	36.0	30.0
Limestone	23.9	50.5	77.0
Monosodium phosphate	0.0	3.2	6.5
Soybean oil	17.7	32.7	47.7
Salt	4.2	2.9	1.6
Vitamin premix[a]	1.1	1.1	1.1
Trace mineral premix[b]	1.1	1.1	1.1
DL-Methionine	2.6	2.6	2.6
Choline chloride	1.0	1.0	1.0
BHT	0.1	0.1	0.1
Celite	10.0	10.0	10.0
Calculated composition			
Metabolizable energy (MJ kg^{-1})	12.34	12.34	12.34
Crude protein	160.5	160.5	160.5
Calcium	10.00	20.00	30.00
Available phosphorus	0.83	1.66	2.50
Total phosphorus	2.78	3.61	4.45
Ca: available phosphorus	12	12	12
Ca: total phosphorus	3.60	5.53	6.74
Digestible threonine	5.56	5.56	5.56
Digestible methionine	4.85	4.85	4.85
Digestible methionine + cysteine	7.08	7.08	7.08
Analyzed values			
Calcium[c]	9.96	20.24	30.38
Total phosphorus[c]	2.76	3.42	4.76
Ca: total phosphorus[c]	3.60	5.91	6.38
Calcium[d]	10.39	20.63	30.97
Total phosphorus[d]	2.79	3.64	4.79
Ca: total phosphorus[d]	3.72	5.66	6.47

[a]Containing per kg: Vit A – 15.000.000 UI; Vit D3 – 1.500.000 UI; Vit E – 15.000 UI; Vit B1 – 2.0 g; Vit B2 – 4.0 g; Vit B6 – 3.0 g; Vit B12 – 0.015 g; Nicotinic acid – 25.0 g; Ac. Pantothenic – 10.0 g; Vit K3 – 3.0 g; Folic acid – 1.0 g; Selenium – 0.25 g; antioxidant – 10.0 g and q.s. vehicle – 1,000 g; [b]Containing per kg: Manganese – 80 g; Iron – 80 g; Zinc – 50 g; Copper – 10 g; Cobalt – 2 g and Iodine – 1 g; [c]Values analyzed referring to feedstuffs containing fine limestone; [d]Values analyzed referring to feedstuffs containing coarse limestone.

We determined the Ca and P levels in the feed based on the protocol developed by the WPSA (2013) to determine P digestibility in broiler chickens, which recommends that the higher Ca and P levels did not exceed the requirement for the birds. All feeds kept a Ca:aP ratio of 12:1, as Rostagno et al. (2017) recommended for laying hens.

The calculated concentrations of total Ca in the diets were 10, 20, and 30 g kg^{-1}. All diets contained 10 g kg^{-1} of celite (acid-insoluble ash – AIA) as a marker. Diets were offered *ad libitum*.

Collection and processing of excreta and ileal digesta

The duration of the experiment included five days for adaptation and five days for total excreta collection. The excreta of all hens were collected twice a day and stored in plastic bags at −18 °C until the end of the collection period (five days). At the end of the 10-day period, all birds were slaughtered by cervical dislocation. The contents of part of the ileum (20 cm) near the cecal region (5 cm above the ileocecal junction) were collected and stored at −18 °C. The excreta were homogenized, and the ileal digesta were lyophilized. The samples were subsequently ground (0.5 mm particles) and stored in plastic jars for the chemical analyses.

Chemical analyses

The *in vitro* solubility of limestones was determined via percentage weight loss, as described by Cheng and Coon (1990b). The experiment was carried out in triplicate. A solution of hydrochloric acid (0.1 N) was heated in water bath for 15 min at 42 °C. Next, 100 mL of HCl solution was added to each limestone sample (2 g), and the mixture was incubated in the water bath for 10 min at 80 rpm. The mixture was then gravimetrically filtered through a paper filter (n° 41) and transferred to the greenhouse for 10 h at 60 °C. Following incubation in the drying oven, the samples were weighed to determine the percentage of weight loss.

Representative samples of diets, excreta, and ileal digesta were analyzed for DM (method 930.15; AOAC International, 2012), Ca, P, Mg, Fl, S and Fe (method 968.08D; AOAC International, 2012), and AIA (Van Keulen and Young, 1977).

Calculations

Total Ca retention and ileal Ca digestibility of limestone were calculated for each diet and replicated according to the following equation:

$$\text{Total retention or ileal Ca digestibility coefficient (\%)} = 100 - [100 \times (AIA_{diet} \times Ca_{excreta\ or\ digesta}) / (AIA_{excreta\ or\ digesta} \times Ca_{diet})],$$

where: AIA_{diet} was the concentration of acid-insoluble ash in the diet (g kg^{-1} DM), $Ca_{excreta\ or\ digesta}$ was the Ca concentration in the excreta or digesta (g kg^{-1} DM), $AIA_{excreta\ or\ digesta}$ was the concentration of acid-insoluble ash in the excreta or digesta (g kg^{-1} DM), and Ca_{diet} was the Ca concentration in the diet (g kg^{-1} DM).

The values from the first equation were used to calculate the content (g kg^{-1} of DM) of total digestible Ca and ileal digestible Ca for each of the diets, as follows:

$$\text{Total retained or ileal digestible Ca (g kg}^{-1}\text{ of DM)} = \text{total retention or ileal Ca digestibility (\%)} \times Ca_{diet}/100$$

The total or ileal Ca content (g kg^{-1} DM) of diets was plotted against the total Ca concentration (g kg^{-1} DM) using the linear regression analysis. The slope of the regression line represented the apparent retention and digestibility coefficients of Ca in limestone.

Statistical analysis

We used a 3 × 2 factorial arrangement of treatments to investigate the effects of three Ca concentrations from two limestone particle sizes on laying hens for each collection method (total and ileal). The data statistical procedures were conducted using PROC NLIN in SAS (Statistical Analysis System, version 9.2). The differences were considered significant at an alpha level of 0.05. A linear regression model was used to assess the effects of Ca levels in the diets on the parameters evaluated.

Results

The Ca concentration analyzed in fine-grained limestone at level one was 0.03 g kg^{-1} lower than the calculated value. At levels two and three, Ca concentrations were 0.25 and 0.38 g kg^{-1} higher than the calculated values, respectively (Table 2). In coarse-grained limestone, the analyzed Ca concentrations at levels one, two, and three were 0.40, 0.67, and 0.97 g kg^{-1} higher than the calculated concentrations, respectively.

The birds remained healthy and showed no leg problems or mortality throughout the trial. Retention coefficients (CaRC) and digestibility (CaDC) of Ca were estimated to calculate the values of Ca retained (CaR) and digested (CaD), respectively. There was no interaction between Ca concentrations in the diet and limestone particle size (fine and coarse), on CaDC (p = 0.062; Table 3). There was interaction between Ca concentration in the diet and limestone particle size on CaD (p = 0.001), CaRC (p < 0.001) and CaR (p < 0.001).

The CaD of fine- and coarse-grained limestone increased linearly with increasing Ca concentrations in the diet (Table 4). The apparent digestibility coefficients of Ca estimated for CaD were 0.72 and 0.35 for fine-grained and coarse-grained limestone, respectively.

The CaRC of fine-grained limestone increased ($Y = 0.012X + 0.412$; $r^2 = 0.55$), whereas the CaRC

Table 3 – Ca digestibility coefficients (CaDC), Ca digestible (CaD), Ca retention coefficients (CaRC), and Ca retained (CaR) of laying hens fed with three levels of Ca and two limestone particle sizes (fine and coarse) submitted to two sampling methods[a].

Limestone Granulometry	Ca level	CaDC	CaD (g kg^{-1} DM)	CaRC	CaR (g kg^{-1} DM)
Fine	Level 1	0.60	5.98	0.56	5.64
	Level 2	0.64	12.15	0.61	11.54
	Level 3	0.68	20.78	0.82	24.98
Coarse	Level 1	0.69	10.00	0.74	10.66
	Level 2	0.67	13.75	0.61	12.58
	Level 3	0.52	16.12	0.60	18.38
SEM[b]		0.02	8.79	0.02	0.96
Probability, $p \leq$					
Level		0.584	< 0.0001	0.053	< 0.0001
Granulometry		0.736	0.717	0.569	0.811
Level × Granulometry		0.062	0.001	< 0.0001	< 0.0001

[a]Each value represents the average of eight repetitions (six birds/repetition); [b]Standard error of the mean.

Table 4 – Linear relationship between calcium (Ca) digestible (CaD) and Ca retained (CaR) *vs.* Ca content in the diet.

		Regression Equations	SE of the slope[a]	SE of the intercept	r^2	Apparent coefficient of Ca
CaD	Fine	$Y = 0.725X - 1.364$	0.070	1.505	0.83	0.72
	Coarse	$Y = 0.355X + 5.475$	0.096	2.212	0.38	0.35
CaR	Fine	$Y = 0.957X - 4.861$	0.056	1.211	0.93	0.96
	Coarse	$Y = 0.475X + 3.421$	0.089	2.073	0.56	0.47

[a]Standard error of regression

of coarse-grained limestone decreased linearly ($Y = -0.008X + 0.827$; $r^2 = 0.18$) with increasing Ca levels added to the diet of laying hens. The CaR of fine- and coarse-grained limestone increased linearly with increasing Ca concentrations in the diet. The apparent retention coefficients of Ca estimated for CaR were 0.96 and 0.47 for fine-grained and coarse-grained limestone, respectively.

Discussion

Studies on broiler chickens show that Ca digestibility is higher when using coarse-grained than fine-grained limestone (Anwar et al., 2016; 2017). The authors attribute this occurrence to the lower *in vitro* solubility of coarse-grained limestone because, according to Zhang and Coon (1997), limestone with lower in *vitro* solubility presents higher *in vivo* solubility. However, our results show that, although coarse-grained limestone displayed lower *in vitro* solubility than fine-grained limestone (15.52 *vs*. 24.49), the highest coefficient corresponded to fine-grained limestone. Roland Sr (1986), Zhang and Coon (1997), and Anwar et al. (2016) believed that coarser limestone particles were retained in the chicken gizzard for a longer time, which can favor slower limestone digestion and prolong Ca availability to the birds (De Witt et al., 2006) due to its low *in vitro* solubility (Anwar et al., 2016). However, considering the percentage of coarse particle size distribution *vs.* fine particles and geometric mean diameter, we can infer that birds retain coarse particles in their gizzard for a limited time, as intact particles of coarse limestone were observed in the excreta and the digesta analyzed in this study. Zhang and Coon (1997) attribute the differences in the results to the differences of *in vitro* and *in vivo* solubilities of limestones, as well as to the intrinsic conditions of the digestive tract for the absorption of Ca in limestones and to the accumulation of limestone in the gizzard. This condition could negatively affect *in vivo* solubility, since Ca could be eliminated by the amount of Ca ingested later and the dissociation of $CaCO_3$ into ionic Ca, producing more Ca available for absorption.

As determined by Anwar et al. (2017), particle size distribution was 27 % for particles from 1,000 to 2,000 µm and 25 % for particles < 500 µm, considered as coarse and fine limestone, respectively. Moreover, the average geometric diameter was 526 µm, considering both limestone sizes. Conversely, our study showed higher values of particle size distribution of limestone (%) and geometric mean diameter [coarse-grained limestone = 1,190 µm (22 %) and 2,000 µm (77 %), with a geometric mean diameter of 1,978 µm; fine-grained limestone = < 595 µm (46 %), with a geometric mean diameter of 480 µm]. These results reinforce the hypothesis that laying hens have less Ca retention in diets with coarse-grained limestone in relation to broilers. Furthermore, we can infer that perhaps the digestion process of limestone particles in laying hens is different from that in broilers, considering the higher proportion of limestone in the diet of laying hens.

The total excreta collection method can present an obstacle in measuring nutrient retention, as the increased risk of sample contamination (Mutucumarana et al., 2014) could underestimate the actual retention coefficient. However, this did not apply to our study, as the apparent retention coefficient from the total collection method was 24 % for fine-grained limestone and 12 % for coarse-grained limestone, higher than that obtained from the ileal collection method. Undigested oligosaccharides in the upper gastrointestinal tract (Abdelqader et al., 2013) were potentially exploited by the microflora in the ceca of chickens. These oligosaccharides decrease the pH due to their microbial fermentation, favoring Ca solubility in water and its absorption (Rémésy et al., 1993) in response to the production of short-chain carboxylic acids (Roberfroid, 2000). Furthermore, Ngunjiri et al. (2019) stated that the diversity of the microbial population of the caecum is more significant than in the ileum. Besides, the birds in our study had the same oviposition cycle. Hens take approximately 24–25 h for complete egg formation, with the last 21 h responsible for shell formation (Johnson, 2015). During the night period, when there is a greater need for Ca, the birds do not feed and keep the intestinal lumen practically empty of Ca; thus, the use of serum Ca is more significant during this period (Bar, 2009). In addition, chickens show lower serum Ca levels between 18h00 and 20h00 and higher Ca levels from 8h00 to 12h00 (Sloan et al., 1974; Lin et al., 2018). According to Roland Sr et al. (1972), serum Ca levels have an inverse effect on Ca excretion. These statements may explain the different results found in our study for Ca absorption and retention values. David et al. (2021) suggest that the ileal and total digesta timing collection may affect the calculated coefficient results. Similarly, in our study, the ileal content samples were collected in the morning period (8h00 to 11h00), when serum Ca levels are higher, and, according to Sloan et al. (1974) and Lin et al. (2018), this time range shows higher serum Ca levels. This elevation of serum Ca levels may have influenced the decrease in Ca uptake. David et al. (2021) also suggest that the collection of total excreta content may occur at both egg formation cycles, favoring the dilution of the effects in the results, corroborating our study. This reinforces the hypothesis that laying hens display a higher retention coefficient of Ca when analyzing the total content of the extract.

Conclusion

The coefficients of retention and apparent digestibility estimated for laying hens fed with fine-grained limestone were higher concerning birds fed with coarse-grained limestone.

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Authors' Contributions

Conceptualization: Diana, T.F.; Calderano, A.A.; Rostagno, H.S.; Tavernari, F.C.; Albino, L.F.T. **Data acquisition**: Diana, T.F.; Marques, M.R.L. **Data analysis**: Diana, T.F.; Veroneze, R. **Design of methodology**: Diana, T.F.; Calderano, A.A.; Rostagno, H.S.; Albino, L.F.T. **Writing and editing**: Diana, T.F.; Calderano, A.A.; Rostagno, H.S.; Tavernari, F.C.; Albino, L.F.T.

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