

THIAGO FERREIRA DIANA

CALCIUM DIGESTIBILITY OF INORGANIC SOURCES FOR LAYING HENS

Thesis presented to the Animal Science Graduate Program of the Universidade Federal de Viçosa, in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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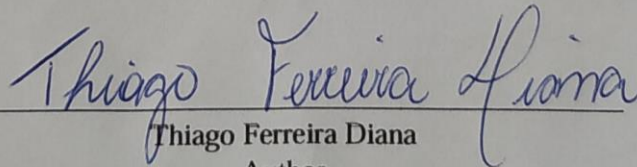
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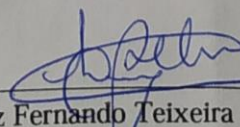
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ABSTRACT

DIANA, Thiago Ferreira, D.Sc., Universidade Federal de Viçosa, February, 2022. **Calcium digestibility of inorganic sources for laying hens.** Adviser: Luiz Fernando Teixeira Albino. Co-advisers: Arele Arlindo Calderano, Fabyano Fonseca e Silva (*in memoriam*) and Horacio Santiago Rostagno.

Two independent experiments were conducted in order to determine the digestibility of calcium from different inorganic sources. **Experiment one:** The apparent calcium (Ca) digestibility coefficient (ADC) and true digestibility coefficient (TDC) of different inorganic calcium sources were determined in laying hens of different ages. Three Ca digestibility tests were carried out, each assessing 240 Lohmann Brown lineage laying hens distributed in a completely randomized design. Nine dietary treatments were arranged in a 3×3 factorial design consisting of three ages (40, 50 and 70 weeks) \times three Ca (dicalcium phosphate (DCP) sources, fine (FL) and coarse (CL)) limestone, comprising eight replicates per treatment of six birds per experimental unit. Regarding the DCP, the ADC was higher ($P < 0.05$) in 40-week-old birds. The DCP ADC for 40-, 50- and 70-week-old birds was 0.889, 0.613 and 0.712, respectively. No effect ($P > 0.05$) of age on the ADC was noted for either FL or CL. Comparing Ca sources, DCP exhibited a higher ($P < 0.05$) ADC (0.889), followed by FL (0.699) and CL (0.515), in 40-week-old birds. DCP (0.712) and FL (0.652) presented ($P < 0.05$) higher ADC compared to CL (0.482), in 70-week-old birds. No effect of Ca sources at 50 weeks on the ADC was observed ($P > 0.05$). Endogenous loss values of 790, 860 and 930 mg.kg^{-1} of consumed dry matter were observed at 40, 50 and 70 weeks, respectively. For the TDC, no interaction ($P > 0.05$) was observed between Ca sources and bird age. The highest TDC value ($P > 0.05$) was found in birds fed DCP (0.786) followed by FL (0.637) and CL (0.534). In addition, birds at 40 weeks of age (0.714) exhibited higher TDC values ($P < 0.05$) compared to animals at 50 weeks of age (0.608). The findings reported herein demonstrate that the true digestibility is greater in the youngest birds and that consumed the DCP and the FL in relation to the birds that consumed the CL. **Experiment two:** The objective of this study was to determine the apparent retention and digestibility coefficients of Ca in limestone of different granulometries, in laying hens. A total of 288 Lohmann Brown laying hens (50 weeks of age; $1,964 \pm 98$ g) were distributed in a completely randomized experimental design with a 3×2 factorial arrangement [three Ca concentrations (10, 20, and 30 g kg^{-1}) \times two limestone granulometries (480 and 1978 μm)]. Eight repetitions per treatment, with six birds per experimental unit. The duration of the experiment included five days each of adaptation

and total excreta collection. At the end of the 10-day period, all birds were slaughtered and the ileal contents were collected. The total or ileal Ca content was plotted against the Ca of diets concentration using linear regression analysis. The slope of the regression line represented the apparent retention (CaR) and digestibility coefficients of Ca (CaD) in limestone. An interaction was found between Ca concentration in the diet and limestone granulometry on CaD ($p = 0.001$) and CaR ($p < 0.001$). The CaD of fine- and coarse-grained limestone increased linearly with increasing Ca concentrations in the diet. The 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 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: Calcitic Limestone. Dicalcium phosphate. Granulometry. Regression.

RESUMO

DIANA, Thiago Ferreira, D.Sc., Universidade Federal de Viçosa, fevereiro de 2022. **Digestibilidade do cálcio de fontes inorgânicas para galinhas poedeiras.** Orientador: Luiz Fernando Teixeira Albino. Coorientadores: Arele Arlindo Calderano, Fabyano Fonseca e Silva (*in memoriam*) e Horacio Santiago Rostagno.

Foram realizados dois experimentos independentes, a fim de determinar a digestibilidade do cálcio de diferentes fontes inorgânicas. **Experimento um:** O coeficiente de digestibilidade aparente de cálcio (Ca) (ADC) e o coeficiente de digestibilidade verdadeira (CDT) de diferentes fontes de cálcio inorgânico foram determinados em galinhas poedeiras de diferentes idades. Foram realizados três testes de digestibilidade do Ca, cada um avaliando 240 poedeiras da linhagem Lohmann Brown distribuídas em delineamento inteiramente casualizado. Nove tratamentos dietéticos foram dispostos em um delineamento fatorial 3×3 composto por três idades (40, 50 e 70 semanas) \times três fontes de Ca (fosfato bicálcico (DCP), calcário fino (FL) e grosso (CL)), compreendendo oito repetições por tratamento de seis aves por unidade experimental. Em relação ao DCP, o ADC foi maior ($P < 0,05$) nas aves de 40 semanas. O DCP ADC para aves de 40, 50 e 70 semanas foi de 0,889, 0,613 e 0,712, respectivamente. Nenhum efeito ($P > 0,05$) da idade no ADC foi observado para FL ou CL. Comparando as fontes de Ca, DCP apresentou maior ($P < 0,05$) ADC (0,889), seguido por FL (0,699) e CL (0,515), em aves de 40 semanas. DCP (0,712) e FL (0,652) apresentaram ($P < 0,05$) maior ADC em relação ao CL (0,482), em aves de 70 semanas. Nenhum efeito de fontes de Ca em 50 semanas foi observado no ADC ($P > 0,05$). Valores de perda endógena de 790, 860 e 930 mg.kg^{-1} de matéria seca consumida foram observados em 40, 50 e 70 semanas, respectivamente. Para o TDC, não foi observada interação ($P > 0,05$) entre as fontes de Ca e a idade das aves. O maior valor de TDC ($P > 0,05$) foi encontrado nas aves alimentadas com DCP (0,786) seguida de FL (0,637) e CL (0,534). Além disso, as aves com 40 semanas de idade (0,714) apresentaram valores de TDC mais elevados ($P < 0,05$) em relação aos animais com 50 semanas de idade (0,608). Os achados aqui relatados demonstram que a verdadeira digestibilidade é maior nas aves mais jovens e que consumiram o DCP e o FL em relação às aves que consumiram o CL. **Experimento dois:** O objetivo deste trabalho foi determinar os coeficientes de retenção aparente e digestibilidade do Ca em calcários de diferentes granulometrias, em galinhas poedeiras. Um total de 288 poedeiras Lohmann Brown (50 semanas de idade; 1.964 ± 98 g) foram distribuídas em um delineamento experimental inteiramente casualizado com arranjo fatorial 3×2 [três concentrações de Ca (10, 20 e 30 g

kg-1) × duas granulometrias de calcário (480 e 1978 μm)]. Oito repetições por tratamento, com seis aves por unidade experimental. A duração do experimento incluiu cinco dias cada de adaptação e coleta total de excretas. Ao final do período de 10 dias, todas as aves foram abatidas e o conteúdo ileal coletado. O teor de Ca total ou ileal foi plotado contra a concentração de Ca das dietas usando análise de regressão linear. A inclinação da linha de regressão representou os coeficientes de retenção aparente (CaR) e digestibilidade do Ca (CaD) no calcário. Foi encontrada interação entre a concentração de Ca na dieta e a granulometria do calcário no CaD ($p = 0,001$) e CaR ($p < 0,001$). O CaD do calcário de granulação fina e grossa aumentou linearmente com o aumento das concentrações de Ca na dieta. O CaR do calcário de granulação fina e grossa aumentou linearmente com o aumento das concentrações de Ca na dieta. Os coeficientes de digestibilidade aparente estimados para poedeiras alimentadas com calcário de grão fino e grão grosso foram 0,72 e 0,35, respectivamente. Os coeficientes de retenção aparente estimados para poedeiras alimentadas com calcário de granulação fina e granulação grossa foram 0,96 e 0,47, respectivamente.

Palavras-chave: Calcário Calcítico. Fosfato Bicalcico. Granulometria. Regressão.

SUMMARY

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CHAPTER 1

1.1. General introduction

With the increased demand for eggs from chickens by consumers, there is a need for constant advances in technology and genetic improvement of these animals. As a consequence of these advances, adjustments in the nutrition of these birds must be made periodically, in order to provide adequate levels of nutrients in the diets. Furthermore, these adjustments ensure better nutrient utilization and lower feed costs, since it is known that around 70 to 75% of the production costs are related to feeding these animals. In this context, calcium (Ca) and phosphorus (P) are the main essential minerals in the nutrition of laying hens, since they participate in almost all metabolic processes of these birds, such as growth, reproduction, energy metabolism, among other physiological processes (Bertechini, 2006), besides being important in bone and eggshell formation (Nunes et al., 2006; Leeson and Summers, 2008).

Ca is one of the main minerals in the formation and mineralization of bone in the form of hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), about 99% of the total Ca in the body of birds is present in the bone skeleton (Scott et al., 1982; Veum et al., 2010) and the rest is found in the extracellular matrix. Furthermore, the fate of Ca in the body of layers depends on their age and physiological state. In growing layers, the Ca is destined for bone formation, whereas in the laying period the Ca is destined for eggshell formation (Scherer et al., 2004). However, the metabolism of Ca is closely related to that of P, along with vitamin D₃, and this interrelationship establishes the homeostasis between Ca and P in the body of these animals (Li et al., 2017).

The synergistic effect between Ca and P is controlled by parathormone (PTH), vitamin D metabolism and calcitonin. This hormonal control guarantees an adequate absorption and

excretion of these minerals by the intestine of the birds, besides allowing adequate levels of them for metabolic processes and bone mineralization (Fernandéz, 1995).

Therefore, in the past, little attention was paid to determining the digestibility and ideal levels of Ca in diets for laying hens. However, current studies have been showing the need for investigation of this mineral, in order to maintain not only the adequate levels of this mineral, but also of others associated with it, considering that Ca interacts with other minerals, which makes the evaluation of its requirement difficult (Dell' Isola and Baião, 2001). Moreover, the granulometry and origin of limestone used in commercial laying diets are of fundamental importance, since a finer granulometry limestone improves the physiological efficiency of the bird, due to the higher solubility attributed to it (Rabon and Roland, 1985). On the other hand, coarser grained limestone takes a longer time to be solubilized, causing it to remain for a longer time in the gizzard of these animals. This permanence of coarser limestone in the gizzard improves the flow of Ca for eggshell formation, due to the lower energy expenditure with the deposition of this mineral in the bones of these birds (Cabó, 1987).

According to Cheng and Coon (1990a), coarser-grained limestone, because it remains longer in the birds' gizzard, its *in vivo* solubility is increased relative to finer-grained limestone. This *in vivo* solubility has a negative correlation with the *in vitro* solubility in Ca availability for laying hens (Cheng and Coon, 1990 b ; Zhang and Coon, 1997 a).

Another important issue in determining the digestibility of Ca is the methods used for its investigation. Basically, there is the ileal digesta collection method (Zhang and Adeola, 2017) and the total excreta collection method, where the apparent digestibility values are obtained, and when the endogenous Ca losses by the animal are determined, the true digestibility values are obtained (Sakomura and Rostagno, 2016).

Several authors have cited no difference between the values collected in both methods for determining Ca digestibility (González-Vega et al., 2013; Zhang et al., 2016). However, these authors used the animal model broilers and or pigs. Because of this, further investigations should be done to prove the effects of collection methods, for example, in laying hens at different stages of development.

1.2. Thesis objectives

The general objective of this thesis was to evaluate the digestibility of inorganic calcium sources in laying hens. The specific objectives were: i) to determine the apparent and true calcium digestibility coefficients of different inorganic Ca sources for layers of different ages; ii) to determine the apparent calcium digestibility coefficient of calcium in calcitic limestone of different granulometries using different sampling methods, in commercial layers.

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CHAPTER 2

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Age and Calcium Sources in Laying Hen Feed Affect Calcium Digestibility

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2.1. Abstract

The apparent calcium (Ca) digestibility coefficient (ADC) and true digestibility coefficient (TDC) of different inorganic calcium sources were determined in laying hens of different ages. Three Ca digestibility tests were carried out, each assessing 240 Lohmann Brown lineage laying hens distributed in a completely randomized design. Nine dietary treatments were arranged in a 3 × 3 factorial design consisting of three ages (40, 50 and 70 weeks) × three Ca (dicalcium phosphate (DCP) sources, fine (FL) and coarse (CL)) limestone, comprising eight replicates per treatment of six birds per experimental unit. Regarding the DCP, the ADC was higher ($P < 0.05$) in 40-week-old birds. The DCP ADC for 40-, 50- and 70-week-old birds was 0.889, 0.613 and 0.712, respectively. No effect ($P > 0.05$) of age on the ADC was noted for either FL or CL. Comparing Ca sources, DCP exhibited a higher ($P < 0.05$) ADC (0.889), followed by FL (0.699) and CL (0.515), in 40-week-old birds. DCP (0.712) and FL (0.652) presented ($P < 0.05$) higher ADC compared to CL (0.482), in 70-week-old birds. No effect of Ca sources at 50 weeks on the ADC was observed ($P > 0.05$). Endogenous loss values of 790, 860 and 930 mg.kg⁻¹ of consumed dry matter were observed at 40, 50 and 70 weeks, respectively. For the TDC, no interaction ($P > 0.05$) was observed between Ca sources and bird age. The highest TDC value ($P > 0.05$) was found in birds fed DCP (0.786) followed by FL (0.637) and CL (0.534). In addition, birds at 40 weeks of age (0.714) exhibited higher TDC values ($P < 0.05$) compared to animals at 50 weeks of age (0.608). The findings reported herein demonstrate that the true digestibility is greater in the youngest birds and that consumed the DCP and the FL in relation to the birds that consumed the CL.

Keywords: Dicalcium Phosphate, Digestion, Endogenous Calcium, Granulometry, Limestone

2.2. Introduction

The main inorganic calcium (Ca) sources used in bird diets are calcitic limestone and calcium phosphates, which exhibit variable Ca bioavailability [1]. However, the determination of Ca digestibility in these sources has received little attention, mainly due to their low cost, abundant availability and surplus global reserves [2] [3].

Recently, the initiative to determine digestible phosphorus (P) in feed has led to further attention concerning Ca digestibility [3]. In addition, young birds tend to absorb Ca more efficiently than older birds [4]. According to Albino et al. [5], older laying hens exhibited a 20% reduction in intestinal Ca absorption, increasing bone Ca mobilization and reducing carbonic anhydrase activity, leading to lower eggshell calcification.

Previous studies on broilers reported low true Ca digestibility coefficients for fine limestone [6] [7]. There was evidence that the Ca digestibility was due to the physical and solubility characteristics of the applied Ca sources [8]. Zhang and Coon [9] reported that laying hens displayed the ability to maintain larger food particles in their gizzards for longer periods of time, thus increasing solubility and in vivo use. Limestone particle size influences in vitro solubility [9] [10] [11] and a negative correlation between in vitro and in vivo Ca solubility in laying hens has been noted [9] [12].

Given the above, the hypothesis of this study was that laying hen age and inorganic Ca source may influence Ca digestibility. Thus, the aim herein was to determine the apparent and true Ca digestibility coefficients of different inorganic Ca sources for laying hens of different ages.

2.3. Material and methods

This research was authorized by the Animal Care and Use Committee of the Universidade Federal de Viçosa (process number 06/2020) and was carried out per the ethical principles from the Brazilian College of Animal Experimentation.

2.3.1. Experimental Design and Diets

Three Ca digestibility tests were carried out for laying hens of different ages (40, 50 and 70 weeks). A total of 240 Lohmann Brown lineage laying hens were used in each trial, distributed in a completely randomized design, comprising eight replicates per treatment of six birds per experimental unit. A 3×3 factorial scheme (three ages \times three Ca sources) was adopted. The birds were distributed according to their body weight (1.938 ± 190 g) and egg production. The interval of each test, the birds were fed with ration according to their requirement, according to the one proposed by Rostagno et al. [13]. The tested Ca sources were dicalcium phosphate and fine and coarse calcitic limestone.

A basal diet containing 1.10 g.Kg^{-1} total Ca and 0.89 g.Kg^{-1} available P was formulated. The tested Ca sources replaced basal diet starch in varying amounts (DCP – basal diet + dicalcium phosphate; FL: basal diet + fine-grained calcitic limestone; CL: basal diet + coarse-grained limestone) (Table 1). A diet free of available Ca and P was also formulated to determine endogenous Ca losses (Table 2).

Table 1. Ingredient composition and analysis (g kg^{-1}) of experimental diets.

Ingredients	Basal	DCP	FL	CL
Corn	598.08	598.08	598.08	598.08
Soybean meal	279.60	279.60	279.60	279.60
Soy oil	10.00	10.00	10.00	10.00

Sugar	40.00	40.00	40.00	40.00
Common Salt	4.97	4.97	4.97	4.97
Starch	60.83	40.83	-	-
Dicalcium phosphate	-	20.00	-	-
Calcite Limestone	-	-	60.83	60.83
Vitamin Supplement ¹	1.10	1.10	1.10	1.10
Mineral Supplement ²	1.10	1.10	1.10	1.10
DL-Methionine	2.91	2.91	2.91	2.91
L-Threonine	0.31	0.31	0.31	0.31
BHT ³	0.10	0.10	0.10	0.10
Choline chloride	1.00	1.00	1.00	1.00
Calculated composition				
Crude Protein	175.9	175.9	175.9	175.9
Metabolisable energy (MJ Kg ⁻¹)	12.14	12.14	12.14	12.14
Calcium	1.1	6.0	24.0	24.0
Available Phosphorus	0.89	4.59	0.89	0.89
Ca: Available Phosphorus	1.23	1.30	26.96	26.96
Analysed composition				
Dry matter	894.9	921.6	909.6	926.6
Calcium	1.23	6.63	27.03	26.66
Available Phosphorus	0.91	4.64	0.94	0.97
Ca: Available Phosphorus	1.35	1.43	28.75	27.49

¹ Containing per kg: Vit. A - 15,000,000 International Unite (IU); Vit. D3 - 1,500,000 IU; Vit E - 15,000 IU; 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; B.C. Pantothenic - 10.0 g; Vit. K3 - 3.0 g; B.C. folic-1.0 g; Zinc bacitracin - 10.0 g; Selenium - 0.25 g and antioxidant - 10.0 g. ² Containing per kg: Manganese- 80 g; Iron - 80 g; Zinc - 50 g; Copper - 10 g and Cobalt - 2 g; Iodine-1 g. ³Butyl Hydroxy Toluene. MJ= Mega Joule.

Table 2. Composition and analysis of ingredients (g kg⁻¹) of the Ca and P free feed.

Ingredients	Amounts
Corn cob	166.90
Starch	800.00
Soy oil	10.00
Potassium carbonate	6.50
Common Salt	4.79
Vitamin Supplement ¹	0.50
Mineral Supplement ²	0.50
DL-Methionine	0.51
L-Lysine HCl	3.22
L-Threonine	0.70
L-Valine	0.42
L-Arginine	2.17
Glycine	1.50
L-Tryptophan	0.42
L-Isoleucine	0.28
BHT ³	0.10
Choline chloride	1.00

Calculated Composition

Crude Protein	70.93
Metabolisable energy (MJ Kg ⁻¹)	12.14
Calcium	0.00
Available Phosphorus	0.00
Total Phosphorus	0.00

Analysed composition

Dry matter	917.9
Calcium	0.00
Total Phosphorus	0.02

¹ Containing per kg: Vit. A - 15,000,000 IU; Vit. D3 - 1,500,000 IU; Vit E - 15,000 IU; 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; B.C. Pantothenic - 10.0 g; Vit. K3 - 3.0 g; B.C. folic-1.0 g; Zinc bacitracin - 10.0 g; Selenium - 0.25 g and antioxidant - 10.0 g. ² Containing per kg: Manganese- 80 g; Iron - 80 g; Zinc - 50 g; Copper - 10 g and Cobalt - 2 g; Iodine-1 g. ³Butyl Hydroxy Toluene. MJ= Mega Joule.

2.3.2. Birds

The chicks used were acquired from a 1-day-old local hatchery and were fixed on the floor in an open shed and, at the 17th week of age, were transferred to a laying shed (60 × 9 m) covered with clay tiles, housed in cages [25 cm wide, 35 cm long and 40 cm high; two birds per cage (three cages combined with the experimental unit)].

During the rearing, rearing and production phases up to 40 weeks of age, the birds were managed as described in the lineage manuals and fed with rations formulated according to the recommendations of Rostagno et al. [13].

In the 40th week, the birds were fed with the experimental rations for nine days, being five days for adaptation to the cage and the experimental diet and four days for total excrement collection.

At each end of the experimental phase (nine days), the birds received the feed formulated according to the recommendations of Rostagno et al. [13], until they are submitted to a new digestibility test.

Ambient temperature ($^{\circ}\text{C}$) was monitored through three maximum and minimum thermometers located throughout the shed. The maximum and minimum mean shed temperatures varied between 29.08°C and 18.33°C , respectively. A 16 h light/8h dark photoperiod was applied. Food and water were provided ad libitum

2.3.3. Sample Collection and Processing

Hen excreta were collected and stored in plastic bags in a freezer (-18°C) until the end of the collection period of each test. The hens received a diet free of calcium and phosphorus to assess endogenous losses. At the end of the collection period, the excreta were thawed, weighed, homogenized, dried in a ventilated oven at 55°C for 72 h and ground and stored in plastic containers for subsequent analyses.

The following data was collected: feed intake (g) (FI), dry matter intake (g) (DMI), Ca intake (g) (CaI), basal feed diet and Ca sources (g), Ca supply by the basal diet and food (g.Kg^{-1}), Ca content in the diets and excreta (g.Kg^{-1}), Ca excretion (g) and Ca excreted by birds receiving a diet with low Ca content (g.Kg^{-1}). These data were used to obtain the apparent and true Ca digestibility coefficients values and apparent dry matter digestibility coefficient, using the equations adapted by Rostagno and Featherston [14].

2.3.4. Calculations

Apparent Ca Digestibility Coefficient (ADC).

$$\text{ADC} = \frac{\text{Ingested Ca (g)} - \text{Excreted Ca (g)}}{\text{Ingested Ca (g)}}$$

True Ca Digestibility Coefficient (TDC).

$$\text{TDC} = \frac{[\text{Ingested Ca (g)} - (\text{Excreted Ca (g)} - \text{Endogenous Ca})]}{\text{Ingested Ca (g)}}$$

Apparent dry matter digestibility coefficient (ADMDC).

$$\text{ADMDC} = \frac{\text{Ingested dry matter (g)} - \text{Excreted dry matter (g)}}{\text{Ingested dry matter (g)}}$$

2.3.5. Chemical Analyses

The mean geometric diameter (MGD) determinations of the feed were performed at the Embrapa—CNPSA Animal Nutrition laboratory, in Concordia, SC, using the Softgran Granucalc® program [15]. This is an alternative method that approximates the applied screen meshes (Table 3).

Table 3. Mean geometric diameter (MGD), geometric standard deviation (GSD), solubility and Ca and P concentrations of the test foods analyzed in the laying bird experiments

Test feeds	MGD (µm)	GSD	Solubility (%)	Ca (g Kg ⁻¹)	P (g Kg ⁻¹)
DCP	560.00	2.58	-	266.10	184.90
FL	558.00	1.98	21.55	365.50	-

CL	1998.50	1.29	15.52	363.50	-
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Ca content was determined by Flame Atomic Absorption Spectrometry [16]. In vitro solubility was determined according to Cheng and Coon [10], through the percentage of weight loss, where 100 mL of 0.1 N hydrochloric acid was added.

2.3.6. Statistical Analyses

The analysis of variance was performed according to the statistical model to a completely randomized design:

$$Y_{ij} = \mu + t_i + \beta_j + (t\beta)_{ij} + \epsilon_{ij},$$

in which Y_{ij} = observed value for treatment i , in repetition j , μ = average of the experiment, t_i = effect of level i of factor t , β_j = effect of level j of factor β , $(t\beta)_{ij}$ = effect of the interaction between t_i and β_j , and ϵ_{ij} = random error associated to each observation.

The data were submitted to an ANOVA test, and the means were compared by the Tukey test at a 5% probability using the statistical package R Core Team [17].

2.4. Results

An interaction ($P < 0.05$) was observed between Ca sources and bird age (weeks), for FI and DMI (Table 4). The highest FI and DMI were found in 40- and 50-week-old birds relative to 70-week-old birds when consuming CL and in 40- and 70-week-old birds when consuming FL relative to DCP and CL ($P < 0.05$).

Like FI and DMI, CaI also showed an interaction ($P < 0.05$) between Ca sources and bird age. Birds at 40 and 50 weeks of age showed higher CaI ($P < 0.05$) when consuming CL compared to birds at 70 weeks of age. The highest CaI ($P < 0.05$) was found in the 40- and

70-week-old birds that consumed FL followed by CL and DCP, and at 50 weeks of age ($P < 0.05$) that consumed FL and CL followed by DCP.

For ADMDC, there was no effect ($P > 0.05$) of Ca sources, bird ages and their interaction.

Concerning the ADC, an interaction ($P < 0.05$) between Ca sources and bird age was detected (Table 5). Regarding DCP, the ADC was higher ($P < 0.05$) in 40-week-old birds, while no effect of age ($P > 0.05$) on ADC was observed for FL and CL ($P > 0.05$).

Comparing Ca sources, DCP exhibited a higher ADC ($P < 0.05$), followed by FL and CL in 40-week-old birds. DCP and FL exhibited a higher ADC ($P < 0.05$) compared to CL in 70-week-old birds, while no effect ($P > 0.05$) of Ca sources on ADC was observed at 50 weeks.

Endogenous losses of 790, 860 and 930 $\text{mg}\cdot\text{kg}^{-1}$ of consumed dry matter were obtained at 40, 50 and 70 weeks of age, respectively.

For TDC, no interaction ($P > 0.05$) was observed between Ca sources and bird age. The highest TDC ($P > 0.05$) was observed in birds fed DCP, followed by FL and CL. In addition, 40-week-old birds exhibited a higher TDC value ($P < 0.05$), compared to 50-week-old birds.

Table 4. Means for feed intake (FI), dry matter intake (DMI), Ca intake (CaI) and apparent dry matter digestibility coefficient (ADMDC) of Ca sources at different ages for laying birds¹

	Sources	Age (weeks)			Means ^{Sources}	SEM	P value		
		40	50	70			Age	Sources	Age x Sources
FI (g/ hen d)	DCP	89.340Ba	90.430Aa	78.334Ba	86.035				
	FL	106.201Aa	101.916Aa	107.041Aa	105.053	10.613	0.001	<0.001	0.004
	CL	94.764ABa	99.139Aa	74.354Bb	96.952				
	Mean ^{Age}	96.768	97.162	86.576					
DMI (g/ hen d)	DCP	81.588Ba	83.352Aa	72.850Ba	79.263				
	FL	96.110Aa	93.131Aa	97.407Aa	95.549	9.722	0.001	<0.001	0.004
	CL	87.258ABa	92.119Aa	69.138Bb	82.838				
	Mean ^{Age}	88.319	89.534	79.798					
CaI (g/ hen d)	DCP	0.543Ca	0.568Ba	0.463Ca	0.524				
	FL	2.681Aa	2.466Aa	2.600Aa	2.582	0.221	<0.001	<0.001	<0.001
	CL	2.380Ba	2.411Aa	1.822Bb	2.204				
	Mean ^{Age}	1.868	1.871	1.628					

	DCP	0.864	0.887	0.874	0.874				
ADMDC	FL	0.866	0.882	0.886	0.878	0.029	0.080	0.495	0.752
	CL	0.861	0.883	0.860	0.868				
	Mean ^{Age}	0.864	0.883	0.874					

Means followed by different lowercase letters on the line indicate a statistically significant difference by the Tukey test (P <0.05).

Means followed by different capital letters in the column indicate a statistically significant difference by the Tukey test (P <0.05).

¹Each value represents the mean of eight replicates (six birds per replicate).

SEM= standard error mean.

Table 5. Means for Ca, Apparent (ADC) and True (TDC) Digestibility Coefficients of Ca sources, at different ages, for laying birds¹

Sources	Age (weeks)			Means ^{Sources}	SEM	P value		
	40	50	70			Age	Sources	Age x Sources
ADC	DCP	0.889Aa	0.613Ab	0.712Ab	0.738			
	FL	0.689Ba	0.556Aa	0.652Aa	0.633	0.011	<0.001	0.040
	CL	0.515Ca	0.550Aa	0.482Ba	0.516			
	Mean ^{Age}	0.698	0.574	0.615				
TDC	DCP	0.892	0.713	0.754	0.786A			
	FL	0.692	0.559	0.662	0.637B	0.013	<0.001	0.208
	CL	0.558	0.553	0.492	0.534C			
	Mean ^{Age}	0.714a	0.608b	0.636ab				

Means followed by different lowercase letters on the line indicate a statistically significant difference by the Tukey test (P <0.05).

Means followed by different capital letters in the column indicate a statistically significant difference by the Tukey test (P <0.05).

¹Each value represents the mean of eight replicates (six birds per replicate).

SEM= standard error mean.

2.5. Discussion

The higher in vitro solubility of fine-grained limestone compared to coarse particles observed in the present study has also been observed in previous assessments [6] [7] [10] [18]. The larger surface area of the FL particle counts may increase the hydrochloric acid reaction and consequently, in vitro solubility [19].

The calculated Ca value in the limestones specified by Rostagno et al. [13] (377.00 g.Kg⁻¹) and by the NRC [20] (380.00 g.Kg⁻¹) were close to those analyzed in the thin (365.50 g.Kg⁻¹) and coarse (363.50 g.Kg⁻¹) limestones assessed in the present study. The present findings are consistent with previously published data, in which limestone Ca concentrations range between 360.00 and 415.00 g.Kg⁻¹ [21] [22] [23]. Anwar et al. [7] state that limestone cannot exceed 400.00 g.Kg⁻¹ of Ca based on the atomic and molecular weights of Ca carbonate, and that the aforementioned values can be attributed to analytical errors, contamination with Ca hydroxide or both.

The higher values found for FI and DMI, in the present study, with the 40 and 50-week-old birds, compared to older birds (70 weeks old), when treated with CL, may be attributed to the larger granulometry of limestone, because according to Portella et al. [24] and Nir et al. [25], older chickens tend to select and ingest larger feed particles. This greater intake of these feed particles may interfere with the dwell time of the feed in the gizzard and the rate of intestinal transit [1], leading to greater satiety of the animal and decreased FI. This finding corroborates that found by Geraldo et al. [26], when evaluating two limestone granulometry (0.135 mm versus DGM = 0.899 mm) during 8 to 12 weeks of age of Lohmann—LSL birds, observed lower IF of older birds that consumed feed with limestone with higher granulometry.

Given the results in this study, within each age (40 and 70 weeks of chicken age), birds treated with FL compared to CL and DCP, showed higher FI and DMI. These results

reinforce that due to the greater consumption of coarser particles and longer time the feed remains in the gizzard, the hens are satiated longer, which reduces FI and consequent reduction in DMI. However, the DCP, by presenting the MGD (560.00 μm) close to the MGD of FL (558.00 μm), should not be attributed to the particle size of the DCP, but in relation to its Ca digestibility coefficient, which may have favored its lower FI and DMI.

The results of this study indicate that laying hens were more efficient in digesting Ca from DCP and FL, which contributed, in part, to the higher TDC values observed for DCP and FL compared to CL. This can be explained by the particle size of these ingredients (Table 3), as DCP and FL exhibit lower MGD, which, in turn, affects intestinal functions and digestive processes [27] [28]. Finer particle size increases the contact surface area of the particles per unit volume, which can improve digestion efficiency and nutrient absorption [9] [30]. In addition, Ca levels in the DCP diet (6.00 g.Kg^{-1}) were lower than in the limestone diet (24.00 g.Kg^{-1}), where it can be demonstrated by CaI. Thus, higher Ca levels may have intensified the lower Ca absorption effect observed in the CL diet [31].

Birds consuming diets with high levels of Ca tend to have a low absorption rate for this mineral. This low absorption rate is associated with saturation of the Ca transport protein [32]. In addition, due to the increase in pH promoted by high Ca levels in the gut, the formation of insoluble calcium phosphate is reduced due to the neutralization in the gut promoted by the increase in pH [33]. Furthermore, in older birds, the capacity to absorb Ca is lower compared to younger birds [34].

Similar results were reported by Mayer [35], who observed a 10% decrease in the apparent Ca digestibility rate (0.376 versus 0.275) with increased limestone particles from 126 to 933 micrometers. However, intact particles (coarse limestone) were notoriously excreted, explaining the low Ca CL digestibility.

The hens were expected to exhibit greater CL digestion compared to FL. Anwar et al. [2] when comparing the effect of calcium source and particle size (fine (<0.5 mm) and coarse (1 - 2 mm)) on the apparent ileal Ca digestibility coefficient for broilers reported higher coefficients for birds that consumed the larger particles, 0.430, versus 0.710 that consumed fine particles. It is possible that the inclusion of coarser particle size particles with low in vitro solubility increased in vivo solubility and caused improvements in nutrient retention and absorption in laying hen [9] [36]. In addition, diets with larger particles positively influence the development and size of poultry gizzards [37] [38], as more rustic diets stimulate intestinal motility [39], due to the stimulus of cholecystokinin release [40]. This, in turn, acts on the release of endogenous enzymes and gastro-duodenal reflux [41] [42], which favors a decrease in the rate of digestion passage [25], and an increase in nutrient digestibility [43], differing from the results of the present study.

The TDC observed in this study was higher in 40-week-old birds compared to 50-week-old birds. A similar result was reported by Sordi et al. [44] when evaluating Ca digestibility for laying hens, who also noted a decrease in the true Ca digestibility coefficient of different DCP and limestone granulometries with increasing bird age. These results, in addition to being influenced by apparent digestibility, may also be due to the average endogenous Ca losses of the hens. Older birds exhibited higher endogenous losses, which may have influenced the reduction of TDC since, in the present study, endogenous loss values of 790, 860 and 930 mg.Kg⁻¹ of consumed dry matter were detected at 40, 50 and 70 weeks of age, respectively. David et al. [3], observed a higher coefficient of true ileal digestibility for limestone (0.510) in broilers compared to DCP (0.320). These results differ from those reported herein, where DCP (0.786) presented a higher coefficient compared to FL (0.637) and CL (0.534). Due to the discrepancies found in the present study in relation to other

published assessments, further studies related to age and Ca source effects on Ca digestibility in laying hens are suggested.

2.6. Conclusion

It can be concluded that currently researches still disagree regarding the ideal granulometry of calcium sources associated to their digestibility, mainly related to limestone. However, in the current study, although the true digestibility was higher in younger birds when treated with DCP and FL in relation to CL, some points still need to be elucidated, such as the proof that smaller limestone particles are more efficient in calcium digestibility, especially for brown laying hens.

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2.8. References

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CHAPTER 3

Manuscript being considered for publication in Scientia Agricola

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

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3.1. Abstract

The objective of this study was to determine the apparent retention and digestibility coefficients of Ca in limestone of different granulometries, in laying hens. A total of 288 Lohmann Brown laying hens (50 weeks of age; $1,964 \pm 98$ g) were distributed in a completely randomized experimental design with a 3×2 factorial arrangement [three Ca concentrations (10, 20, and 30 g kg^{-1}) \times two limestone granulometries (480 and $1978 \mu\text{m}$)]. Eight repetitions per treatment, with six birds per experimental unit. The duration of the experiment included five days each of adaptation and total excreta collection. At the end of the 10-day period, all birds were slaughtered and the ileal contents were collected. The total or ileal Ca content was plotted against the Ca of diets concentration using linear regression analysis. The slope of the regression line represented the apparent retention (CaR) and digestibility coefficients of Ca (CaD) in limestone. An interaction was found between Ca concentration in the diet and limestone granulometry on CaD ($p = 0.001$) and CaR ($p < 0.001$). The CaD of fine- and coarse-grained limestone increased linearly with increasing Ca concentrations in the diet. The 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 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, retention

3.2. Introduction

A reliable determination on the retention and digestibility of calcium (Ca) sources, as well as the determination of an adequate protocol for this process is required to measure Ca

utilization in laying hens accurately. Among all nutrients for laying hens, calcium is the main mineral responsible for the internal and external quality of the egg (Sahin et al., 2018). As eggshell formation directly affects both the retention and ileal digestibility of Ca (Bar, 2009), it is important to understand their underlying mechanisms. In addition, limestone is the main ingredient used as a source of Ca in the diets of laying hens. However, Ca digestibility can vary according to limestone granulometries (limestone with fine granulometry *vs* coarse grain) (Diana et al., 2021). Studies have shown that fine-grained limestone has a higher *in vitro* solubility than coarser-grained limestone and that the higher the *in vitro* solubility, the lower the *in vivo* solubility (Cheng and Coon, 1990a; Zhang and Coon, 1997). Anwar et al. (2016; 2017) studied the true digestibility of Ca from limestone with different granulometries in broiler chicken and observed a lower coefficient value when evaluating fine-grained limestone. However, it is difficult to affirm whether coarser limestone is the most indicated, considering that it is necessary to evaluate the particle size limit of limestone (ideal particle size) used as a source of Ca for laying hens. Moreover, the concentration of limestone included in the diet of laying hens is higher than that of broiler chicken (Adedokun et al., 2018). This can hamper the digestibility of coarse-grained limestone compared to that of fine-grained limestone.

To determine the digestibility of nutrients in birds, the most widely used methods are via the collection and analysis of ileal digesta and total excreta. However, the results observed using these methods can vary (An et al., 2020). By contrast, the total excreta collection method is less costly and does not require the sacrifice of animals (Ravindran and Bryden, 1999). However, to measure Ca digestibility using the total collection method, the elimination of this mineral through urine must be taken into account, which is not necessary for the ileal collection method. The elimination of Ca in the urine is dependent on hormonal control in renal excretion, where the plasmatic amount of P and Ca itself participate in this control. Low

levels of plasma Ca, parathyroid hormone is activated, through Ca-sensitive receptors, the excretion of Ca in the urine and increased absorption of this mineral by the intestine. At high plasma levels of Ca, calcitonin is activated, acting inversely to parathyroid hormone (Leeson and Summers, 2001). Therefore, it is important to adopt the most appropriate method to determine Ca digestibility in laying hens.

Thus, we aimed to determine the apparent digestibility coefficient of Ca in calcitic limestone of different granulometries using different sampling methods, in laying hens.

3.3. Material and methods

Research on animals 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, and 648,74 m altitude), in the state of Minas Gerais, Brazil.

3.3.1. Birds and experimental design

A total of 288 Lohmann Brown laying hens (1.964 ± 98 g) were used. Prior to 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 formulated 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 x 2 [three concentrations of total Ca in the diet x 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 a metabolic cages [25 cm wide, 35 cm long, and 40 cm high; two birds per cage (three cages make up the experimental unit)] for 10 days, and fed the experimental

diets. The shed was maintained at an average maximum and minimum temperature of 28.30 °C and 19.33 °C, respectively, under a 16h00 photoperiod.

3.3.2. Limestone

The fine- and coarse-grained limestone used in this study were purchased from a local commercial source. Both limestones were acquired from the same limestone source in order to answer the proposed objectives (to compare 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 *Laboratório de Análises Físico Químicas* (LAFQ) of *Embrapa Suínos e Aves*, using conventional standard methodology (Zanotto and Bellaver, 1996; *Embrapa Suínos e Aves*, 2013). The Ca, P, Mg, Fl, S and Fe content of each limestone type was also determined.

3.3.3. Diets

Six isoproteic and isoenergetic diets were formulated using fine and coarse limestone as the main source of Ca (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 phosphorus (aP) and total Ca.

We determined the Ca and P levels in the feed by following the principles of the protocol developed by the WPSA (2013) for the determination of P digestibility in broiler chickens, which recommend that the higher Ca and P levels not exceed the bird's requirement. All feeds maintained a Ca:aP ratio of 12:1, as recommended by Rostagno et al. (2017), 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*.

3.3.4. Collection and processing of excreta and ileal digesta

The duration of the experiment included five days each of adaptation and five days 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 further chemical analysis.

3.3.5. Chemical analysis

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 a 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 10h00 at 60 °C. Following incubation in the drying oven, the samples were weighed to determine the percentage weight loss.

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

3.3.6. Calculations

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

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

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

The values obtained 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:

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

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

3.3.7. Statistical analysis

A 3×2 factorial arrangement of treatments was used to investigate the effects of three Ca concentrations from two limestone granulometries on laying hens, for each collection methods (total and ileal). Data were analyzed using the SAS 9.4 GLM procedure (SAS Institute Inc., Cary, NC, USA). 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 evaluated parameters.

3.4. Results

The analyzed Ca concentration 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, with no leg problems and no mortality throughout the trial period. Retention coefficients (CaRC) and digestibility (CaDC) of Ca were estimated to calculate the values of Ca retained (CaR) and digestible (CaD), respectively. No interaction was detected between Ca concentrations in the diet and limestone granulometry (fine and coarse), on CaDC ($p = 0.062$; Table 3). An interaction was found between Ca concentration in the diet and limestone granulometry 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 that of coarse-grained limestone decreased linearly ($Y = -0.008X + 0.827$; $r^2 = 0.18$) with increasing Ca levels included in 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.

3.5. Discussion

Studies on broiler chickens have shown that Ca digestibility was higher when using coarse-grained limestone than when using fine-grained limestone (Anwar et al., 2016; 2017). These 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, the results of the present study showed that, although coarse-grained limestone displayed a lower solubility *in vitro* compared to fine-grained limestone (15.52 vs. 24.49), the highest coefficient corresponded to fine-grained limestone. Roland (1986), Zhang and Coon (1997), and Anwar et al. (2016) believed that coarser limestone particles were retained in the chicken gizzard for longer. This permanence of particles in the gizzard can favor slower limestone digestion and maintain Ca availability to the birds for longer (De Witt et al., 2006) due to its low solubility *in vitro* (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 digesta analyzed in this study. This observation can be explained by Zhang and Coon (1997), these authors attribute the differences in the results not only to the different solubilities *in vitro* vs. the *in vivo* solubility of limestones, but also the intrinsic conditions of the digestive tract for the absorption of Ca in limestones, the accumulation of limestone in the

gizzard, which would negatively affect solubility *in vivo*, since Ca would end up being eliminated by the action of the amount of Ca ingested later and the dissociation of CaCO_3 into ionic Ca, producing more Ca available for absorption.

Particle size distribution, as determined by Anwar et al. (2017), was 27% for particles that were 1000 to 2000 μm and 25% for those that were < 500 μm , considered as coarse and fine limestone, respectively. Moreover, the average geometric diameter was 526 μm , considering both limestone sizes. By contrast, the present study revealed superior values from the analysis of particle size distribution of the limestone (%) and geometric mean diameter (coarse-grained limestone = 1190 μm (22%) and 2000 μm (77%), with a geometric mean diameter of 1978 μm ; fine-grained limestone = < 595 μm (46%), with a geometric mean diameter of 480 μm). These results reinforce the above hypothesis, that laying hens have less calcium 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 former's diet.

The total excreta collection method can present an obstacle in the measurement of nutrient retention, as the increased risk of sample contamination (Mutucumarana et al., 2014) may result in an underestimation of the real retention coefficient. However, this did not apply to the present 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 microflora present in the chickens' ceca. These oligosaccharides contribute to a decrease in pH due to their microbial fermentation, favoring the solubility of Ca 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 greater than that of the ileum. Furthermore, the birds in the present study had the same ovoposition cycle. Hens take approximately 24-25 hours for complete egg formation, with the last 21 hours responsible for shell formation (Johnson 2015). The night period, where there is a greater need for Ca, the birds are not feeding, due to the dark period, keeping the intestinal lumen practically empty of Ca and the use of serum Ca is more significant during this period (Bar, 2009). Also, chickens show lower serum Ca levels between 6 to 8 pm and higher from 8 to 12 am (Sloan et al., 1974; Liu et al., 2018). According to Roland et al. (1972), serum Ca levels have an inverse effect on Ca excretion. These statements may explain the different results found in this study, between Ca absorption and retention values. David et al. (2021) suggest that the timing of ileal and total digesta collection may interfere with the calculated coefficient results. Consistent with this, in the current study, the ileal content samples was collected in the morning period (8 to 11 am), where, which according to Sloan et al. (1974) and Liu et al. (2018), is the time range when serum Ca levels are elevated. This possible 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 have been at both egg formation cycles and this favors in diluting the effects caused in the results, corroborating with the present study. This reinforces the hypothesis that laying hens display a higher retention coefficient of Ca when analyzing the total content of the extract.

3.6. Conclusion

The apparent digestibility coefficients estimated for laying hens fed 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.

3.7. Acknowledgments

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3.8. References

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Table 1 - Particle size analysis, mean geometric diameter, geometric standard deviation, *in vitro* solubility and Ca, P, Mg, Fl, S and Fe content of fine and coarse limestone

Particle size (μm)	Fine (%)	Coarse (%)
2000	0.1	77.1
1190	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	1978
Geometric standard deviation (μm)	2.06	1.31
<i>In vitro</i> 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

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
<i>Calculated composition</i>			
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
<i>Analyzed values</i>			
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 - 3.0g; Folic acid - 1.0 g; Selenium - 0.25 g; antioxidant - 10.0 g and q.s. vehicle - 1000 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.

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 granulometry (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)
	Level 1	0.60	5.98	0.56	5.64
Fine	Level 2	0.64	12.15	0.61	11.54
	Level 3	0.68	20.78	0.82	24.98
	Level 1	0.69	10.00	0.74	10.66
Coarse	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	<.0001	0.053	<.0001
	Granulometry	0.736	0.717	0.569	0.811
	Level x Granulometry	0.062	0.001	<.0001	<.0001

^a Each value represents the average of eight repetitions (six birds/repetition).

^bStandard error of the mean.

Table 4 - Linear relationship between Ca digestible (CaD) and Ca retained (CaR) vs. Ca content in diet (g Kg^{-1} DM)

		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

^aStandard error of regression.

CHAPTER 4

4.1. General conclusions

The use of coarser-grained limestone in diets for laying hens has lower digestibility and calcium retention coefficients compared to finer-grained limestone. In addition, older laying hens tend to decrease the calcium digestibility of these ingredients and increase their endogenous loss.