R. Bras. Zootec., 48:e20180253, 2019
https://doi.org/10.1590/rbz4820180253

Non-ruminants
Full-length research article

Revista Brasileira de Zootecnia

Brazilian Journal of Animal Science
ISSN 1806-9290
www.rbz.org.br

Reduction of calcium levels in rations supplemented with vitamin D_3 or 25-OH-D_3 for broilers

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ABSTRACT - An experiment was carried out to verify the response to the Ca reduction levels of diets with different vitamin D sources on performance, bone mineral deposition, serum concentrations, digestibility, carcass characteristics, and meat quality of broiler chickens in the period from 1 to 42 days reared in thermoneutral environment. A total of 504 male broilers with one day of age and average weight of 43.27±1.08 g were housed in climatic chambers and distributed in a completely randomized design. The study consisted of a 4×2 factorial, with four Ca reduction levels (0, 10, 20, and 30%) and two vitamin D sources (2760 IU of D_3 or 25-OH-D_3). The performance of animals at 21 and 42 days of age was not affected by Ca reduction by up to 30%, regardless of the vitamin source used. Dietary reduction from 10% decreased serum Ca concentrations. The use of vitamin D_3 provided a serum P level greater than the 25-OH-D_3. Calcium reduction decreased serum 25-OH-D_3 levels. No effect of vitamin source or Ca levels on broiler carcass characteristics was observed at 42 days. The vitamin source did not influence meat quality, while Ca reduction of the diet provided lower losses by thawing and cooking and higher initial pH values. The b* color was reduced in diets with lower Ca levels of the diet. Reducing Ca up to 30% does not affect the performance and carcass characteristics, regardless of the vitamin D source used. The quality of broiler meat is improved with the Ca reduction in the diet, but the vitamin used has no effect on such characteristics. We can conclude, based on the results of performance, blood, and bone, that the performance variables are not adequate to determine the actual requirement of Ca, since as it is a priority to maintain performance, bone mineral mobilization occurs, which may compromise the carcass quality of the birds.

Keywords: bone mineralization, digestibility, meat quality, performance

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Received: October 14, 2018
Accepted: April 22, 2019

How to cite: Tizziani, T.; Donzele, R. F. M. O.; Donzele, J. L.; Silva, A. D.; Muniz, J. C. L.; Jacob, R. F.; Brumano, G. and Albino, L. F. T. 2019. Reduction of calcium levels in rations supplemented with vitamin D_3 or 25-OH-D_3 for broilers. Revista Brasileira de Zootecnia 48:e20180253.
https://doi.org/10.1590/rbz4820180253

Introduction

Calcium is an essential nutrient to the body of birds, as it participates in several biochemical functions and in bone formation. The deficiency of this mineral can cause damages; thus, it is essential to adequately meet the nutrient requirements in the different stages of bird development. Its metabolism is closely related to that of phosphorus (P) (Mello et al., 2012), which leads to caution in the formulation of balanced rations for these minerals to obtain maximum dietary utilization, since unbalanced relations can harm performance (Rao et al., 2006) and bone quality. It has been suggested that birds have a high utilization efficiency when fed sub-optimal Ca levels (Li et al., 2012).

Vitamin D plays a key role in Ca metabolism of birds. Animals reared in the absence of natural light require supplementation to meet the requirement of this vitamin. The most common form of addition to rations is cholecalciferol (D_3); however, several studies have used animal isoforms (Fritts and Waldroup, 2003; Han et al., 2013; Han et al., 2016). After absorption, D_3 is transported to the liver, where it is hydroxylated, resulting in the formation of 25-OH-D_3 (Soares Jr et al., 1995). This is the main circulating form in the blood and is, therefore, used as a marker of vitamin D status in animals (Arnold et al., 2015) and an important indicator of mineral metabolism of birds. To become active, this molecule needs one more hydroxylation, which occurs in the kidneys at position 1, thus giving rise to the metabolically active hormone form 1,25-$(OH)_2$-D_3.

Vitamin D is known to act by improving the absorption and utilization of dietary Ca and P (Shafey et al., 1990; Yan and Waldroup, 2006), but few studies have evaluated the effect of different sources on Ca-deficient diets for broilers. In addition to performance, mineral concentration in the bone is an important means to evaluate the requirement and retention of minerals. Another way to verify this influence is through blood markers of bone remodeling such as alkaline phosphatase, besides the mineral level and vitamin D status in the blood (Shafey et al., 1990).

The effects of dietary supplementation with 25-OH-D_3 in rations have been reported to improve muscle protein synthesis and broiler meat quality (Vignale et al., 2015; Bozkurt et al., 2017). On the other hand, there is a shortage of research demonstrating supplementation with different vitamin D sources in diets with Ca reduction on meat quality and carcass characteristics of poultry.

In this sense, the objective of this work was to verify the response of animals to the Ca reduction in diets supplemented with different vitamin D sources on performance, bone mineral deposition, plasma concentrations, carcass characteristics, and meat quality of male broilers from 1 to 42 days reared in thermoneutral environment.

Material and Methods

An experiment was conducted in a laboratory in Viçosa, MG, Brazil (20°45'57.19" S, 42°51'35.42" W, and 682 m altitude). All the experimental procedures adopted were approved by the local ethics committee on animal use (case no. 44/2014), in accordance with the ethical principles of animal experimentation established by the National Council for the Control of Experimentation Animal (CONCEA) and with the current legislation.

A total of 504 one-day-old male Cobb 500 chicks, weighing 43.27±1.08 g, were housed in climatic chambers, where mean temperature and relative humidity were maintained according to the technical recommendations of the strain. Each climatic chamber contained two replicates randomly distributed.

The birds were distributed in a completely randomized experimental design with eight treatments in a 2×4 factorial arrangement [two vitamin D sources (vitamin D_3 or 25-OH-D_3 (Hy-D®) × four levels of Ca reduction (0, 10, 20, and 30%)] in the recommendation of the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011) for the stages 1 to 7, 8 to 21, 22 to 33, and 34 to 42 days of age (Tables 1 and 2), with seven replicates and nine birds per experimental unit. The experimental treatments were obtained by removal of limestone and addition of the inert. The different vitamin D sources were included in the diet along with the mineral-vitamin premix, providing approximately 2,760 IU/kg ration of vitamin D (D_3 or 25-OH-D_3). Experimental rations and water were supplied *ad libitum* throughout the experimental period.

During the experimental period, the chambers were monitored and recorded through dry bulb, wet bulb, and black globe thermometers. The data were subsequently converted into the Black Globe Temperature and Humidity Index (BGHI) as proposed by Buffington et al. (1981), to characterize the thermal environment of the birds. The light program was continuous with 24 h of artificial light.

At the end of the experimental period (42 days of age), three birds from each experimental unit with the weight closest to the average of the cage (10% above or below the mean) were selected and used

for subsequent evaluations. Two of the three birds per experimental unit were subjected to solid fasting for 12 h and weighed. After this period, these birds were sent to the slaughterhouse, where they were desensitized via electrosurgery (with electric current of 60 V), slaughtered by bleeding by cutting the jugular artery, as recommended by Normative Instruction No. 3 (MAPA, 2000), and, after being scalded and plucked, they were eviscerated; the carcasses were weighed for evaluation of carcass and cut yields. A third bird was maintained in a 12-h solid fasting for blood collection by puncture of the brachial vein, collection of breast for meat quality, and tibia and femur analyses.

At the end of each phase (7, 21, 33, and 42 days), feed intake was calculated by the difference between the total ration provided and the leftovers in the feeders and floor of the climatic rooms to obtain the total intake accumulated at 21 and at 42 days. The birds were weighed at the beginning (day 1), at 21 days, and at the end of the experimental period to determine their weight gain (WG) in the periods from 1 to 21 and from 1 to 42 days of age. Feed conversion (FC) was calculated by dividing feed intake (FI) by the accumulated body weight gain in the respective evaluated periods.

For the determination of mineral (Ca and P) concentrations, the left tibia of each bird was used, skinned, and dried in an oven (105 °C). Afterwards, the bones were calcined in a muffle furnace (600 °C) for 6 h for measurement of ash contents and preparation of mineral solution, following the methodology of Silva and Queiroz (2002). The determination of Ca contents was performed by atomic absorption spectrometry, while P of the bones was determined by means of colorimetry. Samples for phosphorus analysis were digested, and the solution was prepared using reagent molybdovanadate using ascorbic acid as reducing agent. The absorbance of the sample was performed at a wavelength of 400 nm.

Table 1 - Composition of experimental diets in natural matter (g/kg)

Item	Experimental diet			
	1-7 days	8-21 days	22-33 days	34-42 days
Ingredient				
Corn	478.88	528.23	553.54	591.44
Soybean meal	437.53	387.13	355.07	320.72
Soybean oil	37.68	42.00	52.08	52.09
Dicalcium phosphate	18.42	15.67	13.93	11.23
Limestone	9.28	9.25	8.37	7.78
Inert (sand)	1.00	1.00	1.00	1.00
Salt	5.08	4.83	4.58	4.45
L-lysine HCl	1.41	1.57	1.47	1.60
DL-methionine	3.27	2.92	2.70	2.45
L-threonine	0.50	0.45	0.31	0.29
Mineral-vitamin mix[1]	5.00	5.00	5.00	5.00
Choline chloride	1.25	1.25	1.25	1.25
Antioxidant	0.10	0.10	0.10	0.10
Anticoccidian	0.50	0.50	0.50	0.50
Growth promoter	0.10	0.10	0.10	0.10
Total	1000.00	1000.00	1000.00	1000.00
Calculated composition[2]				
Metabolizable energy (kcal/kg)	2960	3050	3150	3200
Crude protein (%)	23.92	22.02	20.74	19.48
Lysine (%)	1.324	1.217	1.131	1.060
Methionine + cystine (%)	0.953	0.876	0.826	0.774
Threonine (%)	0.861	0.791	0.735	0.689
Ca (%)	0.920	0.841	0.758	0.663
Available phosphorus (%)	0.470	0.401	0.354	0.309

[1] Quantity per kg of product: vitamin A, 5,600,000 IU; vitamin D, 552,000 IU; vitamin E, 10,000 IU; vitamin B1, 1,550 mg; vitamin B2, 4,000 mg; vitamin B6, 2,080 mg; pantothenic acid, 10,400 mg; vitamin K3, 1,200 mg; folic acid, 650 mg; niacin, 28,000 mg; vitamin B12, 8,000 µg; selenium, 300 mg; antioxidant, 0.50 g; manganese, 150,000 mg; zinc, 140,000 mg; iron, 100,000 mg; copper, 16,000 mg; iodine, 1,500 mg.
[2] Calculated through the food composition of the Brazilian Tables of Poultry and Swine (Rostagno et al., 2011).

The values of the minerals were expressed in terms of percentage of ash in relation to the weight of the dry and defatted bone (Barbosa et al., 2010; Müller et al., 2012), and Ca:P ratio was obtained by dividing the percentage of Ca by that of P in the ashes.

The analyses of bone strength (BS) were performed using the left femur of each bird. The femurs were sustained by the extremities on supports and the load was applied to the center of each bone (diaphysis region) at a constant speed of 10 mm/min. The results were expressed as Kgf/cm^2 referring to the maximum force for the rupture of each bone.

A total excreta collection was carried out in the period of 30 to 33 days of age for the estimation of mineral excretion (Ca and P). The animals were housed in cages containing metal trays covered with plastic for collection. The total collection was carried out twice a day (7.00 and 18.00 h) and the collected material was stored in plastic bags properly identified and conditioned in a refrigerator. At the end of the collection period, the excreta were homogenized, and an aliquot of approximately 300 g was removed and subjected to partial drying in an oven at 60 °C for 72 h. After being processed, the excreta were sent to a specialized laboratory in Mayrinque, SP, Brazil. For the calculation of the retention coefficient, the FI and total excreta of the period were recorded. Mineral retention (%) was obtained through the formula MR = ((mineral ingestion − mineral excretion)/mineral intake) × 100. The values were expressed on a dry matter basis.

The yield (%) of the eviscerated carcasses and noble cuts (breast, drumstick, and thigh) of chickens at 42 days of age were evaluated. In determining the yield, the weight of clean and eviscerated carcass was considered, with feet and head, in relation to fasting live weight. For the noble cuts, yield calculations were performed in relation to the weight of eviscerated carcass with feet and head.

The initial (15 min *post mortem*) and final (24 h *post mortem*) pH were measured using a manual Testo 205 pH meter. A Konica Minolta CR300 colorimeter was used to evaluate the color of the breast meat. The characteristics L* (brightness - dark to light level), a* (intensity of red/green), and b* (intensity of yellow/blue) were evaluated in five different regions of the breast muscle (*Pectoralis major*) 24 h *post mortem*.

Table 2 - Calculated composition of Ca, P, aP, and Ca:aP ratio of experimental rations

	Calcium reduction			
	0%	10%	20%	30%
Ca, 1-7 days (%)	0.920	0.828	0.736	0.644
P, 1-7 days (%)	0.706	0.706	0.706	0.706
aP, 1-7 days (%)	0.470	0.470	0.470	0.470
Ca:aP ratio	1.96	1.76	1.57	1.37
Ca, 8-21 days (%)	0.841	0.757	0.672	0.588
P, 8-21 days (%)	0.639	0.639	0.639	0.639
aP, 8-21 days (%)	0.401	0.401	0.401	0.401
Ca:aP ratio	2.10	1.89	1.68	1.47
Ca, 22-33 days (%)	0.758	0.682	0.606	0.530
P, 22-33 days (%)	0.595	0.595	0.595	0.595
aP, 22-33 days (%)	0.354	0.354	0.354	0.354
Ca:aP ratio	2.14	1.93	1.71	1.50
Ca, 34-42 days (%)	0.663	0.596	0.530	0.464
P, 34-42 days (%)	0.535	0.535	0.535	0.535
aP, 34-42 days (%)	0.309	0.309	0.309	0.309
Ca:aP ratio	2.15	1.93	1.72	1.50

aP - available phosphorus.
The treatments were obtained by the removal of limestone and addition of the inert.

For the evaluation of drip loss (DL), an aliquot of 80-100 g of the breast muscle was used. Samples were suspended in the refrigerator for 48 h. The initial and final weights were obtained to calculate the percentage of loss.

The thawing loss (TL) was obtained by the weight difference of the frozen muscle and muscle after thawing. Breast samples were thawed for 16 h in a refrigerator at approximately 8 °C.

After being thawed and weighed, the breasts were vacuum-packed and cooked in water bath at 70 °C for 30 min. Soon thereafter, they were cooled, dried, and weighed to obtain weight loss by cooking (CL).

The shear force (SF) analysis was performed with the same samples used for CL. The breasts were cut parallel to the muscle fibers in five rectangles (1.0×1.0×1.3 cm) and subjected to mechanical force using a TAXT2i texturometer, coupled with a Warner-Bratzler Shear Force probe with a capacity of 20 kg and isolator speed of 20 cm/min, providing the SF measurement of the sample, in kgf/cm^2).

Blood samples were taken by puncture of the brachial vein using a syringe and needle with anticoagulant (heparin); 5 mL of blood were collected from one bird per repetition. After collection, the plasma was extracted by centrifugation at 3000 rpm for 10 min, then transferred to cryotubes and immediately frozen at −18 °C for the analysis of P, Ca, total alkaline phosphatase (TAP), bone alkaline phosphatase (BAP), and 25-OH-D_3.

For the analysis of TAP, P, and Ca, the automatic equipment for biochemistry Mindray BS200E was used, using Bioclin determination kits. The analyses of BAP and 25-OH-D_3 were performed in a Beckman Coulter Access® immunoassay system using bone alkaline phosphatase (Ostase®) and 25-OH-D_3 (25(OH) Vitamin D total®).

The obtained data were subjected to analysis of variance in a completely randomized design, using the PROC GLM of SAS software (Statistical Analysis System, University Edition), considering the level of 5% probability. Dunnett's test was used for contrast with the control group. For the vitamin sources, the level of 5% probability was considered by the t test.

The statistical model used was:

$$Yij = \mu + \alpha i + \beta j + \gamma ij + \varepsilon ij,$$

$$\text{with } i = 1, 2, 3, 4 \text{ and } j = 1, 2,$$

in which Y = mean of the experimental unit factor i and factor j, μ = constant inherent to all experimental units, αi = effect of level i of factor α, βj = effect of level j of factor β, γij = effect of interaction between factors α and β, and εij = error of the experimental unit factor i and factor j.

Results

There was no interaction (P>0.05) between vitamin D sources and Ca levels on performance (WG, FI, and FC) of broiler chickens in the periods of 1 to 21 and 1 to 42 days of age (Table 3). Thus, it was evidenced that the performance response standard of birds due to Ca reduction levels of the diets did not vary among the vitamin D sources evaluated.

The reduction of dietary Ca levels, regardless of the vitamin D source used, did not influence (P>0.05) the performance of birds in the different evaluated periods.

There was no interaction (P>0.05) between vitamin D sources and Ca reduction levels of the diets for any of the blood variables analyzed at 42 days of age (Table 4). However, it was verified that, although it did not vary among the vitamin D sources, the plasma Ca level decreased (P<0.05) due to the reduction of its level in the diets.

There was no alteration (P>0.05) in serum P levels due to the reduction of dietary Ca levels; however, birds fed vitamin D_3 had higher levels (P<0.05) of this mineral in the blood than those fed diets containing the 25-OH-D_3 metabolite.

In relation to TAP and BAP, it was observed that, regardless of the vitamin D source and reductions in Ca levels of the diets evaluated, serum levels did not vary (P>0.05).

The Ca reduction of the rations by 30% decreased (P<0.05) the serum level of 25-OH-D_3 of broilers. On the other hand, the influence of vitamin D source on the metabolite concentration in the blood was not verified, although an increase in absolute values, over 20%, was observed with the use of 25-OH-D_3 in relation to D_3.

No interaction was observed (P>0.05) for the variables of Ca, P, and ash deposition in the tibiae and BS of broilers at 42 days of age (Table 5). On the other hand, there was a decrease (P = 0.0012) in Ca deposition of animals fed rations from 20% Ca reduction. Similar behavior of the data was observed for bone ash content in the tibiae of broilers, with lower content than those of control when Ca content was reduced by 30%. The observed change in ash deposition probably reflects the observed changes in Ca content, since no Ca reduction effect was observed in rations varying the Ca to available P ratio (Ca:aP

Table 3 - Performance of broilers in the initial (1-21days) and total (1-42 days) rearing per

Variable	Calcium reduction (%)				Vitamin D source		CV %	Variation source (P-value)		
	0	10	20	30	D_3	25-OH		Ca	Vitamin	Ca × vit
1-21 days of age										
WG (g)	995	987	1003	1002	998	995	3.30	0.5455	0.7494	0.1174
FI (g)	1250	1252	1276	1267	1263	1260	3.33	0.3005	0.7929	0.7732
FC	1.28	1.29	1.28	1.30	1.29	1.29	2.76	0.7879	0.9702	0.2231
1-42 days of age										
WG (g)	3080	3087	3089	3032	3064	3080	2.05	0.0684	0.3716	0.7565
FI (g)	4511	4546	4482	4501	4507	4514	1.92	0.2736	0.7813	0.7221
FC	1.48	1.47	1.47	1.47	1.48	1.47	1.74	0.7866	0.3032	0.8692

WG - weight gain; FI - feed intake; FC - feed conversion.
Means with different letters in the same row differ (5%) by Dunnett's test, for levels, and by the t test, for vitamin source.

Table 4 - Serum Ca and P concentrations, total alkaline phosphatase (TAP), bone alkaline phosphatase (BAP), and 25-OH-D_3 of broilers at 42 days of age

Variable	Calcium reduction (%)				Vitamin D source		CV %	Variation source (P-value)		
	0	10	20	30	D_3	25-OH		Ca	Vitamin	Ca × vit
Ca (mg/dL)	8.22a	7.42b	7.07b	6.54b	7.29	7.33	9.55	<0.0001	0.8209	0.0927
P (mg/dL)	5.76	5.61	5.74	5.68	5.84a	5.55b	4.88	0.4545	0.0002	0.3857
TAP (U/L)	1103	1536	1273	1364	1415	1224	45.45	0.2938	0.2392	0.3598
BAP (µg/L)	4.51	4.43	4.94	4.92	4.70	4.70	20.36	0.5054	0.9948	0.4949
25-OH (ng/mL)	11.78a	9.01a	11.29a	6.94b	8.82	10.70	37.32	0.0203	0.1119	0.7248

CV - coefficient of variation.
Means with different letters in the same row differ (5%) by Dunnett's test, for levels, and by the t test, for vitamin source.

Table 5 - Effect of Ca reduction and vitamin D source on deposition of Ca, P, and ashes in the tibiae of broilers at 42 days of age

Variable	Calcium reduction (%)				Vitamin D source		CV %	Variation source (P-value)		
	0	10	20	30	D_3	25-OH		Ca	Vitamin	Ca × vit
Ca (%)	15.53a	15.07a	14.89b	14.57b	15.08	14.94	4.02	0.0012	0.3941	0.1403
P (%)	7.99	7.90	7.91	7.71	7.83	7.93	4.73	0.2416	0.3302	0.1704
Ashes (%)	38.81a	37.94a	37.48a	36.71b	37.59	37.88	5.09	0.0421	0.5647	0.1287
BS (Kgf)	10.58a	8.00a	8.02 a	6.57b	8.37	8.21	38.68	0.0166	0.8589	0.9569

BS - bone strength; CV - coefficient of variation.
Means with different letters in the same row differ (5%) by Dunnett's test, for levels, and by the t test, for vitamin source.

ratio; fixed aP), on the deposition of P in the bone of birds. The vitamin sources used in the diets did not affect (P>0.05) Ca, P, and ash contents in the tibiae and BS of the broilers.

The 30% reduction in dietary Ca level influenced (P<0.05) negatively the BS of the bones of birds. However, the vitamin source used had no effect on this bone characteristic.

On the other hand, there was an interaction (P<0.0001) between the factors studied on Ca:P ratio (Table 6) deposited on the tibiae of broilers. In rations whose Ca requirements were fully met, the highest ratio was found in the group of animals receiving 25-OH-D_3. The Ca:P ratio deposited in the tibiae of broilers was not altered with the Ca reduction of the rations when the D_3 form was used as the vitamin D source. In contrast, the deposited Ca:P ratio was lower from the 10% Ca reduction with the use of the 25-OH-D_3 metabolite.

There was no interaction (P>0.05) between the factors studied for the variables of mineral concentration in the excreta and mineral retention. The reduction of dietary Ca levels from 10% caused lower Ca values in the excreta of birds (Table 7). Consequently, an increase in mineral retention was observed with the lowest Ca level of the feed. Conversely, the P concentration in the excreta and mineral retention coefficient were not influenced by Ca reduction (fixed aP) of the rations.

The vitamin D source used in the diet influenced the mineral retention variables and the mineral concentration in the excreta. Lower Ca and P concentrations were observed in the excreta when the D_3 form was used, resulting in a higher retention of these minerals.

The results of carcass characteristics showed that no interaction effect (P>0.05) was observed between the vitamin sources and Ca levels of the rations (Table 8) at slaughter (42 days). The Ca reduction up to 30% of the established requirement did not affect (P>0.05) the carcass, breast , drumstick, and thigh yields, and the vitamin source had no influence (P>0.05) on these variables.

Likewise, there was no interaction (P>0.05) between vitamin D sources and dietary Ca levels of diets for any of the qualitative characteristics of broiler meat at 42 days of age (Table 9).

When evaluating the factors separately, it was verified that the vitamin D source used in the diets did not change (P>0.05) any of the variables analyzed for meat quality. Similar behavior was found for the Ca reduction of the rations, in which the reduction in up to 30% did not compromise meat quality.

Table 6 - Deviation of the interaction between Ca reduction level and vitamin D source of the rations on the Ca:P ratio deposited in the tibiae of broilers

Vitamin D source	Calcium reduction (%)			
	0	10	20	30
Vitamin D_3	1.91Ba	1.92Aa	1.92Aa	1.91Aa
25-OH-D_3	1.94Aa	1.90Bb	1.86Bb	1.86Bb

Means with different lowercase letters in the same row differ by Dunnett test (5%).
Means with different uppercase letters in the column differ by the t test (5%).

Table 7 - Effect of the Ca reduction and vitamin D source in the concentration of Ca and P in the excreta and coefficient of mineral retention of broilers

Variable	Calcium reduction (%)				Vitamin D source		CV %	Variation source (P-value)		
	0	10	20	30	D_3	25-OH		Ca	Vitamin	Ca × vit
Ca (g/kg)	16.00a	13.69b	11.99b	9.16b	11.99b	13.43a	6.59	<.0001	<.0001	0.2225
CaR (%)	61.39a	63.43a	64.09a	66.48b	66.22a	61.48b	6.10	0.0118	<.0001	0.0628
P (g/kg)	11.24	11.99	11.84	11.54	11.04b	12.09a	6.06	0.1686	<.0001	0.0622
PR (%)	65.42	64.34	64.36	62.41	65.90a	62.36b	5.66	0.1844	0.0006	0.3413

CaR - calcium retention; PR - phosphorus retention; CV - coefficient of variation.
Means with different letters in the same row differ (5%) by Dunnett's test, for levels, and by the t test, for vitamin source.

Table 8 - Yield of carcass and noble cuts of broilers from 1 to 42 days fed diets with Ca level reduction (variable Ca:aP ratio)

Variable	Calcium reduction (%)				Vitamin D source		CV %	Variation source (P-value)		
	0	10	20	30	D_3	25-OH		Ca	Vitamin	Ca × vit
CY (%)	84.97	84.65	84.37	85.19	84.64	84.95	4.94	0.6053	0.4337	0.7311
BY (%)	37.32	36.47	36.82	37.19	37.18	36.71	3.64	0.3600	0.4245	0.5874
DY (%)	11.27	11.55	11.26	11.49	11.37	11.37	3.21	0.2233	0.9387	0.9822
TY (%)	13.58	13.73	13.49	13.50	13.52	13.63	5.48	0.6300	0.4407	0.6212

CY - carcass yield; BY - breast yield; DY - drumstick yield; TY - thigh yield; CV - coefficient of variation.
Means with different letters in the same row differ (5%) by Dunnett's test, for levels, and by the t test, for vitamin source.

Table 9 - Meat quality of broilers fed reduced Ca levels

Variable	Calcium reduction (%)				Vitamin D source		CV %	Variation source (P-value)		
	0	10	20	30	D_3	25-OH		Ca	Vitamin	Ca × vit
DL (%)	2.60	2.95	2.89	2.92	2.97	2.71	25.52	0.5512	0.1988	0.3386
TL (%)	5.78a	5.41a	4.72b	4.68b	5.15	5.15	12.55	<0.0001	0.9934	0.7431
CL (%)	11.18a	10.89a	9.87b	10.24b	10.51	10.58	9.46	0.0042	0.7940	0.0897
SF (Kgf/cm^2)	1.97	2.01	2.07	1.91	1.98	1.99	13.10	0.4278	0.8623	0.6293
L*	59.31	58.98	59.89	59.34	59.47	59.29	2.73	0.5233	0.6715	0.3519
a*	4.63	4.58	4.17	4.41	4.45	4.44	18.81	0.4687	0.9607	0.9571
b*	16.17a	16.06a	16.00a	15.28b	15.91	15.75	4.45	0.0088	0.3895	0.6919
pHi	6.55	6.52	6.55	6.54	6.52	6.55	1.37	0.7409	0.100	0.4841
pHf	6.06	6.04	6.05	6.03	6.02	6.06	1.62	0.8937	0.1062	0.3645
Ti (°C)	38.84	38.68	38.93	38.52	38.64	38.84	2.81	0.7566	0.5006	0.8131
Tf (°C)	10.47	10.69	10.90	10.96	10.82	10.69	8.78	0.5092	0.6002	0.0642

DL - dripping loss; TL - thawing loss; CL - cooking loss; SF - shear force; L* - brightness; a* - intensity from red to green; b* - intensity from yellow to blue; pHi - initial pH; pHf - final pH; Ti - initial temperature; Tf - final temperature; CV - coefficient of variation.
Means with different letters in the same row differ (5%) by Dunnett's test, for levels, and by the t test, for vitamin source.

As for color, no change in L* and a* values were observed; however, birds fed a 30% Ca reduction presented a lower b* value (P<0.05) compared with those fed the required Ca content.

The level of dietary Ca reduction did not alter pHi (15 min *post mortem*) and pHf (24 h *post mortem*) values of the breast muscle of broilers. Similarly, pH values were not altered by the vitamin source used in the diets. The initial and final temperatures evaluated in the breast of broilers were not determinant of pH change, since no difference between the treatments (P>0.05) was observed for these variables.

Discussion

In relation to the environmental conditions inside the climatic chambers, Santos et al. (2009), Valério et al. (2003), and Medeiros et al. (2005) characterized the thermal environment with BGHI of 80 to 86, 74 to 80, and 69 to 77 as thermal comfort for broiler chickens in the periods of 1 to 8, 8 to 21, and 22 to 42 days of age, respectively. In this sense, it can be stated that in this study, the birds were kept in a thermoneutral environment throughout the experimental period.

The results of the present study demonstrated that the reduction of dietary Ca level by up to 30% during the initial phase (1-21 days) and total period (1-42 days) maintained the performance, regardless of the vitamin source evaluated. Singh et al. (2013) found that the reduction of 25 and 33% in the periods from 1 to 21 and 22 to 42 days, respectively, did not affect the performance of broiler chickens. Similarly, Rao et al. (2006), Wilkinson et al. (2014), and Hamdi et al. (2015) did not find difference in WG in the initial and growth period for chickens fed Ca-deficient diets. Complementary to this, FC was not affected in the periods evaluated by dietary Ca and probably reflects maintenance observed in WG

and FI of the birds, demonstrating that the Ca reduction in the initial diets did not affect performance and use of nutrients in the subsequent rearing stages. This suggests the high efficiency in the use of Ca at low levels in the diet, which may be the result of the regulation of Ca transporters when this is below the animal requirement (Li et al., 2012), providing maintenance of animal performance.

In this study, considering that aP was not limiting, the Ca:aP ratios ranged from close to 2:1 to 1.4:1 at the lower levels of Ca in the different phases. However, this imbalance was not sufficient to impair the animal performance. Singh et al. (2013), Lalpanmawia et al. (2014), and Delezie et al. (2015) indicated that the high Ca:P ratios of rations causes changes mainly in FI of the birds. This is due to the ability of Ca to form insoluble complexes with P, which compromises the use of these minerals by birds (Tamim et al., 2004; Selle et al., 2009; Han et al., 2016). Additionally, the reduction of these minerals should be done in a balanced way for the proper development of the birds (Delezie et al., 2012; Akter et al., 2016; Han et al., 2016; Gautier et al., 2017), once that imbalance between dietary Ca and P can cause damage to the performance and bone development of birds (Li et al., 2012). This was evidenced in the work of Wilkinson et al. (2014), in which the intake of a separate Ca source by broilers was higher in those animals fed low Ca level and close Ca:aP ratio (0.91:1), suggesting that birds have the ability to regulate intake by the dietary Ca level to minimize dietary mineral imbalance while maintaining an appropriate Ca:aP intake, provided they have an additional Ca source available.

The vitamin D source did not alter the performance of birds in the different evaluated periods. Bozkurt et al. (2017) also did not observe improvement in WG at 10, 24, and 38 days when they used 25-OH-D_3 as a vitamin D source. Contrary to what was observed in this experiment, Yarger et al. (1995) and Fritts and Waldroup (2003) found that the use of 25-OH-D_3 provided an increase in WG of broiler chickens. Factors such as vitamin dose, dietary mineral level, genetics, bird age, and conditions under which the studies were conducted may interfere with the magnitude of the observed results.

Serum Ca concentrations were lower when dietary Ca levels were reduced compared with the basal diet. These results probably reflect the homeostasis of this mineral in the organism of birds. Calcium-deficient diets or increased requirement by the animal result in a reduction in plasma Ca concentrations (Proszkowiec-Weglarz and Angel, 2013). However, the vitamin source used did not change Ca concentration in the blood. Under normal conditions, Ca concentration is maintained within narrow limits, by means of integrated hormonal regulation involving the intestine, kidneys, and bones, while that of P, which is mainly carried out by renal function, is regulated with less rigor (Sie et al., 1974).

The P concentrations in the blood were not altered by Ca reduction in the diet, maintaining its serum level. The Ca level in the diet may influence the availability and consequent absorption of P from the diet, especially when at high levels (Driver et al., 2005). This response pattern was not observed in this experiment, since aP levels were calculated to meet the requirement of birds in all rearing phases. On the other hand, the fact that rations present reduced Ca levels may have prevented interaction with P in the gastrointestinal tract, which would reduce the availability of this mineral as observed by Plumstead et al. (2008). However, vitamin D_3 increased serum P levels in comparison with 25-OH-D_3, contrary to the results observed by Bozkurt et al. (2017). Studies have suggested that the effects of 25-OH-D_3 on dietary P use (Applegate et al., 2003) are not entirely clear. Edwards Jr (2002) demonstrated an adverse effect with the use of 25-OH-D_3 on the plasma P concentrations, as verified in the present study. Although the variation in Ca did not interfere with the plasma P level, it was evident that the reduction of 30% Ca in diets resulted in a decrease in the serum level of 25-OH-D_3. On the other hand, the use of the 25-OH-D_3 metabolite in rations resulted in a non-significant increase of 21.3% in the absolute value of the serum level of this metabolite. Based on the lower absolute value of the serum 25-OH-D_3 concentration when vitamin D_3 was used in the diet, it may have occurred due to a probable increase in the 25-OH-D_3 hydroxylation into 1,25-$(OH)_2$-D_3. This possible increase in hydroxylation, due to the positive action of 1,25-$(OH)_2$-D_3 in bone mineralization, would be consistent with the fact that P concentration in the blood had a higher absolute value in animals fed vitamin D_3.

No significant difference was observed in serum TAP and BAP concentrations among the vitamin D sources used in rations, nor did the Ca reduction altered the activity of these enzymes. Total alkaline

phosphatase is measured by its activity and corresponds to the sum of the various isoforms (hepatic, bone, and intestinal) present in serum (Kaplan, 1972; Saraiva and Lazaretti-Castro, 2002), whereas BAP corresponds to the specific isoform of bone involved in bone mineralization. High values probably reflect changes in bone metabolism. Roberson and Edwards Jr (1994) reported that TAP activity was not influenced by the addition of phytase when used in low Ca levels and high P levels in broiler feeds. These results are supported by the work of Nakagi et al. (2013), who concluded that the highest synthesis and expression of this enzyme is observed in P-deficient diets. Additionally, as reported in review, Schmidt et al. (2007) suggested that the elevation of enzymes related to bone metabolism is more sensitive in young animals. Therefore, the supply of P nutritional requirements in all rearing stages and the fact that the blood tests were carried out at 42 days of age explain the absence of variation between treatments, results confirmed by the maintenance of P in the blood of birds.

The main form of vitamin D circulating in the blood is 25-OH-D_3 and is, therefore, considered as the vitamin status marker. This metabolite undergoes renal hydroxylation to take the active hormonal form 1,25-$(OH)_2$-D_3 (Soares Jr et al., 1995; Yarger et al., 1995) by the action of the 1α-hydroxylase enzyme, responsible for this activation (Christakos et al., 2016). This isoform is highly regulated by Ca and P homeostasis through calciotrophic hormones and the active form 1,25-$(OH)_2$-D_3. In a recent study, Bozkurt et al. (2017) observed that 25-OH-D_3 supplementation increases vitamin D status in broilers in diets that meet the mineral requirement. On the other hand, Ca-deficient diets, as in this study, may increase the demand for vitamin D, stimulating the enzymatic action in the kidneys, via parathormone (PTH), favoring the conversion of 25-OH-D_3, which may have led to reduction in serum levels with a reduction of 30% of the dietary Ca requirement.

Bone mineralization is considered an important response in determining the requirement of minerals such as Ca and P. The results found in this experiment indicate that the dietary Ca reduction impairs bone characteristics of the broilers. This reduction affected not only Ca deposition, but also bone mineralization, observed with the lowest amount of bone ash (−5.4%) in the animals that received rations with a 30% Ca reduction compared with the control. Gautier et al. (2017) observed a reduction in total ash content when they reduced the Ca content of the broiler diet in the initial growth period (2-23 days) by 60%. On the other hand, Rousseau et al. (2016) observed no effect of Ca reduction in 40 and 46% in the phases of 10-22 and 23-35 days of age, respectively, on the content of bone ash. Thus, with the results of the mineral concentrations in the ashes, it was evidenced that the maintenance in bone deposition seemed priority to its maintenance of the plasma Ca level, since the dietary Ca reduction and consequent plasma Ca reduction did not influence plasma and bone contents of P. Additionally, it has been verified that levels above the requirement (Akter et al., 2016) and high dietary Ca:aP ratios (Gautier et al., 2017) negatively interfere in the homeostasis of these minerals, directing the decrease in bone quality.

As for the Ca:P ratio observed in the tibiae of birds, there was an interaction between the vitamin D sources and Ca reduction levels, in which the use of vitamin D_3 did not alter the Ca:P ratio in the bones, whereas with the use of 25-OH-D_3, the ratio was influenced at the 20 and 30% reduction levels. Although it did not alter the Ca deposition between the vitamin sources, the absolute lower Ca concentration in the ash of birds fed 25-OH-D_3, even though it was not significant, was enough to change the Ca:P ratio deposited in bones, evidencing that the reduction of the ratio deposited in the higher levels of restriction with the use of 25-OH-D_3 as a vitamin source occurred due to the compromise of the Ca deposition and, consequently, in the ash. This hypothesis is confirmed by the results of Ca concentration and ash in the tibiae of the animals.

Therefore, it was evidenced that the 30% Ca reduction in the diet compromised the bone ash concentration and, consequently, bone resistance. This involvement of BS, among other factors, may result in an increase in the number of leg injuries of broiler chickens (Wilkinson et al., 2011) in pre-slaughter management and carcass condemnations in slaughterhouses.

It was observed that the removal of Ca from rations from 10% reduced the concentration of this mineral in bird excreta. On the other hand, there was an increase in mineral retention at the 30% reduction

level in diet. The higher Ca retention of rations when sub-optimal levels are added to the diet has been demonstrated as an adaptive response of broilers (Rousseau et al., 2016) through increased digestibility (Wilkinson et al., 2014) and consequent absorption, modulated by increased expression of intestinal transporters. The vitamin source of the rations was determinant in the Ca and P retention in broilers, in which the D_3 form presented better results for both variables. In this sense, it is clear that both Ca and P share a common regulatory mechanism, mediated possibly by 1,25-$(OH)_2$-D_3. While increased P retention may be the cause of higher P values observed in blood, higher Ca retention was not sufficient to maintain blood concentrations and bone deposition in animals receiving lower Ca levels in feed.

Therefore, it can be verified that the use of Ca by the animals is priority for growth in detriment to bone deposition. The different response between performance and bone characteristics is explained by the higher Ca and P requirement for bone than for soft tissue growth (Larbier and Leclerq, 1992), since approximately 99 and 80% of Ca and P, respectively, of the composition of the animals are found in bone tissue.

The carcass characteristics were not altered by the evaluated variables (Table 9). Thus, the deposition of muscle tissue was maintained even in Ca-deficient diets, as evidenced by the maintenance of WG, which may explain the absence of variation in the yields of noble cuts and carcass yields. The vitamin source was not determinant in presenting changes in carcass and in noble cuts of broilers at 42 days. However, the work of Vignale et al. (2015) demonstrated that 25-OH-D_3 can improve the yield of breast muscle in chickens through the stimulus via mTor. The fact that vitamin D dosage was similar between sources may have limited possible action on muscle deposition.

Water holding capacity (WHC) is an important analysis in determining meat quality as it affects the appearance and yield of the product. Dripping loss, TL, and CL are a means of measuring WHC. In the present study, meat quality was altered only when there were changes in the dietary Ca content. Lower TL and CL were observed with the reduction of dietary Ca levels. On the other hand, the vitamin source did not alter any of the losses, different from the results observed by Bozkurt et al. (2017), who verified that the use of 25-OH-D_3 reduced CL of the breast of chickens. The higher losses observed in this study may be associated, among other factors, with the higher Ca contents of the rations. Although the intramuscular Ca concentration is unlikely to be altered and was not evaluated in the present study, it may alter *post-mortem* muscle proteolysis. The increase of sarcoplasmic Ca^{2+} is responsible for acting by activating muscle metabolism and accelerating lactate production and subsequent *post-mortem* accumulation in the muscle (Barbut et al., 2008), altering the ability of muscle proteins to retain water. This modification, in turn, may affect the action of the enzyme complex calpain-calpastatin and, consequently, WHC.

Regarding color, the L* and a* values were not altered; however, lower b* values were observed with the reduction of dietary Ca content. Higher water losses also reflect the reduction of pigments in the meat (Çelen et al., 2016), which may be associated with the results observed when Ca levels were close to the requirement. In contrast, when the reduction was 30% in dietary Ca content, the observed b* values were lower.

However, it should be noted that the data obtained do not reflect anomalies in the final meat quality, such as PSE (pale, soft, and exudative), since no variation was observed in the L* value and in the pH of the meats. Therefore, given the lack of literature on such evaluations, further studies are suggested as to how the dietary Ca level may be linked to these qualitative modifications of chicken meat.

Conclusions

The calcium reduction up to 30% of the requirement does not affect the performance of broiler chickens and does not alter the carcass characteristics. The increase in calcium retention, when levels of reduction are higher, is not enough to maintain broiler blood homeostasis, consequently impairing bone quality. Meat quality is affected by the level of dietary calcium. The vitamin source used does not interfere with performance, carcass characteristics, and meat quality; however, serum phosphorus concentration

is higher with the use of vitamin D_3. Thus, it can be inferred from the results of performance, blood, and bone, that the performance variables are not adequate to determine the actual requirement of calcium, since as it is a priority to maintain performance, bone mineral mobilization occurs, which may compromise the carcass quality of the birds.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: T. Tizziani, R.F.M.O. Donzele and J.L. Donzele. Data curation: T. Tizziani. Formal analysis: T. Tizziani and A.D. Silva. Funding acquisition: R.F.M.O. Donzele. Investigation: T. Tizziani, R.F.M.O. Donzele and J.L. Donzele. Methodology: T. Tizziani and J.L. Donzele. Project administration: J.L. Donzele. Resources: T. Tizziani, R.F.M.O. Donzele, A.D. Silva, J.C.L. Muniz and R.F. Jacob. Supervision: T. Tizziani, J.L. Donzele, A.D. Silva and J.C.L. Muniz. Validation: T. Tizziani. Visualization: T. Tizziani and R.F. Jacob. Writing-original draft: T. Tizziani, G. Brumano and L.F.T. Albino. Writing-review & editing: J.L. Donzele and T. Tizziani.

Acknowledgments

We acknowledge the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) for the financial support to carry out this work.

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