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Original Article

25-Hydroxycholecalciferol As an Alternative to Vitamin D3 in Diets with Different Levels of Calcium for Broilers Reared Under Heat Stress

■Author(s)

Marques MFG[I] https://orcid.org/0000-0002-9373-7882
Oliveira RFM[I] https://orcid.org/0000-0002-9689-0284
Donzele JL[I] https://orcid.org/0000-0002-1196-1240
Albino LFT[I] https://orcid.org/0000-0002-2753-2010
Tizziani T[I] https://orcid.org/0000-0002-2902-0051
Faria LF[I] https://orcid.org/0000-0002-9655-2023
Muniz JCL[I] https://orcid.org/0000-0003-4837-4215
Dalólio FS[I] https://orcid.org/0000-0001-7669-6364
Lozano C[II] https://orcid.org/0000-0003-1615-6758
Silva CC[II] https://orcid.org/0000-0003-3822-5611

[I] Department of Animal Science, the Federal University of Viçosa, Viçosa MG, 36570000, Brazil.
[II] DSM Nutritional Products, Av. Engº Billings, 1729, São Paulo City, São Paulo State, 05321-010, Brazil.

■Mail Address

Corresponding author e-mail address
Felipe Santos Dalólio
Avenida Peter Henry Rolfs, s/n - Campus Universitário, Viçosa – MG, 36570-900 – Brasil.
Phone: (5538) 9 98643655
Email: felipesantos181@hotmail.com

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ABSTRACT

The objective was to evaluate the effect of dietary 25-hydroxycholecalciferol (25(OH)D_3) or vitamin D_3 (VitD_3) and different total calcium (Ca) levels on the performance, carcass characteristics, blood, enzymatic, and bone biochemistry of broilers reared under heat stress between 1 and 42 days of age. A total of 504 male, Cobb 500, broiler chickens were distributed in a completely randomized design in a 2 × 4 factorial arrangement (VitD_3 or 25(OH)D_3 × four Ca levels (100, 90, 80 and 70% of the recommendations of Rostagno *et al*. (2011)), eight treatments, seven replicates and nine broilers per cage. Feed intake and feed conversion ratio did not (p>0.05) vary when levels of Ca were reduced and vitamin D_3 sources were supplemented in the diets from 1 to 21 days for broilers chickens. 25 (OH)D_3 increased weight gain results (p<0.05). From 1 to 42 d, no differences (p>0.05) were observed on performance, carcass yields and meat quality, bone deposition of Ca and P, and alkaline phosphatase concentration. Higher serum (p<0.05) concentrations of Ca and P were found in broilers fed with 25(OH)D_3. The replacement of VitD_3 with 25(OH)D_3 and the Ca reduction of 30% in diets did not negatively affect performance, carcass characteristics, and Ca and P deposition in the tibia of broilers at 42 days of age, under heat stress.

INTRODUCTION

Heat stress causes physiological and metabolic changes in broiler chickens by negatively affecting performance, immunosuppression, metabolic disturbances, and mortality rates (Mujahid *et al*., 2005). In the last decades, a noticeable increase in broiler production has occurred in tropical countries. These are regions characterized by high incidence of solar radiation, high temperatures, and high relative humidity, compromising broilers well-being, especially in the growing-finishing phase, when they are more sensitive to heat stress (Rao *et al*., 2016). Thus, studies have been carried out to determine strategies to mitigate the negative effects caused by heat stress, with vitamin and mineral supplementation presenting itself as a relevant nutritional strategy.

Calcium (Ca) is an essential nutrient for broilers, as it participates in several biochemical functions and in bone formation. The deficiency of Ca causes bone demineralization, so fulfilling modern broilers' requirements through their lifecycle is paramount. The metabolism of Ca is closely linked to phosphorus (P), which leads to prudence in the formulation of balanced diets for these minerals to obtain maximum dietary utilization, as unbalanced diets may impair bone quality and performance (Rao *et al*., 2006). Nevertheless, it has been suggested that broilers have high efficiency in the use of these nutrients when fed sub-optimal levels of Ca (Li *et al*., 2012).

Vitamin D plays a key role in mineral metabolism. Due to the lower conversion efficiency in the skin, dietary vitamin D_3 is needed for broilers. Cholecalciferol (D_3) is the common way of adding vitamin D_3 to diets. However, it is currently possible to use isoforms or vitamin D_3 metabolites in diets for broilers (Fritts & Waldroup, 2003; Garcia *et al*., 2013; Han *et al*., 2016; Hsiao *et al*., 2018; Tizziani *et al*., 2019). Following absorption, vitamin D_3 is hydroxylated in the liver, resulting in the formation of the 25(OH) D_3 metabolite, the main circulating form in blood, used as a vitamin D marker in animals (Arnold *et al*., 2015). To become active, this molecule needs one more hydroxylation, which occurs in the kidneys at position 1, thus originating the metabolically active hormone form 1,25$(OH)_2D_3$. Vitamin D plays a crucial role in the absorption and utilization of dietary Ca and P. Few studies have evaluated the effect of different sources of this vitamin on diets with sub-levels of Ca for broilers reared under heat stress. In addition to performance, the Ca and P concentration in bones has been an important parameter to evaluate the retention of these minerals. To verify this effect, blood markers of bone remodeling such as alkaline phosphatase and plasma mineral levels have been also used (Shafey *et al*., 1990). The lack of results in relation to the use of 25(OH)D_3 in diets for broilers reared under heat stress and the inconsistency found in scientific literature make it necessary to conduct further studies in this regard.

In this sense, the present study aims to evaluate the effect of 25(OH)D_3 on performance, carcass yield, meat quality, and bone characteristics of broilers fed diets containing different levels of calcium reared under heat stress in the period from 1 to 42 days of age.

MATERIAL AND METHODS

All the procedures used in this study were approved by the Animal Ethics Committee of the Federal University of Viçosa (UFV), under case number 27/2012. The experiment was conducted at the Animal Bioclimatology Laboratory in the Animal Science Department of the Agricultural Sciences Center at UFV, in Viçosa, Minas Gerais, Brazil.

A total of 504 Cobb 500 broiler chicks vaccinated against Marek's disease were reared from 1 to 42 days of age, with an initial weight of 43 ± 0.33 g. The broilers were placed in climatic chambers with air temperature of 32.9 ± 0.48°C and relative humidity of 64.0 ± 7.14% from 1 to 21d and 31.1 ± 0.31°C and 79.0 ± 6.45% from 22 to 42d of age. The animals were distributed in a completely randomized design, in a 2 × 4 factorial arrangement (two sources of vitamin D: VitD_3 or 25(OH)D_3 (Hy-D®, DSM Nutritional Products) × four calcium levels), with eight treatments, seven replicates, and nine broilers per experimental unit. The experimental unit was represented by the cage.

The diets were formulated to meet the requirements of broilers for all nutrients except calcium, as recommended by Rostagno *et al*. (2011) for the pre-starter (1 to 7 days), starter (8 to 21 days), grower (22 to 33 days), and finisher (34 to 42 days) phases, (Tables 1 and 2). The Ca levels used were: 100, 90, 80 and 70% of the recommendations of Rostagno *et al*. (2011).

At the first day of age, broilers were weighed and transferred to climatic chambers built in metal batteries (0.85 × 0.85 m) and tiled floor with a tray type feeder and baby drinkers. From the 8th day, manual feeders and nipple drinkers were used. The environmental conditions of the climatic chambers were controlled by an electronic system and recorded by thermal sensors associated with data loggers. The light program used was 23 hours of artificial light and 1 hour of dark from 1 to 21d, and 20 hours of artificial light and 4 hours of dark from 22 to 42d, using three 45-W fluorescent lamps per room.

Experimental diets provided in the mash form and water were provided *ad libitum* over the experimental period, with water being replaced three times a day (7:00 a.m., 12:00 a.m. and 6:00 p.m.). On day 21, the two broilers with the farthest weights from the mean in each experimental unit were discarded. At the end of the experimental period (day 42), three broilers from each experimental unit (cage) with the closest weight to the average (± 10%) were used for the subsequent analyzes. These broilers were fasted for 12 hours and weighed. After this period, broilers were sent to the slaughterhouse with artificial blue light, where they were desensitized using electrical stunning (with 60 V electric current) and bled by cutting the jugular vein. After scalding, birds were defeathered and two broilers were used to evaluate carcass yield. One broiler from each experimental unit was used for blood collection and analyses of meat quality and bone mineral content from the tibia.

At 21 and 42 days, feed intake (FI) was calculated considering the difference between the total feed provided and feed leftovers. Broilers were weighed at 1d, 21d, and 42d to determine the weight gain of broiler chickens. Feed conversion ratio (FCR) was obtained by dividing the FI by the accumulated weight gain (WG) in the period.

Table 1 – Centesimal and calculated composition of experimental diets in natural matter basis.

Ingredients (g kg^{-1})	Experimental diets			
	1-7 d	8-21 d	22-33 d	34-42 d
Corn (78.8 g kg^{-1})	478.88	528.23	553.54	591.44
Soybean meal (450.0 g kg^{-1})	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	1.00	1.00	1.00	1.00
Common salt	5.08	4.83	4.58	4.45
L-Lysine HCl (78.5%)	1.41	1.57	1.47	1.60
DL-Methionine (99%)	3.27	2.92	2.70	2.45
L-Threonine (98.5%)	0.50	0.45	0.31	0.29
Mineral-Vitamin mixture[1]	5.00	5.00	5.00	5.00
Choline chloride (60%)	1.25	1.25	1.25	1.25
BHT	0.10	0.10	0.10	0.10
Coxistac	0.50	0.50	0.50	0.50
Avilamycin	0.10	0.10	0.10	0.10
Total	1000.00	1000.00	1000.00	1000.00
Calculated composition				
Metabolizable energy, kcal kg^{-1}	2960	3050	3150	3200
Crude protein, g kg^{-1}	239.2	220.2	207.4	194.8
Digestible lysine, g kg^{-1}	13.24	12.17	11.31	10.60
Digestible methionine + cystine, g kg^{-1}	9.53	8.76	8.26	7.74
Digestible threonine, g kg^{-1}	8.61	7.91	7.35	6.89
Total calcium, g kg^{-1}	9.20	8.41	7.58	6.63
Non-phytate phosphorus, g kg^{-1}	4.70	4.06	3.69	3.13
Digestible phosphorus, g kg^{-1}	3.95	3.52	3.24	2.84
Sodium, g kg^{-1}	2.20	2.10	2.00	1.95

[1]Minimum composition per kg of feed: Copper: 8.6 mg copper carbon-amino-phosphochelate; Iron: 43.7 mg iron carbon-amamino-phosphochelate; Manganese: 56.4 mg manganese carbon-amino-phosphochelate; Selenium: 0.34 mg Selenium carbon-amino-phosphochelate; Zinc: 43.7 mg Zinc carbo-amino-phosphochelate; Vitamin A: 8250 IU; Vitamin E: 31 IU; Vitamin K3: 1.65 mg; Vitamin B1: 2.2 mg; Vitamin B2: 5.5 mg; Vitamin B6: 3.08 mg; Vitamin B12: 13 µg; Folic Acid: 2.5mg; Nicotinic Acid: 33 mg; Pantothenic Acid: 11.03 mg; Biotin: 0.8 mg; Choline: 33 mg; Vitamin D: 2760 IU as Vitamin D_3 or $25(OH)D_3$.

Table 2 – Calculated composition of calcium (Ca), phosphorus (P), non-phytate phosphorus (nPP), and Ca:nPP ratio of experimental diets.

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

The absolute weight (kg) and the yield (%) of the eviscerated carcasses and cuts (breast, thigh and leg quarter) were evaluated at day 42. Carcass yield was calculated as the percent of live BW [(carcass weight / live BW) × 100%]. Yield for each carcass component was calculated as the percent of eviscerated carcass [(carcass portion / eviscerated carcass weight) × 100%] per bird.

The pH analysis was measured 15 minutes *post-mortem* at three different points on the right side of the breast muscle using a Test® 205 portable pH-meter. These carcasses were then identified and maintained in a cooling chamber for 24 hours at 4°C for re-reading of the final pH using the same pH-meter.

After that, the evaluation of the coloration of the breast meat of the broilers was carried out using a Minolta Chroma meter CR-300. Meat color was evaluated in terms of lightness (L*), redness (a*), and yellowness (b*), with three repetitions per point, in three different regions of the inner part of the upper, middle, and lower breast muscle (pectoralis major), after exposure of the carcass reading surface to air for 30 minutes for myoglobin oxygenation.

For the analysis of drip water loss (DWL), a sample of the left breast muscle of approximately 80-100 g was cut per experimental unit and immediately weighed. The sample was placed in the net bag and hung in a waterproof bag filled with air, so that the sample did not have contact with the bag. After a 24-hour period of cooling (1-5°C), the sample was dried and weighed again. The result of the drip water loss was expressed as a percentage of the initial weight. The left breast muscle of the broiler was used for the determination of cooking weight loss (CWL). It was refrigerated, weighed to obtain the weight before cooking and subsequently packed and transferred to a water bath at 85°C for 30 minutes for steaming. After this procedure, the samples were taken out of the bath, cooled to room temperature, and weighed again. Differences between the initial and final weight of the samples corresponded to the loss of water from cooking. Analysis of the shear force was performed with the same fillets used in the determination of weight loss by cooking. For this, the samples were trimmed and cut into three rectangles (1.0 × 1.0 × 1.3 cm). The analysis described above was performed using a TAXT2i texturometer, coupled with the Warner-Bratzler Shear Force-mechanical probe, with a 20 kg capacity and load break speed of 20 cm/minute, providing shear force (SF) of the sample in force-kilogram ($kgf.cm^{-2}$).

On day 42, five millimeters of blood were collected from one broiler per replicate by puncture of the jugular vein using an anticoagulant tube (heparin). After collection, the plasma was extracted by centrifugation at 3,000 rpm for 10 minutes, then transferred to cryotubes and immediately frozen at -18°C for further analysis of phosphorus, calcium, and total alkaline phosphatase (TAP). For the analysis of phosphorus, calcium, and TAP, a Mindray automatic equipment for biochemistry (modelBS200E) was used, using Bioclin determination kits.

To determine the concentrations of minerals - calcium and phosphorus - the left tibia of each broiler was removed, stripped, placed in the oven at 65°C for 72 hours, and then degreased in a Soxhlet extractor, as described by Silva & Queiroz (2002). Afterwards, they were taken to the oven at 65°C to dry for a period of 72 hours. After drying, the samples were crushed in a ball mill for further preparation of the mineral solution, according to Silva & Queiroz (2002). The values of the minerals were expressed as a percentage of the weight of the dry and defatted bone, and the calcium: phosphorus (Ca:P) ratio was obtained by dividing the percentage of calcium by that of phosphorus in the dry matter.

The statistical model used was: $Yijk = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$, where: Y_{ijk} is the observation k of the i^{th} $VitD_3$ or $25(OH)D_3$ within the j^{th} Ca levels; μ is the overall mean; α_i is the effect of the i^{th} $VitD_3$ or $25(OH)D_3$; β_j is the effect of the j^{th} Ca levels; $(\alpha\beta)_{ij}$ is the interaction of the i^{th} $VitD_3$ or $25(OH)D_3$ i with the j^{th} Ca levels; and ε_{ijk} is the residual random error.

The results were analyzed according to a randomized design with a 2 × 4 factorial arrangement ($VitD_3$ or $25(OH)D_3$ × four Ca levels) using SAS (2002). Data were submitted to analysis of variance (ANOVA) and Tukey test was used to evaluate differences between means. All possible interactions among and between the main effects were evaluated using the general linear model procedure of SAS software. *p*-values < 0.05 were considered statistically significant.

RESULTS

No interaction was found ($p>0.05$) between sources of vitamin D_3 and the levels of reduction of Ca on broiler performance in the 1 to 21d and 1 to 42d periods (Table 3). However, the supplementation with $25(OH)D_3$ increased ($p<0.05$) the WG of broilers from 1 to 21d of age compared to supplementation with $VitD_3$.

No interaction ($p>0.05$) was found between the sources of vitamin D_3 and Ca levels in carcass and carcass portions yields at 42d (Table 4).

Reductions in levels of Ca were observed ($p>0.05$), regardless of sources of vitamin $D_{3,}$ had no interaction with breast meat quality (Table 5).

No interaction ($p>0.05$) occurred between sources of vitamin D_3 and Ca levels on the serum concentrations of Ca, P, and total alkaline phosphatase (TAP) at 42d (Table 6). No reduction of dietary Ca levels was found in serum concentrations of Ca, P, and TAP ($p>0,05$). However, the supplementation with $25(OH)D_3$ increased ($p<0,05$) serum concentrations of Ca and P as compared to dietary $VitD_3$.

No interaction ($p>0.05$) occurred between vitamin D_3 sources and Ca levels on the concentration of Ca and P in the tibia (Table 7).

DISCUSSION

The reduction in the levels of dietary Ca, regardless of the sources of vitamin $D_{3,}$ did not influence ($p>0.05$) broiler performance in all rearing phases. These results are consistent with those obtained by Li *et al*. (2012) and Tizziani *et al.* (2019), who did not observe any

Table 3 – Feed intake (FI), weight gain (WG), and feed conversion ratio (FCR) of broiler chickens reared under heat stress at 1 to 21 and 1 to 42 days of age, fed diets supplemented with different vitamin D sources and decreased levels of calcium.

Ca reduction (%)	1 to 21 days of age			1 to 42 days of age		
	FI (g)	WG (g)	FCR (g/g)	FI (g)	WG (g)	FCR (g/g)
0	1200	905	1.32	3613	2354	1.53
10	1221	907	1.34	3715	2352	1.58
20	1217	901	1.35	3603	2348	1.53
30	1213	880	1.38	3562	2313	1.54
Vitamin D source						
D_3	1211	884 a	1.37	3613	2321	1.55
$25(OH)D_3$	1214	912 b	1.33	3633	2363	1.53
[1]CV (%)	5.61	5.05	5.96	4.72	5.62	3.95
			p – valor			
Ca reduction	0.8587	0.3740	0.3291	0.1244	0.8227	0.1705
Vitamin D source	0.8481	0.0226	0.0568	0.6646	0.2318	0.2312
Ca x Vit D	0.5117	0.2374	0.3139	0.4662	0.2603	0.5846

Means with different letters in the same column differ from each other by the Tukey's test at 5%;

[1]CV (%) = coefficient of variation.

Table 4 – Yield of carcass and noble cuts (breast, thigh and leg quarter) of broiler chickens at 42 days of age, fed diets supplemented with different vitamin D sources and decreased levels of calcium, and reared under heat stress.

Ca reduction (%)	Carcass yield and noble cuts (%)			
	Carcass	Breast	Thigh	Leg quarter
0	70.31	34.32	12.09	14.32
10	70.04	33.95	12.25	14.57
20	69.44	34.22	12.22	14.67
30	70.04	34.28	12.22	14.20
Vitamin D source				
D_3	69.59	34.15	12.23	14.49
$25(OH)D_3$	70.32	34.23	12.15	14.39
[1]CV (%)	2.80	6.79	5.59	7.34
		p – valor		
Ca reduction	0.9106	0.7975	0.8256	0.3198
Vitamin D source	0.4808	0.8867	0.5380	0.5986
Ca x Vit D	0.7993	0.0809	0.4147	0.3175

[1]CV (%) = coefficient of variation.

effect on the performance of the broilers reared in thermo comfort when fed diets containing low levels of Ca. However, Li *et al*. (2012) verified that, when they provided Ca levels above broiler requirements, a reduction in FI, and consequently in WG, occurred. Therefore an increase in the efficiency of Ca absorption might have occurred in the present study. After all, sub-optimal levels of Ca may enhance the activity of the 1α-hydrolase enzyme in broilers' kidneys and, consequently, in plasma levels of $1,25(OH)_2D_3$, resulting in an increased expression of the Ca transporter protein in the intestine (Blahos *et al*., 1987).

In relation to the sources of vitamin D_3, there was no difference (p>0.05) in FI and FCR of broilers from 1 to 21d. These results are in agreement with those reported by several authors (Ledwaba & Roberson, 2003; Fritts & Waldroup, 2005; Rao *et al*., 2006; Castro *et al*., 2018;

Table 5 – Meat quality of broiler chickens at 42 days of age, fed diets supplemented with different vitamin D sources and decreased levels of calcium and reared under heat stress.

Ca reduction (%)	Breast meat quality variables								
	L*	a*	b*	pH_{15min}	pH_{24h}	DWL (g)	TWL (%)	CWL (%)	SF ($kgf.cm^{-2}$)
0	58.58	5.85	16.00	6.30	5.94	2.33	13.25	12.06	1.66
10	59.56	5.92	17.19	6.38	5.99	2.36	12.18	10.96	1.73
20	58.98	5.95	17.04	6.29	5.94	2.33	13.15	11.97	1.60
30	60.52	5.70	17.40	6.34	5.99	2.20	13.14	11.76	1.89
Vitamin D source									
D_3	59.43	5.95	16.88	6.32	5.98	2.37	12.58	12.20	1.72
$25(OH)D_3$	59.39	5.76	16.93	6.33	5.95	2.24	13.28	11.17	1.72
[1]CV (%)	6.24	17.77	9.93	1.87	2.04	14.07	20.69	22.63	18.40
					p – valor				
Ca	0.5455	0.9221	0.1378	0.2125	0.4502	0.5583	0.6863	0.6816	0.1068
VitD	0.9631	0.4823	0.8983	0.6704	0.4259	0.1421	0.3319	0.1537	0.9685
Ca x Vit D	0.2829	0.7410	0.3189	0.9337	0.3520	0.9514	0.2204	0.1488	0.2671

[1]CV (%) = coefficient of variation.

Table 6 – Serum concentrations of calcium, phosphorus and total alkaline phosphatase (TAP) of broiler chickens at 42 days of age fed diets supplemented with different vitamin D sources and decreased levels of calcium and reared under heat stress.

Ca reduction (%)	Serum concentrations		
	Ca, mg/dL	*p*, mg/dL	TAP, µL
0	7.78	5.30	403.53
10	8.04	5.54	409.06
20	8.50	5.60	389.53
30	7.97	5.32	467.39
Vitamin D source			
D_3	7.75 b	5.24 b	436.24
25(OH)D_3	8.40 a	5.64 a	398.51
[1]CV (%)	11.53	9.98	31.86
		p – valor	
Ca reduction	0.2350	0.3427	0.4318
Vitamin D source	0.0114	0.0089	0.2935
Ca x Vit D	0.5214	0.4817	0.8886

Means with different letters in the same column differ from each other by the Tukey's test at 5%;

[1]CV (%) = coefficient of variation.

Table 7 – Percentage of calcium, phosphorus, and calcium and phosphorus ratio in the tibia of broilers at 42 days of age fed diets supplemented with different vitamin D sources and decreased levels of calcium and reared under heat stress.

Ca reduction (%)	Variables		
	Ca, %	*p*, %	Ca: P
0	12.97	7.01	1.84
10	12.65	6.90	1.83
20	12.71	6.85	1.85
30	13.26	7.15	1.85
Vitamin D source			
D_3	12.84	7.02	1.82
25(OH)D_3	12.96	6.93	1.86
[1]CV (%)	6.44	7.34	5.90
		p – valor	
Ca reduction	0.2047	0.1160	0.9501
Vitamin D source	0.6002	0.3306	0.3219
Ca x Vit D	0.9501	0.1750	0.9986

[1]CV (%) = coefficient of variation.

Tizziani *et al.,* 2019), where no significant difference in the performance of broilers due to the sources of vitamin D_3 were observed. Although not significant, a reduction of 2.92% was found in the absolute value of FCR of broilers fed diets containing 25(OH)D_3 (p=0.0568), possibly indicating a trend. However, it was observed that the supplementation with 25(OH)D_3 increased (p<0.05) the WG of broilers aged 1 to 21d as compared to supplementation with VitD_3. Similary, Fritts & Waldroup (2003) found an increase in the WG of broilers reared in thermal comfort at 1 to 21d when 25(OH)D_3 was used, as compared to VitD_3 inclusion. The best efficiency in broiler growth with 25(OH)D_3 might be related to its greater absorption efficiency in comparison to VitD_3.

The higher absorption efficiency of 25(OH)D_3 is explained by its greater polarity (already hydroxylated). Additionally, during the starter phase, broilers' enzyme systems are not completely mature to perform hydroxylation in the liver, which does not affect the efficiency of already hydroxylated metabolites such as 25(OH)D_3. Hsiao *et al*. (2018) observed greater expression of calbindin and β-glucuronidase in the duodenum of 21-day-old broilers reared in thermal comfort when fed diets supplemented with 1,25$(OH)_2D_3$ and 25(OH)D_3, as compared to VitD_3. In contrast, Vazquez *et al*. (2018) observed no effect on the performance of broilers reared in thermal comfort in the period from 1 to 21 days when fed 25(OH)D_3 associated with VitD_3. These authors indicated that the high level of VitD_3 supplemented (5000 IU/kg) contributed to the lack of effect.

Regarding the 1-42 day-of-age period, sources of VitD_3 did not influence (p>0.05) the performance of broilers reared under heat stress. Similar results were found with broilers reared in thermal comfort by Fritts & Waldroup (2005), Roberson *et al*. (2005), Angel *et al*. (2006), Castro *et al*. (2018), and Tizziani *et al.* (2019). However, Santiago *et al*. (2016) observed an increase in the WG of broilers reared in thermal comfort when receiving 25(OH)D_3 supplementation in the 1-42 day-of-age period via drinking water. The way vitamins (feed or water) were supplied, as well as the dosage used and thermal conditions, justifies the divergence of results among the studies. Results differing from those obtained in this study were found by Morris *et al*. (2014), when evaluating sources of vitamin D (25(OH)D_3 and VitD_3) for broiler chickens reared in thermal comfort challenged by mycotoxins. They observed an increase on performance and a reduction on the inflammatory response by reducing the gene expression of interleukin 1-ß in the liver when the broilers were challenged with lipopolysaccharide injection and fed 25(OH)D_3. This suggests that broilers have a more efficient inflammatory response when 25(OH)D_3 is fed.

In the present study, no effect was observed (p>0.05) on the carcass yield and cuts of broilers reared under heat stress and fed diets supplemented VitD_3 or 25(OH)D_3. Similarly, Araújo *et al*. (2002) and Tizziani *et al*. (2019) found no effect on carcass yield and cuts of broiler chickens reared in thermal comfort fed diets with Ca reduction of 25 and 30%, respectively. Angel *et al*. (2006) and Brito *et al*. (2010) did not observe effects on the carcass yield and thigh and leg quarter yield, respectively, when evaluating sources of vitamin D ($25(OH)D_3$ or $VitD_3$) for broilers. On the other hand, Vignale et al. (2015) reported increases in the breast yield of broilers due to the substitution of $VitD_3$ with $25(OH)D_3$. These effects would be related to the fact that $25(OH)D_3$ increases the gene expression of the nuclear VDR receptor in the duodenum and muscle of the broilers, which increases the transcription and activation of mTOR, causing an increase in breast meat production (Hutton *et al*., 2014; Hsiao *et al*., 2018). Additionally, the high level of $25(OH)D_3$ used by Vignale *et al*. (2015) (5500 IU/kg) compared to that of the present study (2760 IU/kg) and the heat stress may justify the divergence of results.

In the present study, no effect (p>0.05) was detected on the meat quality of the breast muscles of broilers reared under heat stress and fed diets supplemented with $VitD_3$ and $25(OH)D_3$. These results show that sub-optimal levels of Ca up to 30% below the requirement did not compromise the parameters of breast meat quality. According to Johnson *et al*. (1990), the level of Ca used in the diet does not generally influence the quality parameters of broiler breast meat, since the activity of calpain, the main enzyme responsible for muscle proteolysis, requires a concentration of free cellular Ca that is not normally altered by its concentration in the diet.

Garcia *et al*. (2013) evaluated $25(OH)D_3$ and other vitamin D_3 metabolites in broiler diets, and did not observe differences in the pH and quality of chicken breast meat either. According to Qiao *et al*. (2001) and Fletcher (2002), meat pH exerts a direct action on the proteins and pigments in their constitution, in addition to being the main parameter that influences the characteristics of water loss, tenderness, and meat coloring. It can be said that the data obtained from meat quality are consistent with those of pH. Considering what was proposed by Woelfel *et al*. (2002), the meat luminosity found in this study (< 59.8 L*), and an initial pH >5.76, the results can be classified as values of good quality meat.

Similarly to this study, Han *et al*. (2016) found that the variation in dietary Ca levels with different sources of vitamin D ($25(OH)D_3 \times 1\alpha\text{-OH-}D_3$) did not influence the serum levels of Ca and P of broiler chickens. Based on the fact that the increase in serum concentration of TAP is associated with the occurrence of rickets (Sahay & Sahay, 2012), it can be said that a reduction of up to 30% of Ca in the diet does not compromise bone mineralization in modern broilers.

In relation to vitamin D_3 sources, an increase (p<0.05) in the serum level of Ca and P was observed when $25(OH)D_3$ was used. These results might be related to a greater efficiency of Ca and P absorption. According to Henry (2011), as serum concentrations of Ca and P decrease, the activity of the enzyme 1-α-hydroxylase increases in the kidneys, increasing the hydroxylation of $25(OH)D_3$ and plasma concentrations of $1,25(OH)_2D_3$, which increased absorption of these minerals. Aburto *et al*. (1998) associated the increase in the serum level of Ca with the supplementation of $25(OH)D_3$ in broilers' diets. However, Hsiao *et al*. (2018) did not observe an increase in the Ca and P serum levels of broilers fed diets containing different sources and active metabolites of vitamin D_3. Tizziani *et al*. (2019) also observed no effect on the serum concentration of Ca and P of broilers fed diets with a 30% reduction in Ca and supplementation of $VitD_3$ and $25(OH)D_3$.

No variation (p>0.05) was observed in the TAP serum concentration due to the evaluated sources of vitamin D_3. Although no significant variation occurred, supplementation with $25(OH)D_3$ in the diets resulted in an average reduction of 8.64% in the absolute values of TAP in the serum of the broilers. According to Malloy & Feldman (2010), the concentration of TAP varies in inverse proportion with the concentration of $25(OH)D_3$ in the serum. Thus, it can be inferred that an increase of this vitamin D_3 metabolite took place in the serum of the broilers, which would agree with the previous report of improvement in the absorption of Ca and P.

Reduction in the dietary levels of Ca did not affect (p>0.05) concentrations of Ca and P in the tibia of the broilers. These results are different from those obtained by Li *et al*. (2012), who reported a reduction in Ca concentration in the tibia due to a 45.50% decrease in the levels of Ca (1.10 × 0.60%), when its relationship with nPP was kept fixed in diets. In the present study, the maximum reduction in dietary Ca was 30%. Furthermore, the ratio of Ca:nPP was not kept fixed. These factors explain the divergence in concentrations of Ca and P in the tibia of the broilers as compared to the results observed by Li *et al*. (2012).

During exposure to high temperatures, broilers increase respiratory rates to dissipate heat by latent mechanisms. If a higher respiratory rate is maintained, respiratory alkalosis occurs, reducing the blood Ca content, and decreasing the Ca mobilization of bone reserves (Daghir, 2008); affecting content of Ca in the tibia. However, this fact was not observed in the present study. The sources of vitamin D_3 did not influence (p>0.05) the percentages of Ca and P in the tibia of the broilers reared under heat stress. These data agree with those observed by Castro *et al*. (2018) and Tizziani *et al.* (2019), since there was no effect of the source of vitamin D_3 used, associated or not with $25(OH)D_3$ supplementation. In contrast, Ledwaba & Roberson (2003) and Atencio *et al*. (2005) reported higher efficiency of $25(OH)D_3$ in increasing the ash concentration in the tibia of broiler chickens in relation to vitamin D_3. The differences in the level of inclusion of vitamin D_3 sources and the different environmental conditions between the studies might be the factors contributing to the inconsistency of the results.

In summary, the substitution of $VitD_3$ by $25(OH)D_3$ in diets increased weight gain from 1 to 21d and serum concentrations of Ca and P. The supplementation of $25(OH)D_3$ in all phases did not negatively compromise performance, carcass characteristics, and Ca and P deposition in the tibia of broilers at 42d, when reared under heat stress. The Ca reduction of 30% in diets in all phases, regardless of the source of vitamin D_3 supplemented, did not compromise performance, carcass characteristics, and Ca and P deposition in the tibia of broilers at 42 d, when reared under heat stress.

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