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

ABSTRACT

The objective was to evaluate the effect of dietary 25-hydroxycholecalciferol (25(OH)D₃) or vitamin D₃ (VitD₃) 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 (VitD₃ or 25(OH)D₃ × 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 D₃ sources were supplemented in the diets from 1 to 21 days for broilers chickens. 25 (OH)D₃ 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)D₃. The replacement of VitD₃ with 25(OH)D₃ 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, immnosuppression, 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).
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Vitamin D plays a key role in mineral metabolism. Due to the lower conversion efficiency in the skin, dietary vitamin D₃ is needed for broilers. Cholecalciferol (D₃) is the common way of adding vitamin D₃ to diets. However, it is currently possible to use isoforms or vitamin D₃ 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 D₃ is hydroxylated in the liver, resulting in the formation of the 25(OH)D₃ metabolite, the main circulating form in blood, used as a vitamin D marker in animals (Arnold et al., 2016; Hsiao et al., 2019).

In this sense, the present study aims to evaluate the effect of 25(OH)D₃ 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 x 4 factorial arrangement (two sources of vitamin D: VitD₃ or 25(OH)D₃, (Hy-D®, DSM Nutritional Products) x 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.
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Table 1 – Centesimal and calculated composition of experimental diets in natural matter basis.

| Ingredients (g kg⁻¹) | 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 mixture¹ | 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    |
| Avidalacyn          | 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

¹Minimum composition per kg of feed: Copper: 8.6 mg copper carbon-amino-phosphochelate; Iron: 43.7 mg iron carbon-amino-phosphochelate; Manganese: 56.4 mg manganese carbon-amino-phosphochelate; Selenium: 0.34 mg Selenium carbon-amino-phosphochelate; Zinc: 43.7 mg Zinc carbon-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 D3: 2760 IU as Vitamin D3 or 25(OH)D3.

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

| Reduciton of Ca | Ca, 1-7 d (%) | P, 1-7 d (%) | nPP, 1-7 d (%) | Ca:nPP ratio |
|----------------|--------------|--------------|---------------|--------------|
| 0 %            | 0.920        | 0.706        | 0.470         | 1.96         |
| 10 %           | 0.828        | 0.706        | 0.470         | 1.76         |
| 20 %           | 0.736        | 0.706        | 0.470         | 1.57         |
| 30 %           | 0.530        | 0.706        | 0.470         | 1.37         |

| Ca, 8-21 d (%) | P, 8-21 d (%) | nPP, 8-21 d (%) | Ca:nPP ratio |
|----------------|--------------|----------------|--------------|
| 0.841          | 0.639        | 0.401          | 2.10         |
| 0.757          | 0.639        | 0.401          | 1.89         |
| 0.672          | 0.639        | 0.401          | 1.68         |
| 0.588          | 0.639        | 0.401          | 1.47         |

| Ca, 22-33 d (%) | P, 22-33 d (%) | nPP, 22-33 d (%) | Ca:nPP ratio |
|----------------|--------------|----------------|--------------|
| 0.758          | 0.595        | 0.354          | 2.14         |
| 0.682          | 0.595        | 0.354          | 1.93         |
| 0.606          | 0.595        | 0.354          | 1.71         |
| 0.530          | 0.595        | 0.354          | 1.50         |

| Ca, 34-42 d (%) | P, 34-42 d (%) | nPP, 34-42 d (%) | Ca:nPP ratio |
|----------------|--------------|----------------|--------------|
| 0.663          | 0.535        | 0.309          | 2.15         |
| 0.596          | 0.535        | 0.309          | 1.93         |
| 0.530          | 0.535        | 0.309          | 1.72         |
| 0.530          | 0.535        | 0.309          | 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: \( Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \epsilon_{ijk} \), where: \( Y_{ijk} \) is the observation \( k \) of the \( i \)th VitD₃ or 25(OH)D₃ within the \( j \)th Ca levels; \( \mu \) is the overall mean; \( \alpha_i \) is the effect of the \( i \)th VitD₃ or 25(OH)D₃; \( \beta_j \) is the effect of the \( j \)th Ca levels; \( (\alpha\beta)_{ij} \) is the interaction of the \( i \)th VitD₃ or 25(OH)D₃ with the \( j \)th Ca levels; and \( \epsilon_{ijk} \) is the residual random error.

The results were analyzed according to a randomized design with a 2 × 4 factorial arrangement (VitD₃ or 25(OH)D₃ × 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 D₃ 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)D₃ increased \((p<0.05)\) the WG of broilers from 1 to 21d of age compared to supplementation with VitD₃.

No interaction \((p>0.05)\) was found between the sources of vitamin D₃ 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 D₃, had no interaction with breast meat quality (Table 5).

No interaction \((p>0.05)\) occurred between sources of vitamin D₃ 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)D₃ increased \((p<0.05)\) serum concentrations of Ca and P as compared to dietary VitD₃.

No interaction \((p>0.05)\) occurred between vitamin D₃ 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 D₃, 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
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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-hydroxylase enzyme in broilers’ kidneys and, consequently, in plasma levels of 1,25(OH)2D3, resulting in an increased expression of the Ca transporter protein in the intestine (Blahos et al., 1987).

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

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

|                  | 1211  | 884 a | 1.37      | 3613 | 2321 | 1.55      |
| Vitamin D source | 1214  | 912 b | 1.33      | 3633 | 2363 | 1.53      |

1CV (%) 5.61 0.3740 0.3291 0.1244 0.8227 0.1705

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 0.472 5.62 3.95

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

1CV (%) = 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

|                  | 69.59   | 34.15   | 12.23 | 14.49       |
| 25(OH)D3         | 70.32   | 34.23   | 12.15 | 14.39       |

1CV (%) 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

1CV (%) = coefficient of variation.

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*   | pH15min | pHpH | DWL (g) | TWL (%) | CWL (%) | SF (kgf.cm−2) |
| 0                | 58.58 | 5.85 | 16.00 | 6.30    | 7.94 | 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

|                  | 59.43 | 5.95 | 16.88 | 6.32    | 5.98 | 2.37    | 12.58   | 12.20   | 1.72        |
| 25(OH)D3         | 59.39 | 5.76 | 16.93 | 6.33    | 5.95 | 2.24    | 13.28   | 11.17   | 1.72        |

1CV (%) 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

1CV (%) = coefficient of variation.
The best efficiency in broiler growth with 25(OH)D₃ might be related to its greater absorption efficiency in comparison to VitD₃.

The higher absorption efficiency of 25(OH)D₃ 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)D₃. 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)₂D₃ and 25(OH)D₃ as compared to VitD₃. 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)D₃ associated with VitD₃. These authors indicated that the high level of VitD₃ supplemented (5000 IU/kg) contributed to the lack of effect.

Regarding the 1–42 day-of-age period, sources of VitD₃ 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)D₃ 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)D₃ and VitD₃) for broiler chickens reared in thermal comfort challenged by mycotoxins. They observed an increase in 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)D₃. This suggests that broilers have a more efficient inflammatory response when 25(OH)D₃ 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 VitD₃ or 25(OH)D₃. 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

### 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.

| Vitamin D source | Ca, mg/dL | P, mg/dL | TAP, μL |
|------------------|-----------|----------|---------|
| D₃               | 7.75 b    | 5.24 b   | 436.24  |
| 25(OH)D₃         | 8.40 a    | 5.64 a   | 398.51  |
| 1CV (%)          | 11.53     | 9.98     | 31.86   |

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

1CV (%) = 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 (%) | 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 |

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

1CV (%) = coefficient of variation.

Tizziani et al., 2019, where no significant difference in the performance of broilers due to the sources of vitamin D₃ 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)D₃ (p=0.0568), possibly indicating a trend. However, it was observed that the supplementation with 25(OH)D₃ increased (p<0.05) the WG of broilers aged 1 to 21d as compared to supplementation with VitD₃. Similarly, Fritts & Waldroup (2003) found an increase in the WG of broilers reared in thermal comfort at 1 to 21d when 25(OH)D₃ was used, as compared to VitD₃ inclusion.
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)D3 or VitD3) for broilers. On the other hand, Vignale et al. (2015) reported increases in the breast yield of broilers due to the substitution of VitD3 with 25(OH)D3. These effects would be related to the fact that 25(OH)D3 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)D3 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 VitD3 and 25(OH)D3. These results show that suboptimal 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)D3 and other vitamin D3 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)D3 × 1α-OH-D3) 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 D3 sources, an increase (p<0.05) in the serum level of Ca and P was observed when 25(OH)D3 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)D3 and plasma concentrations of 1,25(OH)2D3, which increases absorption of these minerals. Aburto et al. (1998) associated the increase in the serum level of Ca with the supplementation of 25(OH)D3 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 D3. 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 VitD3 and 25(OH)D3.

No variation (p>0.05) was observed in the TAP serum concentration due to the evaluated sources of vitamin D3. Although no significant variation occurred, supplementation with 25(OH)D3 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)D3 in the serum. Thus, it can be inferred that an increase of this vitamin D3 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 D3 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 D₃ used, associated or not with 25(OH)D₃ supplementation. In contrast, Ledwaba & Roberson (2003) and Atencio et al. (2005) reported higher efficiency of 25(OH)D₃ in increasing the ash concentration in the tibia of broiler chickens in relation to vitamin D₃. The differences in the level of inclusion of vitamin D₃ 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 VitD₃ by 25(OH)D₃ in diets increased weight gain from 1 to 21 d and serum concentrations of Ca and P. The supplementation of 25(OH)D₃ in all phases did not negatively compromise performance, carcass characteristics, and Ca and P deposition in the tibia of broilers at 42 d, when reared under heat stress. The Ca reduction of 30% in diets in all phases, regardless of the source of vitamin D₃ 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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