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Performance and bone health of broilers reared under artificial lighting and supplemented with different levels of vitamin D_3

Tainá Silva Brandão Lopes[1], Mariana Diniz Costa Vasconcelos[2], Bruno Teixeira Antunes Costa[1], Lorena Salim Sousa[1], Bruno Machado Bertassoli[2], Natália de Melo Ocarino[2], Rogéria Serakides[2], Leonardo José Camargos Lara[1], Itallo Conrado Sousa Araújo[1]*

[1] Universidade Federal de Minas Gerais, Escola de Veterinária, Departamento de Zootecnia, Belo Horizonte, MG, Brasil.
[2] Universidade Federal de Minas Gerais, Escola de Veterinária, Departamento de Clínica e Cirurgia Veterinárias, Belo Horizonte, MG, Brasil.

***Corresponding author:**
italloconradovet@hotmail.com

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ABSTRACT - This study aimed to investigate the effects of different levels of vitamin D_3 in broiler diets on performance and bone health. A total of 360 one-day old male Cobb500® broiler chicks were subjected to five treatments of different levels of vitamin D_3 in diets during two rearing phases: 0 IU/kg in both phases; 625 IU/kg in starter and 500 IU/kg in grower phase (25% of commercial inclusion); 1,250 IU/kg in starter and 1,000 IU/kg in grower phase (50% of commercial inclusion); 1,875 IU/kg in starter and 1,500 IU/kg in grower phase (75% of commercial inclusion); and 2,500 IU/kg in starter and 2,000 IU/kg in grower phase (100% of commercial inclusion). The traits studied weekly were feed intake, body weight, feed conversion, and viability. At 21 and 35 days of age, tibiae and femurs were removed, dissected, and evaluated for dry matter (DM), ash (%MM), calcium (%Ca), phosphorus (%P) and breaking strength. At 35 days of age, the bones were subjected to histopathological analysis for macro- and microscopic morphological evaluation. Data were subjected to regression analysis, using $\alpha = 0.05$. The variables of percentage %MM, %Ca, %P, and breaking strength experienced a positive linear effect up to the supplementation levels of approximately 25% of inclusion. The histopathological analysis found that the group that received the diet with 100% inclusion of vitamin D_3 presented lesions compatible with osteopetrosis and tibial dyschondroplasia. The observed results showed that for isonutritive diets, reduced levels of vitamin D_3 guaranteed performance during the evaluated period while the use of 100% of vitamin D_3, as commonly used, can cause bone diseases and harm the welfare of broilers.

Keywords: bone quality, broiler chicken, cholecalciferol, osteopetrosis, tibial

1. Introduction

Poultry breeding programs, over several years, have selected a series of desirable phenotypic characteristics, such as high feed efficiency and rapid muscle development (Zuidhof et al., 2014). However, the high rate of muscle tissue growth compromises the health of bone tissue, causing diseases such as tibial dyschondroplasia (TD), which generates economic losses due to increased mortality during rearing and increased rates of fracture and breakage in the slaughterhouse (Guo et al., 2019).

To minimize the occurrence of bone diseases, nutritional programs that aim to use vitamins, including vitamin D_3 ($VitD_3$) or calcitriol, have been developed. Vitamin D_3 is important for bone metabolism and

for the control of plasma calcium (Ca) levels, preventing the occurrence of diseases such as rickets, fibrous osteodystrophy, and TD (Souza and Vieites, 2014). It can be obtained by food or metabolized by photolysis reaction, in which ultraviolet rays induce its synthesis from 7-dehydrocholesterol or pro-vitamin D_3. When consumed in the inactive form, there is a need for two hydroxylation until the biologically active form is synthesized. The first hydroxylation occurs in the liver, where 25-hydroxycholecalciferol [25-(OH) D_3] or calcidiol is formed, while the second occurs in the kidneys, forming 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] or calcitriol, which is the biologically active form of $VitD_3$ (Soares et al., 1995).

Currently, it is common to build completely closed sheds for rearing broilers, which makes the metabolization of $VitD_3$ by photolysis less common, increasing the risks of disorders arising from the failure to meet the $VitD_3$ levels required by birds (Leyva-Jimenez et al., 2018). For this reason, $VitD_3$ supplementation in commercial diets for broilers has been used to prevent bone problems (Whitehead et al., 2004). According to Garcia et al. (2013), supplementation by feed can occur both in the form of the cholecalciferol precursor and in the more active forms, calcitriol and its precursor calcidiol. Supplementation levels vary according to the reference, and the recommendation of the Nutritional Research Council (NRC) is 200 IU of $VitD_3$/kg of feed throughout the breeding cycle (NRC, 1994), while the Brazilian Table for Poultry and Swine suggests 3,385 IU/kg of feed for the pre-starter phase, 3,054 IU/kg for the starter phase, 2,409 IU/kg for the growth phase, and 1,763 IU/kg in the final phase (Rostagno et al., 2017). Higher levels are indicated by the Cobb strain manual, which recommends 5,000 IU/kg of feed during the entire production cycle (Cobb, 2018a). However, the industry works with safety margins that can exceed 5 to 10 times the levels recommended by the NRC, with levels close to 2,500 IU/kg of feed in the starter phase and 2,000 IU/kg in the growth phase (Sakkas et al., 2019).

On the other hand, excess $VitD_3$ can cause bone diseases, such as osteopetrosis and osteochondrosis or TD (Aguirre et al., 2005). Therefore, investigations into $VitD_3$ levels that promote the best performance of broilers have been the subject of studies for several years (Edwards et al., 1992; Fritts and Waldroup, 2003; Castro et al., 2018; Lopes et al., 2024). However, the level of $VitD_3$ that promotes the best performance, in terms of feed efficiency and performance, will not always promote bone health (Leyva-Jimenez et al., 2019).

Our hypothesis posits that the recommended levels of $VitD_3$ far exceed the actual needs of male broiler chickens when they are raised without exposure to sunlight. Given the above, the objective of the present study was to evaluate the effects of different levels of $VitD_3$ supplementation in the diet of broilers from 1 to 35 days old, reared under artificial lighting, on bone quality and health.

2. Material and Methods

The experiment was conducted in Belo Horizonte, MG, Brazil (19°55' S and 43°56' W). The current experimental protocol was approved by the Ethical Principles in Animal Experimentation Committee (case number 113/2018).

2.1. Animals, diets, and experimental design

Three hundred sixty one-old-day Cob500® male broiler chickens with an average weight of 43 g were obtained from a commercial local hatchery and distributed in a completely randomized design with five treatments, and six replicates of 12 broilers each. The birds were allocated in cages of 1 m^2 from 1 to 35 days and received water and feed *ad libitum*. All cages were in a single climatized room with 56 m^2, windowless, with walls covered by polyurethane panels and equipped with a central temperature, humidity, and wind cycle controller panel. No access to sunlight was provided to the birds. The light program used was artificial light with fluorescent lamps (Sylvania model T8 comfort, 32 W power, and 4000 k), in a schedule of 8 h of darkness and 16 h of light/day throughout the rearing phase (Bayram and Özkan, 2010). Besides the central control panel, the average air temperature at the bird's level was monitored by an indoor digital thermo-hygrometer (Dual Zona Electronic Thermometer,

VWR, São Paulo, SP, product no. 82021-166), and the ambiance parameters were adjusted according to the Cobb 500 management guide (Cobb, 2018b).

Treatments were levels of $VitD_3$ (Cholecalciferol, 500,000 IU/g, Xiamen Kingdowamay, China) in diets: 0 IU of $VitD_3$/kg in both phases (0% of commercial inclusion); 625 IU/kg in the starter and 500 IU/kg in grower phase (25% of commercial inclusion); 1,250 IU/kg in the starter and 1,000 IU/kg in grower phase (50% of commercial inclusion); 1,875 IU/kg in the starter and 1,500 IU/kg in grower phase (75% of commercial inclusion); and 2,500 IU/kg in the starter and 2,000 IU/kg in grower phase (100% of commercial inclusion) (Table 1).

The diets were based on corn and soybean meal and formulated for starter (1 to 21 days) and grower (22 to 35 days) phases. The diets were formulated considering the nutritional values of the raw ingredients according to Rostagno et al. (2017). The nutritional levels were established according to Castro et al. (2018), except for the $VitD_3$ level, which was included according to each treatment studied. The physical form used in both phases was mash. The diet composition and chemical analysis are detailed in Table 2.

Table 1 - Vitamin D_3 levels used in starter and grower phases

$VitD_3$ (%)[1]	Starter phase (1 to 21 d)	Grower phase (22 to 35 d)
0	0 IU/kg	0 IU/kg
25	625 IU/kg	500 IU/kg
50	1,250 IU/kg	1,000 IU/kg
75	1,875 IU/kg	1,500 IU/kg
100	2,500 IU/kg	2,000 IU/kg

[1] Percentage of inclusion of vitamin D_3 in relation of to the levels used commercially, with 2,500 IU/kg of feed for the starter phase and 2,000 IU/kg of feed for the grower phase.
Cholecalciferol, 500,000 IU/g (Xiamen Kingdowamay, China).

Table 2 - Diet composition and chemical analysis of the diets on as fed basis (g/kg, unless otherwise in indicated)

Ingredient	Starter (1-21 d)	Grower (22-35 d)
Yellow corn	604.0	678.3
Soybean meal, 450 g/kg CP	300.0	233.3
Meat and bone, 400 g/kg CP and 62 g/kg P	63.6	53.3
Soybean oil	18.0	20.0
Limestone	0.00	0.9
NaCl	3.8	4.0
Mineral-vitamin supplement[1]	3.0	4.0
DL-Methionine, 980 g/kg	3.5	3.0
L-Lysine HCl, 980 g/kg	2.7	2.5
L-Threonine	1.4	0.5
Nutritional levels (analyzed)		
Metabolizable energy (MJ/kg)	12.66	13.06
Crude protein	219.6	189.2
Ca	9.4	8.2
Available P	4.8	4.0
Digestible lysine	12.1	10.2
Digestible methionine	7.0	5.8
Digestible methionine and cystine	10.0	5.5
Digestible threonine	9.1	7.9
Digestible tryptophan	2.2	2.0
Sodium	2.0	2.2

[1] Mineral-vitamin supplement (starter phase): vitamin A, 13,685 IU; vitamin D3, 3,157 IU; vitamin E, 350 mg; vitamin K3, 4,410 mg; vitamin B1, 2,415 mg; vitamin B2, 8,662.5 mg; vitamin B6, 5,460 mg; vitamin B12, 21,315 µg; biotin, 96,250 µg; niacin, 53,900 mg; folic acid, 1,228.5 mg; pantothenic acid, 13,860 mg; selenium, 0.3 mg; iodine, 1.0 mg; iron, 300 mg; copper, 10.0 mg; manganese, 90.0 mg; zinc, 80.0 mg. Mineral-vitamin supplement (grower phase): vitamin A, 9,775 IU; vitamin D3, 2,255 IU; vitamin E, 25.0 mg; vitamin K3, 3,150 mg; vitamin B1, 1,725 mg; vitamin B2, 6,187.5 mg; vitamin B6, 3,900 mg; vitamin B12, 15,225 µg; biotin, 68,750 µg; niacin, 38,500 mg; folic acid, 0.87 mg; pantothenic acid, 9,900 mg; selenium, 0.21 mg; iodine, 1.0 mg; iron, 30.0 mg; copper, 10.0 mg; manganese, 90.0 mg; zinc, 80.0 mg.

2.2. Performance

Broilers and feed provided were weighed weekly to obtain feed intake (FI), body weight (BW), and feed conversion (FC). Mortality was daily recorded, and viability (VIA) was calculated considering the number of broilers that died during experimental period in relation to the initial number of broilers in each replicate.

2.3. Broiler chicken bone quality

At 21 and 35 days, one broiler from each replicate, a total of six birds per treatment, was euthanized by cervical dislocation to collect the left and right tibiae and femurs. The soft and connective tissue was manually removed using surgical tweezers and scissors (Castro et al., 2018). The weight (g) and height (cm) of the right tibiae were then measured using an analytical scale (AY220, Shimadzu, Kyoto, Japan) and caliper (Universal analogic caliper, Cat. No. 539-104, Mitutoyo, Tokyo, Japan). The right tibiae were then used to determine the ash percentage according to Silva and Queiroz (2002). Bones were dried at 100 °C for 24 h followed by 76 h refluxing in a Soxhlet with petroleum ether. The fat-free bones were ashed at 600 °C for 6 h, cooled in a desiccator, and weighed to obtain bone ash weight. The bone ash was used to make a standard solution. For that, the ashes were dissolved in a 50% hydrochloric acid solution and filtered with a paper filter (Whatman 1001-0155 filter paper, Sigma-Aldrich, Burlington, MA) in a 100-mL volumetric flask. Distilled water was added to obtain 100 mL of the final solution. The final solution was then used to obtain the percentage of Ca and P by quantitative titration according to AOAC (2005). The left tibiae were used for biomechanical essay for maximum breaking strength according to the methods described by Castro et al. (2018), using a universal machine (model EMIC DL 3000), in a three-point flexing test with a 2000 N load cell. The values obtained were recorded and stored by the Instron Series IX software.

The Seedor index was determined by measuring the tibia at its longest length and dividing the value by tibia weight, obtained using an analytical scale (AY220, Shimadzu, Kyoto, Japan) accordingly to Seedor et al. (1991).

2.4. Broiler chicken bone health

At 35 days of age, the left tibia of one bird per pen was collected for histopathological analysis according to Caputo et al. (2010). The tibia was collected, and the soft and connective tissue was completely removed by using surgical tweezers and scissors. Then, the bones were fixed in 10% buffered formalin for 48 h and maintained in 21% formic acid for 30 days until complete decalcification. The tibiae were separated by group and displayed in a table above a bucky, and cranio-caudal and medium-lateral radiological projections were taken (RX Compacto 500, VMI, 500 mA, Lagoa Santa, MG). The bone luminescence was evaluated to confirm the complete decalcification. After that, each bone was sectioned longitudinally to reveal the hypertrophy and proliferation zones of the growth plate. In that way, it was possible to analyze macroscopically the growth plate linearity, the amount of trabecular bone, and cartilage in the metaphyseal and epiphyseal regions, a methodology adapted from Castro et al. (2018). The longitudinal sections of the bones were photographed (Nikon D3400, Cat. no. N1510, Tokyo, Japan) before inclusion in paraffin. The tibiae were then dehydrated by a sequenced 1-h passage in an increasing series of ethanol (70, 80, 90, and 95%), and a last 4-h passage on 100% alcohol. Further, they were subjected to diaphanization in pure xylol for 2 h before including on paraffin. Finally, 4-µm-thick serial sections were obtained by using a microtome (Micom GmbH, Cat. no. 9901110, Zeiss, Walidorf, Germany) and stained using the hematoxylin-eosin (HE) technique (Feldman and Wolfe, 2014). Each slide was evaluated by optical microscopy and photographed with a Spot Color Insight digital camera coupled to a light microscope (BX-40, Olympus, Tokyo, Japan, - 5X, 10X, 20X, 40X, 100X magnification, PM-30 photomicrographic system), and pictures were captured using the Image Pro Plus® version 4.6 program. The microscopy technique was used to obtain a descriptive histopathological aspect of all bone segments (Table 3).

Table 3 - Parameters evaluated for the descriptive bone histopathological analysis and reference papers[1]

Bone section	Parameters evaluated
Epiphyseal plate	Presence of the secondary ossification center.
	Clarity of distinction and organization between the proliferative, maturation, and hypertrophic zones.
	Vascularization.
	Morphological changes of the chondrocytes.
	Presence of bone end plates.
Metaphysis	Pattern, organization, and mineralization of bone trabeculae.
	Presence of spindle-shaped cells (mesenchymal cells).
	Presence of multinucleated cells (osteoclasts).
	Areas of osteoclastic hyperplasia.
Diaphysis	Presence of chondroid hearts.
	Areas of indistinction between fibroblasts and osteoblasts.
	Areas of non-mineralized bone matrix accumulation.
	Increased volume of osteocytes.
	Presence of trabecular bone.
	Presence of fibrous connective tissue on cortical bone.

[1] Long et al. (1984); Powers et al. (1987); Thompson and Robinson (1989); Thorp et al. (1991).

2.5. Statistical analysis

For all performance evaluations, each cage containing 12 birds was considered an experimental unit in all evaluated periods. For bone quality variables, each bird was considered an experimental unit, with five repetitions of each treatment. Data were first tested for homogeneity of variances and normality of studentized residuals. The data was subjected to three regression models (P<0.05): the quadratic model described by Robbins et al. (1979), the exponential model described by Noll and Waibel (1989), and the model of segmented lines or Linear Response Plateau (LRP) described by Braga (1992), according to the best adjustment obtained for each studied variable. All statistical procedures were performed using R software (R Core Team, 2016).

The statistical model (quadratic or linear) used to test the effect of treatments was:

$$Y_{ij} = \beta_0 + \beta_1 x_i + \beta_2 x_i^2 + \varepsilon_{ij}$$

or

$$Y_{ij} = \beta_0 + \beta_1 x_i + \varepsilon_{ij},$$

in which Y_{ij} = dependent variables in i and replications j, β's = regression coefficients, x_i = placement times (i = 0, 25, 50, 75, and 100%), and ε_{ij} = residual random error.

3. Results

3.1. Performance

The performance from 1 to 7 days was not influenced by $VitD_3$ levels (P>0.05); however, there was an effect of $VitD_3$ levels on performance at 14, 21, and 35 days. The quadratic regression was also significant for performance parameters but with a lower R^2, so the linear regression was chosen for these parameters.

Supplementation with $VitD_3$ changed FI at 14, 21, and 35 days of age. At 14 days, a positive linear result of 0.0026 kg was observed for intake up to the dose of 26.53% of $VitD_3$. For $VitD_3$ levels above 26.53%, the FI reached a plateau at 0.523 kg during this phase (FI14, kg = 0.4538664 + 0.002612009x; X ≥ 26.53; Y = 0.5231; R^2 = 0.65; P<0.001). That is, no differences for FI were observed among the 25,

50, 75, and 100% groups in this phase (Table 4). From 1 to 21 days of age, the increase was 0.0177 kg up to the dose of 25.51% of $VitD_3$, dosage in which the FI reached a plateau at 1.248 kg for this phase, (FI21, kg = 0.7945 + 0.0177x; X ≥ 25.51; Y = 1.224; R^2 = 0.95; P<0.001) (Table 4). A similar result was observed considering from 1 to 35 days of age when a positive linear effect of 0.0929 kg was observed on FI up to the supplementation level of 24.98% of $VitD_3$, when it reached a plateau at 3.668 kg (FI35, kg = 1.342833 + 0.092973333x; X ≥ 24.98; Y = 3.668; R^2 = 0.98; P<0.001) (Table 4).

The dietary $VitD_3$ level influenced BW. At 14 days of age, supplementation provided a positive linear result of 0.0035 kg up to 25.83% of the $VitD_3$ dose used commercially (BW14, kg = 0.3983157 + 0.003518874x; X ≥ 25.83; Y = 0.489; R^2 = 0.74; P<0.001) (Table 4). That is, at that supplementation level, BW reached a plateau at 0.489 kg for this phase. An increase of 0.0035 kg, up to 25.88% of the dose used commercially was observed at 21 days of age, when BW reached a plateau at 1.044 kg in this stage (BW21, kg = 0.5818434 + 0.01783293x; X ≥ 25.88; Y = 1.0443; R^2 = 0.96; P<0.001) (Table 4). At 35 days of age, BW increased by 0.0736 kg up to 24.90% of the dose used commercially, and the treated groups had a uniform BW of 2.547 kg in this stage (BW35, kg = 0.7124537 + 0.07366882x; X ≥ 24.89; Y = 2.547; R^2 = 0.99; P<0.001) (Table 4).

The effects of $VitD_3$ supplementation were also observed on FC. At 14 days of age, supplementation improved FC by 0.003 up to 23.88% of $VitD_3$ dose used commercially. That is, the vitamin D supplementation reached its plateau for FC at 1.026 for this phase with a $VitD_3$ level of 23.88% (FC14 = 1.098333 – 0.003x; X ≥ 23.88; Y = 1.0266; R^2 = 0.31; P<0.001) (Table 4). A similar result was observed at 21 and 35 days of age, in which a decrease of 0.0068 was observed up to 25.32% of the dose used commercially, and of 0.0189 up to 24.76% of the dose used commercially. At 21 days, the $VitD_3$

Table 4 - Effect of vitamin D_3 ($VitD_3$) levels on performance of broilers from 1 to 35 days of age

Treatment	Performance		
	Feed intake (kg)	Body weight (kg)	Feed conversion ratio
		1 to 14 days	
0 IU $VitD_3$/kg	0.485	0.398	1.22
625 IU $VitD_3$/kg	0.516	0.486	1.06
1,250 IU $VitD_3$/kg	0.526	0.483	1.08
1,875 IU $VitD_3$/kg	0.522	0.493	1.05
2,500 IU $VitD_3$/kg	0.520	0.489	1.06
SEM	0.0122	0.0163	0.0489
P-value (L)	<0.001	<0.001	<0.001
P-value (Q)	<0.001	<0.001	<0.001
		1 to 21 days	
0 IU $VitD_3$/kg	0.794	0.581	1.36
625 IU $VitD_3$/kg	1.239	1.027	1.19
1,250 IU $VitD_3$/kg	1.249	1.043	1.21
1,875 IU $VitD_3$/kg	1.259	1.041	1.20
2,500 IU $VitD_3$/kg	1.238	1.045	1.18
SEM	0.0775	0.0775	0.0326
P-value (L)	<0.001	<0.001	<0.001
P-value (Q)	<0.001	<0.001	<0.001
		1 to 35 days	
0 IU $VitD_3$/kg	1.342	0.712	1.85
500 IU $VitD_3$/kg	3.673	2.552	1.39
1,000 IU $VitD_3$/kg	3.632	2.520	1.40
1,500 IU $VitD_3$/kg	3.784	2.573	1.42
2,000 IU $VitD_3$/kg	3.591	2.561	1.36
SEM	0.3879	0.3062	0.0979
P-value (L)	<0.001	<0.001	<0.001
P-value (Q)	<0.001	<0.001	<0.001

SEM - standard error of the mean; L - linear regression; Q - quadratic regression.

supplementation reached a plateau for FC at 1.151 (FC21 = 1.325 – 0.00686667x; X ≥ 25.32; Y = 1.151; R^2 = 0.77; P<0.001) (Table 4) and at 1.396 at 35 days (FC35 = 1.85667 – 0.0189x; X ≥ 24.76; Y = 1.3961; R^2 = 0.88; P<0.001) (Table 4).

There was an increasing linear effect of 1.48% up to 24.08% of the recommended dose for VIA at 35 days (VIA, % = 61.42 + 1.4872x; X ≥ 24.08; Y = 97.24; R = 0.84; P<0.001). Viability stabilized at 97.24% at levels above this (Table 5).

Table 5 - Effect of vitamin D_3 ($VitD_3$) levels on broiler viability from 1 to 35 days of age

Treatment	Viability (%)
0 IU $VitD_3$/kg	61.43
625/500 IU $VitD_3$/kg	98.61
1,250/1,000 IU $VitD_3$/kg	100.00
1,875/1,500 IU $VitD_3$/kg	97.22
2,500/2,000 IU $VitD_3$/kg	94.52
SEM	6.6067
P-value (L)	<0.001
P-value (Q)	<0.001

SEM - standard error of the mean; L - linear regression; Q - quadratic regression.

3.2. Bone quality

In addition to performance, bone parameters, such as percentage of ash, percentage of Ca, percentage of P, breaking strength, and Seedor index were also influenced by $VitD_3$ supplementation. The quadratic regressions were also significant for bone quality variables but with a lower R^2, so the linear regression was chosen for these parameters.

At 21 and 35 days, the tibia ash percentage increased by 0.059% up to the dose of 26.08%, and by 0.269% up to the dose of 28.85% of dietary $VitD_3$, respectively. At 21 days, bone ash percentage reached a plateau at 41.81% (%MM21, % = 26.296 + 0.59488x; X ≥ 26.08; Y = 41.81; R^2 = 0.88; P<0.001), and at 38.09% at 35 days (%MM35, % = 30.316 + 0.26968x; X ≥ 28.85; Y = 38.09; R^2 = 0.71; P<0.001) (Table 6). This means that for dosages higher than 26.08% at 21 days and 28.85% at 35 days, no differences were observed for bone ash percentage.

The tibial mineral content was influenced by dietary vitamin D dosage. Tibia Ca percentage was influenced by $VitD_3$ supplementation. At 21 days, it reached a plateau at 19.84% when the supplement dose was 19.26% of $VitD_3$ (%Ca21, % = 10.7 + 0.47448x; X ≥ 19.26; Y = 19.84; R^2 = 0.68; P<0.023). At 35 days, this same parameter reached a plateau at 18.90% when the supplemented dosage was 48.81% of $VitD_3$ (%CA35, % = Y = 13.53767 + 0.10988x; X ≥ 48.81; Y = 18.90; R^2 = 0.26; P<0.001). For tibial P percentage at 21 days, the plateau was observed at 4.28% of P percentage when the dietary $VitD_3$ was 19.12% (%P21, % = 0.904 + 0.17648x; X ≥ 19.12; Y = 4.28; R^2 = 0.66; P<0.001). At 35 days, P percentage reached a plateau at 4.04% of P with a dietary $VitD_3$ dosage of 38.76% (%P35, % = 1.514 + 0.06536x; X ≥ 38.76; Y = 4.04; R^2 = 0.46; P = 0.0015) (Table 6).

The groups supplemented with $VitD_3$ showed an increased breaking strength rate of 0.57 kgf up to 21 days reaching 21.31% of the $VitD_3$ dose used commercially and an increase of 0.91 kgf up to 35 days reaching the dose of 27.03%. Thus, femur breaking strength reached a plateau at 15.83 kgf when the $VitD_3$ supplementation level was 21.21% (BS21, kgf = 3.6875 + 0.57x; X ≥ 21.31; Y = 15.83; R^2 = 0.87; P<0.001) at 21 days. At 35 days, it reached a plateau at 30.62 kgf with a dietary $VitD_3$ level of 27.03% (BS35, kgf = 5.946 + 0.9128x; X ≥ 27.03; Y = 30.62; R^2 = 0.80; P<0.001) (Table 6). The Seedor index was also influenced by the treatment at 21 and 35 days of age (P<0.001). At 21 days of age, dietary

supplementation with $VitD_3$ increased the Seedor index by 0.54 with a plateau at 115.76 when the $VitD_3$ dosage was 26.46% (SI21 = 100.6692 + 0.540852x; X ≥ 26.43; Y = 115.76; R^2 = 0.61; P<0.001). At 35 days, the increase was by 2.21 until the plateau at 209.36, when the $VitD_3$ was 26.34% of the commercially used dose (SI35 = 15.024 + 2.21488x; X ≥ 26.34; Y = 209.36; R^2 = 0.82; P<0.001) (Table 6).

Table 6 - Effect of vitamin D_3 ($VitD_3$) levels on broiler bone quality at 21 and 35 days of age

Treatment	Variable				
	Ash (%)	Ca (%)	P (%)	Bone strength (kgf)	Seedor index
			21 days		
0 IU $VitD_3$/kg	26.29	10.7	0.91	5.90	97.68
625 IU $VitD_3$/kg	41.16	22.56	5.32	17.86	120.92
1,250 IU $VitD_3$/kg	41.28	20.07	3.44	16.05	126.24
1,875 IU $VitD_3$/kg	40.85	19.21	4.24	15.42	116.00
2,500 IU $VitD_3$/kg	43.30	20.24	4.08	16.79	115.56
SEM	2.7235	2.0498	0.8125	2.2131	4.8223
P-value (L)	<0.001	0.0233	<0.001	<0.001	<0.001
P-value (Q)	<0.001	0.0017	<0.001	<0.001	<0.001
			35 days		
0 IU $VitD_3$/kg	31.76	13.66	1.51	5.95	151.04
500 IU $VitD_3$/kg	37.05	16.03	3.14	28.77	206.04
1,000 IU $VitD_3$/kg	37.87	19.15	3.81	36.55	210.34
1,500 IU $VitD_3$/kg	39.62	19.97	4.06	28.64	206.14
2,000 IU $VitD_3$/kg	36.19	17.83	4.16	28.04	211.66
SEM	1.4985	1.7680	0.6002	4.7202	10.641
P-value (L)	<0.001	<0.001	<0.001	<0.001	<0.001
P-value (Q)	<0.001	<0.001	<0.001	<0.001	<0.001

SEM - standard error of the mean; L - linear regression; Q - quadratic regression.

3.3. Bone macroscopic and histopathological description

The macroscopic examination (Figure 1) revealed that the tibiae of animals that did not receive $VitD_3$ supplementation (0% group) was smaller than the tibiae of animals of the other groups. The treatments of 25, 50, and 75% supplementation of $VitD_3$ had similar macroscopically appearing tibiae. The 100% supplementation group, however, had two tibiae with islands of cartilaginous tissue entering the metaphysis, characteristic of tibial dyschondroplasia. Regarding the histopathological analysis, both the absence and dietary supplementation with $VitD_3$, depending on the dose, promoted important bone changes.

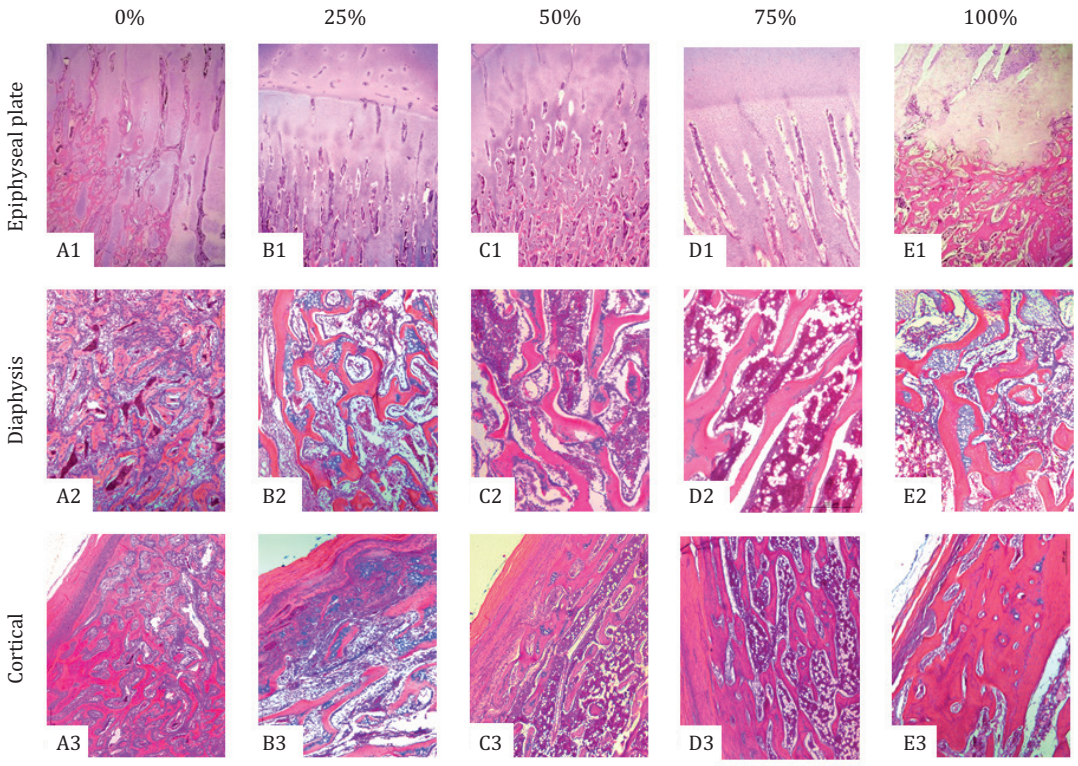
3.4. Group 0 (without $VitD_3$ supplementation)

The tibiae of broilers with no $VitD_3$ supplementation presented histological alterations compatible with rickets, generalized fibrous osteodystrophy, and osteochondrosis (tibial dyschondroplasia). The tibial epiphysis was totally cartilaginous and without the formation of secondary ossification centers. The epiphyseal plaque was thick, irregular, and with few narrow vascular channels, with little differentiated chondrocytes and without distinction between zones. The hypertrophic zone was narrow or even non-existent in most of the epiphyseal plaque, as a sign of cell maturation failure. A distal terminal bone plate was present at several points on the epiphyseal plate, as a sign of interruption of endochondral growth. In some places, cartilage entered the metaphysis and presented chondrocytes with pycnotic nuclei and empty chondrocyte gaps, which are characteristic of individual cell degeneration and necrosis, respectively (Figure 2 A1).

Group 0% - without vitamin D_3 supplementation; Group 25% - with 500 IU/kg of feed; Group 50% - with 1,000 IU/kg of feed; Group 75% - with 1,500 IU/kg of feed; Group 100% - with 2,000 IU/kg of feed.
Yellow arrow on the second and fifth tibiae from 100% group, from the top to the bottom, show islands of cartilaginous tissue.

Figure 1 - Tibia longitudinal cut from boilers at 35 days old after demineralization.

The bone trabeculae of the metaphysis and diaphysis were present in small numbers and were thin, fragmented, poorly mineralized, and misaligned. There was also an accumulation of osteoid on the margins of several trabeculae and between them. The trabeculae presented osteocytes with bulky nuclei and were housed in wide gaps, as a characteristic of intense osteocytic osteolysis. There were also many osteoclasts, several of which were housed within Howship gaps in the trabecular margin, which is characteristic of intense osteoclasis. The bone trabeculae were mostly covered by more than one layer of osteoblasts that had large nuclei. The presence of cartilaginous tissue in the center of the trabeculae (chondroid heart), common in animals in the growth phase, was practically nonexistent in these bones (Figure 2 A2).



From A1 to E1: optical microscopy of epiphyseal plate of 0, 25, 50, 75, and 100% of vitamin D_3 supplementation, respectively (HE x10).
From A2 to E2: optical microscopy of diaphysis of 0, 25, 50, 75, and 100% of vitamin D_3 supplementation, respectively (HE x 25).
From A3 to E3: optical microscopy of cortical of 0, 25, 50, 75, and 100% of vitamin D_3 supplementation, respectively (HE x 50).

Figure 2 - Optical microscopy from tibiae of 35-day-old broilers.

The cortex was thin, immature, poorly mineralized, and predominantly formed by trabecular tissue, with rare primary osteons. There were sites of cortical discontinuity and proliferation of fibrous connective tissue from the periosteum (Figure 2 A3).

3.5. Group 25% of commercial $VitD_3$

The tibiae of broilers supplemented with 500 IU of $VitD_3$/kg (25% of commercial inclusion) presented histological alterations compatible with rickets and generalized fibrous osteodystrophy. Unlike the 0% group, a secondary ossification center was present. However, similar to the 0% group, the epiphyseal plaque had poorly differentiated chondrocytes and without distinction between zones. The hypertrophic zone was narrow or even non-existent in most of the epiphyseal plaque, as a sign of cell maturation failure (Figure 2 B1).

The bone trabeculae of the metaphysis and diaphysis were present in small numbers and were thin, fragmented, and misaligned, but more mineralized compared with the 0% group. The bone trabeculae presented osteocytes with bulky nuclei and were housed in wide gaps, as a characteristic of intense osteocytic osteolysis. There were also osteoclasts within Howship gaps, as a sign of osteoclasis. Most of the bone trabeculae were covered by more than one layer of osteoblasts that had large nuclei and some with cartilage remnants (chondroid heart) in the center (Figure 2 B2).

The cortex was thin, immature, and predominantly formed by trabecular tissue, without the presence of osteons. There were sites of discontinuity of the cortex with the proliferation of fibrous connective tissue from the periosteum (Figure 2 B3).

3.6. Group 50% of commercial $VitD_3$

The tibiae of animals supplemented with 1,000 IU of $VitD_3$/kg (50% of commercial inclusion) presented histological alterations compatible with generalized fibrous osteodystrophy and osteochondrosis (tibial dyschondroplasia). Unlike the 0% group, the secondary ossification center was present in this group, and a large part of the epiphysis was already replaced by bone tissue. However, the epiphyseal plaque, although with better differentiation compared with the 0% and 25% groups, still had a narrow hypertrophic zone. The cartilage entered the metaphysis in some places and presented chondrocytes with pycnotic nuclei, as a characteristic of cell degeneration (Figure 2 C1). Like the 25% group, the cortex was thin, immature, and predominantly formed by trabecular tissue without the presence of osteons. There were sites of cortical discontinuity, with fibrous connective tissue proliferating from the periosteum (Figure 2 C3).

3.7. Group 75% of commercial $VitD_3$

The tibiae of broilers supplemented with 1,500 IU of $VitD_3$/kg (75% of commercial inclusion) showed no changes. The epiphysis had a secondary ossification center, and a large part of it was already replaced by bone tissue with coalescent trabeculae of normal thickness. The epiphyseal plaque was uniform, well differentiated, with many vascular channels, and a well-differentiated hypertrophic zone (Figure 2 D1).

Below the epiphyseal plate, in the area corresponding to the primary spongy bone, and across the metaphysis and diaphysis, the bone trabeculae were coalescent, aligned, and mineralized. In the center of the trabeculae, the osteocytes had bulky nuclei and were housed in wide gaps. In the periphery of the trabecula, osteocytes were small with narrow gaps, as a sign of osteocytic osteolysis within the normal range. Most of the trabeculae were covered by a layer of osteoblasts, sometimes with a large oval core, and sometimes with a fusiform nucleus. Rare osteoclasts were observed (Figure 2 D2). In contrast to the previous groups, the cortex in this group was thick, continuous, and with a large number of osteons (Figure 2 D3).

3.8. Group 100% of commercial $VitD_3$

The tibiae of broilers supplemented with 2,000 IU of $VitD_3$/kg (100% of commercial inclusion) presented histological changes compatible with osteopetrosis and osteochondrosis (TD). The epiphysis had a secondary ossification center, and a large part of it was already replaced by bone tissue with coalescent trabeculae of normal thickness. The epiphyseal plaque was irregular, differentiated in some points, with vascular invasion, and undifferentiated in other places. At these points of undifferentiation, the cartilage of the epiphyseal plate entered the metaphysis and presented chondrocytes with pycnotic nuclei and empty chondrocyte gaps, as a characteristic of individual cell degeneration and necrosis, respectively (Figure 2 E1).

Below the epiphyseal plate, in the area corresponding to the primary spongy bone and in the metaphysis, the bony trabeculae were thick, coalescent, aligned, and mineralized. But in some sites of trabecular bone tissue, there was an increase in the amount of bone, with the formation of bone-like structures, as a characteristic of osteopetrosis. In the diaphysis, there was osteonic tissue within the spinal canal (enostosis). Osteocytes were mostly small and housed in narrow gaps, as a characteristic of inhibiting resorption by osteocytic osteolysis. There were also foci of osteocytes with pycnotic nuclei, as a characteristic of cell degeneration. Most trabeculae were covered by one or more layers of osteoblasts, sometimes with a large oval nucleus, and sometimes with a fusiform nucleus. There was an increase in the number and size of chondroid hearts in the center of some trabeculae, as a characteristic of inhibition of resorption by osteocytic chondrolysis (Figure 2 E2).

4. Discussion

This study found performance and bone quality to be similar when using 25, 50, 75, and 100% of the $VitD_3$ levels commonly used by the poultry industry. This means that using 625 IU of $VitD_3$ in the starter and 500 IU of $VitD_3$ in the grower phase showed the same results as 2,500 IU in the starter and 2,000 IU in the grower phase. Interestingly, the histopathological analysis demonstrated differences among the $VitD_3$ supplementation levels regarding bone health. Between the different $VitD_3$ levels, only birds supplemented with 75% of the $VitD_3$ dose used by the industry (i.e., 1,875/1,500 IU of $VitD_3$) did not present morphological characteristics linked to bone pathologies. That could indicate that bone visual analysis methods, such as histopathology, can be useful for identifying changes in bone morphology that are not perceptive when using quantitative methods, such as bone ash percentage.

Broilers fed without $VitD_3$ supplementation presented worsened performance from 1 to 35 days compared with the 25, 50, 75, and 100% of $VitD_3$ groups by 63.46%. These results were previously observed (Han et al., 2017) and indicate that the $VitD_3$ levels used by the industry do not improve the performance of broilers reared under 100% of artificial lighting when compared with the lower level (i.e., 25% of the industry levels) used in this trial. The potential of using reduced dietary vitamin D levels for healthy birds reared in a completely closed environment without performance and bone health impairment was previously described (Leyva-Jimenez et al., 2019; Tizziani et al., 2019). Vitamin D_3 improves performance by causing an increase in Ca and P absorption in the duodenum (Wasserman and Taylor, 1966; Han et al., 2018; Wu et al., 2022). In its active form, the 1,25-hydroxicholecalciferol bonds to its specific enterocyte intranuclear receptor (VDR) leading to an increased gene expression of calcium-active transporters at the apical membrane of the enterocyte (Han et al., 2018). Furthermore, $VitD_3$ increases the Ca^+ ions transport from the apical to the basal membrane, being a coordinator of Ca uptake from the intestinal lumen into the plasma (Fatemi et al., 2021). However, other scenarios such as dietary Ca and P levels (Brito et al., 2010; Gómez-Verduzco et al., 2013) and protozoan (Shanmugasundaram et al., 2019; Lopes et al., 2024) and bacterial challenges (Morris et al., 2014; Nunes et al., 2020) can change the dietary vitamin D level required to maintain health and performance. Based on the current results, when the dietary Ca and P levels reach the requirement of birds, the dietary $VitD_3$ levels can be safely reduced while maintaining performance.

In the present study, bone Ca and P percentage responded positively to the dietary $VitD_3$ level. An interesting observation is that bone Ca and P percentage was the only parameter that showed an increased dietary $VitD_3$ requirement along the bird's age. Bone parameters are known for being more sensitive to dietary $VitD_3$ levels changes compared with performance traits (Edwards et al., 1994). Additionally, this observation could be related to the fact that while the birds grow, they require a developed skeletal tissue base for mass deposition to avoid an imbalance between muscle and bone tissues (Bozkurt et al., 2017). This bone-muscle metabolic balance can raise Ca and P requirements to produce hydroxyapatite, which results in a higher $VitD_3$ requirement.

The analysis of the macroscopic evaluation of tibiae at 35 days revealed that the group subjected to the diet with no $VitD_3$ had smaller bones than the other groups (Figure 1). This corresponds to the results of the Seedor index, which demonstrated that the group without inclusion of $VitD_3$ had lower bone density than the other groups and that inclusion levels above 26% did not increase bone density. The results of ash content of the tibiae at 35 days indicated that, at this stage, supplementation with $VitD_3$ increased the percentage of Ca up to the dose of 48.81% and P up to the dose of 38.75% of $VitD_3$ used commercially. Supplementation levels above these did not increase ash percent.

Previous studies have indicated that femur breaking strength improves when higher levels of $VitD_3$ supplementation are used (Khan et al., 2010). However, the results of the present study show that, at 35 days of age, $VitD_3$ has a positive effect on bone breaking resistance when supplemented up to 27% of the recommended level, with no positive effects for higher levels, which is similar to the findings of other studies (Castro et al., 2018; Leyva-Jimenez et al., 2019). The parameters represented by ash percentage, Seedor index, and breaking strength were similar among the groups receiving 25, 50, 75, and 100% of $VitD_3$ dose used by the poultry industry. However, histopathological analysis was

of fundamental importance in demonstrating the differences between these groups regarding bone health.

The histopathological findings demonstrate that the use of $VitD_3$ inclusion levels below 75% of that level used commercially, although promoting similar performance, can cause changes in bone homeostasis and make it susceptible to pathologies, such as TD, and osteopenic changes, such as rickets and fibrous osteodystrophy. However, bone changes were also observed in the group with the highest $VitD_3$ inclusion levels. Unlike groups with less than 75% of $VitD_3$, the bone changes observed in the 100% group were characterized by an excessive increase in the amount of bone tissue, a disorder called osteopetrosis. In this group, bones with TD were also observed, showing that the levels currently practiced by the industry can be harmful to bone health. At first, it may be thought that the increase in the amount of bone tissue, observed in bones with osteopetrosis, can increase bone resistance; however, osteopetrosis is a bone disease that can progress to osteonecrosis, leading to bone fragility and fractures (Bollerslev and Andersen, 1989).

Rickets, seen in birds of the group with no dietary $VitD_3$, is an osteopenic disease caused by Ca, P, and/or $VitD_3$ deficiency. This disease is characterized by the expansion of the epiphyseal plaque proliferation zone and failure of bone mineralization (Long et al., 1984). The main consequence is the differentiation and irregular maturation of the epiphyseal plate and osteoid (non-mineralized bone tissue) gathering, causing bone deformities and fractures (Serakides, 2016). Hasky-Negev et al. (2008) demonstrated that hypovitaminosis D inhibits the gene expression of proteins that induce chondrocyte differentiation, which in turn are important to stimulate the blood vessels penetration into cartilage during endochondral growth. Without vessels to allow cellular respiration, it will lead to an increase in the proliferative zone and a reduction in the hypertrophic zone, findings present in this group.

However, more than one bone disease can coexist, as some of them can have a common cause. This is the case of rickets and generalized fibrous osteodystrophy, both caused by Ca and/or $VitD_3$ deficiency (Long et al., 1984; Varshney et al., 2018). The association of the two diseases was observed in broilers of groups 0, 25, and 50 of the $VitD_3$ dose used commercially. Fibrous osteodystrophy can be caused by Ca deficiency in the blood, resulting from hypovitaminosis D, even if dietary Ca levels are within recommended values (Uhl, 2018). In this case, impaired intestinal Ca absorption causes serum hypocalcemia, which in turn stimulates parathyroid glands to produce parathyroid hormone (Uhl, 2018). Secondary nutritional hyperparathyroidism continually increases bone resorption (Toyoda et al., 2004). In response, there is a replacement of the reabsorbed bone tissue with connective tissue, which characterizes generalized fibrous osteodystrophy (Olson et al., 2015).

Tibial dyschondroplasia was another bone change observed in groups 0, 50, and 100% of the $VitD_3$ dose used commercially. It is a manifestation of osteochondrosis and is characterized by the cartilage cells failure to growth and differentiate, also a low trabecular bone vascularization (Riddell, 1975; Jahejo and Tian, 2021). In broilers, TD can be caused by several factors, such as changes in the levels of calcium, $VitD_3$ and rapid growth (Whitehead et al., 2004). Through the VDR receptor present in chondrocytes, $VitD_3$ regulates cell metabolism, or more precisely the differentiation and organization of chondrocytes during endochondral ossification (Wang et al., 2014). The reduction in plasma Ca levels stimulates parathyroid hormone (PTH) secretion by parathyroid glands, which, in turn, inhibits cell differentiation of the epiphyseal plaque and consequently causes TD (Liu et al., 2012). So, the dietary $VitD_3$ seems to play a key role on bone TD occurrence, and the effect of $VitD_3$ on reducing TD incidence has been previously reported (Whitehead et al., 2004; Khan et al., 2010).

However, TD can also be related to overfeeding, occurring in animals that receive diets with high levels of P (Edwards and Veltmann, 1983) and $VitD_3$ (Haschek et al., 1978). Overfeeding, with high mineral or $VitD_3$ levels can cause hypercalcitoninism with consequent inhibition of chondrocyte differentiation, causing TD and osteopetrosis due to reduced bone resorption (Serakides, 2016). These changes were observed in the animals that received the highest value of $VitD_3$, that is, 100% of the $VitD_3$ dose used commercially. Besides its key role on TD prevention, the dietary $VitD_3$ levels seems to be a major factor that can activate different metabolic pathways with contrasting consequences for bone tissue. A previous *in vitro* study demonstrated that $VitD_3$ can inhibit the differentiation of mice cartilage cells

(Yamaguchi and Weitzman, 2012). However, the mechanisms by which $VitD_3$ can cause TD in broilers need to be further studied.

Regarding the diagnosis of osteopetrosis in the 100% group, it is likely that it was also due to hypercalcitoninism caused by excess Ca due to hypervitaminosis D or the direct action of excess $VitD_3$. Although the literature on osteopetrosis is scarce in broilers, osteopetrosis due to hypervitaminosis D has already been described in cattle (Krook et al., 1975), small cattle (Woodard et al., 1993), and pigs (Chineme et al., 1976).

Rickets and osteoarthritis are considered osteopenic diseases that cause fractures, as they reduce bone strength, while osteopetrosis is an osteomegalic disease, characterized by increased bone strength (Serakides, 2016). Therefore, the bone maximum resistance analysis used in this study did not demonstrate differences among the 25, 50, 75, and 100% groups. The main reason why this occurred is that the resistance analysis is applied to the diaphysis, where osteonic or cortical tissue predominates. The changes were observed with higher frequency and severity in the epiphysis and metaphysis, where trabecular bone tissue predominates. These results show that maximum breaking strength has its limitations when it is not associated with an imaging analysis, such as histopathology. Although these diseases are widespread and affect the entire skeleton, they first and most severely affect trabecular bone tissue where the speed of bone synthesis and resorption is higher (Krook, 1983), being necessary to target these bones when applying imaging analysis. In addition, TD is an alteration of bone growth that is not always linked to a decreased bone resistance when using a 3-point bending test (Santos et al., 2022) as the one used in this study. However, TD can develop concurrently with epiphysiolysis, where the degenerated and necrotic epiphyseal plate causes fissures that leads epiphysis and diaphysis to separate. This process mimics a fracture or break during processing in the industry and that, very similar to fractures, can also cause economic losses.

5. Conclusions

Aiming to balance performance and bone health, the results shown here elucidate that, for isonutritive diets, it is not necessary to use the $VitD_3$/kg levels currently recommended and used commercially, since supplementation with 75% of the dose of $VitD_3$/kg used commercially, that is, 1,875 IU of $VitD_3$/kg of feed in the starter phase and 1,500 IU of $VitD_3$/kg of feed in the growth phase, guarantees the maximum performance associated with bone health.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: Lopes, T. S. B.; Serakides, R.; Lara, L. J. C. and Araújo, I. C. S. **Data curation:** Lopes, T. S. B.; Vasconcelos, M. D. C.; Costa, B. T. A.; Sousa, L. S. and Ocarino, N. M. **Formal analysis:** Lopes, T. S. B. and Araújo, I. C. S. **Funding acquisition:** Araújo, I. C. S. **Investigation:** Lopes, T. S. B.; Vasconcelos, M. D. C.; Costa, B. T. A.; Sousa, L. S.; Bertassoli, B. M.; Ocarino, N. M. and Serakides, R. **Methodology:** Bertassoli, B. M.; Serakides, R. and Araújo, I. C. S. **Project administration:** Araújo, I. C. S. **Resources:** Araújo, I. C. S. **Supervision:** Araújo, I. C. S. **Visualization:** Lara, L. J. C. **Writing – original draft:** Lopes, T. S. B.; Bertassoli, B. M.; Ocarino, N. M.; Serakides, R.; Lara, L. J. C. and Araújo, I. C. S. **Writing – review & editing:** Lopes, T. S. B. and Araújo, I. C. S.

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