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Bone characteristics of broiler chickens fed diets supplemented with *Solanum glaucophyllum*[1]

Características ósseas de frangos de corte alimentados com rações suplementadas com *Solanum glaucophyllum*[1]

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Abstract

The aim of this study was to assess the characteristics of the tibiotarsus of male broilers at 21 and 35 days of age. The percentages of collagenous proteins (CP), non-collagenous proteins (NCP), ash, and minerals (calcium, phosphorus, potassium and sodium), as well as weight of dried and defatted tibiotarsus *in natura* were determined. A total of 648 Cobb® male broilers were used in a random block design study with 6 treatments, 6 replicates, and 18 birds per experimental unit. The treatments consisted of diet supplementation with 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 µg of active vitamin D_3 per kg of feed. The birds were weighed at 21 and 35 days of age and one bird per repetition with the mean weight of the experimental unit was slaughtered to collect the tibiotarsus. The organic and mineral composition of the bone was affected by the addition of active vitamin D_3 to the feed. Our results indicate that a dose of up to 1.50 µg of $1,25(OH)_2D_3$/kg of feed is ideal for male broilers between 8 and 35 days of age.

Key words: 1,25-dihydroxycholecalciferol, ash, collagen, minerals, tibiotarsus

Resumo

O objetivo do trabalho foi avaliar as características dos tibiotarsos de frangos de corte machos aos 21 e 35 dias de idade. Foram determinados os percentuais ósseos de proteínas colagenosas (PC), proteínas não colagenosas (PNC), cinzas e minerais (cálcio, fósforo, potássio e sódio), além dos pesos dos tibiotarsos *in natura,* secos e desengordurados. Foram utilizados 648 frangos de corte machos da marca comercial Cobb® em um delineamento em blocos ao acaso com seis tratamentos e seis repetições com 18 aves por unidade experimental. Os tratamentos consistiram na suplementação de 0,0; 0,5; 1,0;

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1,5; 2,0 e 2,5 µg de vitamina D_3 ativa/kg de ração. Aos 21 e 35 dias de idade as aves foram pesadas e um frango/repetição com o peso médio do lote foi retirado e abatido para se obter os tibiotarsos. A composição orgânica e mineral dos ossos foi afetada pelo fornecimento suplementar de vitamina D_3 ativa nas rações. Recomenda-se até 1,50 µg de $1,25(OH)_2D_3$/kg de ração para frangos de corte machos de 8 a 35 dias de idade.

Palavras-chave: 1,25-dihidroxicolecalciferol, cinzas, colágeno, minerais, tibiotarsos

Introduction

The poultry industry has seen significant developments in recent decades due to the use of genetic improvement programs that have led to accelerated animal growth, improved feed conversion, and altered proportions of different carcass muscles, all which require increased bone support in the birds (COOK, 2000). However, undesirable characteristics such as metabolic diseases, body fat deposition, and locomotor disorders are associated with the advances provided by traditional breeding programs (LEDUR et al., 2007).

Locomotive problems are commonly observed in poultry production due to the very high rate of muscle growth, which initially occurs at an early stage on an immature skeletal support structure (GONZALES; MENDONÇA JÚNIOR, 2006). According to Araújo et al. (2012), bone disorders are caused by changes in normal growth and homeostasis.

Bone is composed approximately of 70% minerals, 22% proteins, and 8% water. The organic matrix of bone is responsible for its elasticity and accounts for approximately 25% of its dry matter. The inorganic components that provide rigidity make up approximately 75% of its dry weight and are composed of calcium phosphate and calcium carbonate. Nearly 30% of the organic portion of the extracellular matrix is composed of collagenous proteins, of which 90% are collagen type I. The remaining 10% are proteoglycans and non- collagenous proteins such as osteocalcin, osteonectin and some growth factors (JUNQUEIRA; CARNEIRO, 2004).

Non-collagenous proteins (NCP) have multiple roles in bones, such as the regulation of collagen fiber mineralization and modulation of cell division, migration, differentiation, and maturation (YOUNG, 2003). Several studies have reported that Gla-proteins, which are constituents of NCPs, are able to inhibit bone mineralization and promote osteoclast activity (VERMEER et al., 1995).

Bone strength has been mainly associated with adequate formation and maintenance of its organic matrix, collagen cross-links, deposition, and resorption. However, mineral content and adequate matrix formation in early life are also important (OVIEDO-RONDÓN; WINELAND, 2011). There is an interaction between the organic and mineral components of the bone, since the higher the mineral content is, the higher the elastic modulus and hardness are, although the bone will be more susceptible to cracks (CURREY, 2003). On the other hand, the organic matrix made of collagen contributes to bone resilience, modulates tissue resistance and regulates growth features by providing support to the mineral matrix and contributing to bone traction resistance (MÜLLER, 2010; VELLEMAN, 2000). Calcium (Ca) is the most abundant mineral in the animal body and is prominent in the skeleton, body fluids, and tissues. It is required for the formation and maintenance of bone structure, nerve impulse transmission, blood coagulation, muscle contraction, and enzyme and hormone activation (MACARI et al., 2002).

In addition to calcium, phosphorus (P) is also found in the bone, which contributes to the formation of hydroxyapatite crystals, which is the main component of the inorganic bone matrix.

In addition, it is involved in cellular activities as components of the phospholipid cell membrane and nucleic acids and also plays a role in energy transport and enzyme regulation (ARAÚJO et al., 2002).

In addition to Ca and P, vitamin D is essential for bone metabolism and is directly responsible for the growth of the skeleton that supports the birds and allows for maximum production performance (BRITO et al., 2010). Vitamin D promotes Ca mobilization from bones, in the presence of the parathyroid hormone. To be metabolically active, it must undergo transformations, which occur in the liver and kidneys where hydroxyl groups are added, producing 25-hydroxicholecalciferol ($25(OH)D_3$) and 1,25- dihydroxycholecalciferol ($1,25(OH)_2D_3$) (SOUZA; VIEITES, 2014).

The use of $1,25(OH)_2D_3$ has been studied in poultry production with the aim of improving the relationship between growth and bone tissue strength, thus reducing locomotor problems (SOUZA et al., 2013; VIEITES et al., 2014). The hydroxylated metabolites of vitamin D_3 and 7-dehydrocholesterol have been identified in several plants, in particular in those belonging to the Solanaceae family (JÄPELT; JAKOBSEN, 2013). *Solanum glaucophyllum* (SG) is composed of 54.3% carbohydrates, 24.9% proteins, 4.1% water, 17.1% minerals (BACHMANN et al., 2013b), and $1,25(OH)_2D_3$ glycosides (8.6 to 100 µg/g of dried leaves). However, these percentages depend on the environmental conditions under which the plant is grown and on its genetic makeup. The molecular distribution of glycosylated moieties in SG is 1 - 12 hexose units per aglycone (EUROPEAN FOOD SAFETY AUTHORITY, 2015).

The aim of this study was to assess the organic and mineral composition as well as the resistance to breakage of the tibiotarsus of male broilers fed diets supplemented with *Solanum glaucophyllum.*

Materials and Methods

The study was conducted in the Poultry Farming Sector of the Federal Institute of Education, Science and Technology (IFMT - São Vicente Campus), in the municipality of Santo Antônio de Leverger, Mato Grosso. The study included 648 Cobb® male broilers in a randomized block design with six treatments (0, 50, 100, 150, 200, and 250 g of Panbonis® per ton of feed, corresponding to 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 µg of supplementary active vitamin D_3 per kg of feed, respectively) each done in six replicates. The experimental unit (pen) included 18 birds. Distribution into blocks allowed for greater uniformity of pen positioning in the plots of the experimental battery brooder. The commercial product used Panbonis® as the source of active vitamin D_3 (Technofeed, Switzerland), was included in the experimental feed as a substitute of the inert material (sand), and was composed of 10 mg/kg of the vitamin (standardized *Solanum glaucophyllum*).

The experimental diets were formulated according to recommendations by Rostagno et al. (2005) for ages ranging from 8 to 21 days and 22 to 35 days (Table 1). The commercial source of active vitamin D_3 replaced the inert material (washed sand) in the diets.

A continuous light program (24 h) was used throughout the entire experimental period. The birds received feed and water *ad libitum* via tubular semi-automatic feeders and nipple-type drinkers. At 21 and 35 days of age, one bird per pen (experimental unit) was weighed and slaughtered by cervical dislocation and the tibiotarsus was removed (Registered at the Ethics Committee for Animal Use of the IFMT - CEUA-IFMT/SVC, process no. 23197.001804/2013-02). All tissue was removed from the bones and the samples were labeled and frozen (-20°C).

Laboratory testing of bone organic and mineral composition was performed at the Laboratory of Animal Biochemistry (LBA) in the Department of

Biochemistry and Molecular Biology of the Federal University of Viçosa (UFV). *In natura* bone was used to determine the Seedor index. The tibiotarsus was weighed on analytical scales (±0.0001g) and measured using a digital pachymeter (0-150 mm to an accuracy of 0.01 mm). The Seedor index was obtained by dividing bone weight (mg) by bone length (mm), and taking into account measures bone density (SEEDOR et al., 1991), therefore the higher the index, the higher the density of the bone piece.

Table 1. Experimental feeds and calculated composition in the age ranges from 8 to 21 and 22 to 35 days.

Ingredients (%)	Feed (8 to 21 days)	Feed (22 to 35 days)
Corn	57.956	60.8238
Soybean meal	35.0591	31.4815
Soybean oil	2.8600	3.7700
Dicalcium phosphate	1.8136	1.6702
Limestone	0.8325	0.7911
Salt	0.4450	0.4240
DL - Methionine (99%)	0.2691	0.2458
L - Lysine HCl (50,7%)	0.3420	0.3323
L - Treonina (99%)	0.0731	0.0613
Choline chloride (60%)	0.1000	0.1000
Vitamin and mineral mix[1,2]	0.2000	0.2000
Virginiamycin (10%)	0.0150	0.0150
Salinomycin (12%)	0,.0500	0.0500
Antioxidant[3]	0.0100	0.0100
Washed sand (inert)	0.0250	0.0250
Total	100	100
Calculated Composition		
Metabolizable energy (Kcal)	3050	3147
Crude protein (%)	21.1400	19.7300
Calcium (%)	0.8990	0.8370
Available phosphorus (%)	0.4490	0.4180
Sodium (%)	0.2180	0.2080
Chlorine (%)	0.2977	0.2863
Methionin + digestible cistina (%)	0.8440	0.7910
Digestible lysine (%)	1.1890	1.0990
Digestible threonine (%)	0.7730	0.7140
Digestible tryptophan (%)	0.2416	0.2222

[1] Supplement POLIAVE CORTE CQT INICIAL (Tortuga®):Vitamin D_3 - 1,339,000.00 UI/kg; Vitamin A - 5,546,000.0 UI/kg;Vitamin E - 12,430.00 UI/kg; Vitamin K3 - 944.00mg; Vitamin B1 - 1,005.00 mg; Vitamin B6 - 1,245.00 mg; Vitamin B2 - 2,250.00 mg; Vitamin B12 - 6,000.00 mcg; Biotin - 50.00 mg; Niacin - 15,000.00 mg; Pantothenic acid - 5,890.00 mg; Folic acid - 495.00 mg; Iron - 24,800.00 mg; Selenium - 150.00mg; Copper - 4,280.00 mg; Iodine - 500.00 mg; B.H.T - 1,000.00 mg; Biotin - 50.00 mg; Manganese 33,300.00 mg; Zinc - 25,680.00mg. [2]Supplement POLIAVE CORTE CQT Final (Tortuga®): Vitamin D_3 - 658,300.00 UI/kg; Vitamin A - 2,726.000.00 UI/kg; Vitamin E - 4,990.00 UI/kg; Vitamin K3 - 464.00mg; Vitamin B1 - 502.00 mg; Vitamin B6 - 623 mg; Vitamin B2 - 1,125.00 mg; Vitamin B12 - 3,000.00 mcg; Biotin - 15.00 mg; Niacin - 7,500.00 mg; Pantothenic acid - 2,940.00 mg; Folic acid - 153.00 mg; Iron - 24,800.00mg; Selenium - 150.00mg; Copper - 4,280.00mg; Iodine - 500.00mg; B.H.T - 1,000.00mg; Manganese- 33,300.00mg; Zinc - 25,680.00mg; Iodine - 500.00 mg.
[3] Butylated hydroxytoluene (BHT).

The left tibiotarsus was used to determine bone resistance as well as collagenous (CP) and non-collagenous protein (NCP) contents. It was thawed to room temperature and subsequently subjected to a bending test using an INSTRON universal mechanical device, model 4204. All bones were tested in the same position, with the extremities resting on two supports placed an appropriate distance from each other, and the load was applied in the central area (bone diaphysis) at a constant rate of 10 mm/min, as specified by the American Society of Agricultural Engineers (ASAE, 1992). The value corresponding to bone rupture was expressed as kilograms-force (kgf). Immediately after bone resistance was determined, the tibiotarsus was sectioned lengthwise and the bone marrow was removed with distilled water jets. Then, bone fat was extracted with petroleum ether in a Soxhlet apparatus for 12 h to determine the concentration of CPs and NCPs, according to the method proposed by Barbosa et al. (2010).

The tibiotarsi were demineralized with a solution of ethylenediaminetetraacetic acid (EDTA) disodium salt to extract NCPs. Extraction completion was confirmed with oxalic acid. NCPs were quantified with the Bradford method (BRADFORD, 1976) using bovine serum albumin (BSA) as the standard.

After defatting and demineralization, the bones were thoroughly washed with distilled and deionized water to prepare them for EDTA extraction, and CPs were quantified using the Kjeldahl method in order to estimate total nitrogen (TN). The CP content was obtained by multiplying TN by 6.25 (SILVA; QUEIROZ, 2002). The percentages of CPs and NCPs were calculated in relation to the weight of the dried defatted bone.

In order to determine the concentration of minerals, the right tibiotarsus was thawed and transferred to an oven at 105 °C for 6 h, and subsequently defatted with petroleum ether in a Soxhlet apparatus for 4 h in order to obtain the weight of dried defatted bone. It was then ashed in a muffle furnace at 600 °C for 6 h to determine ash content and subsequent preparation of mineral solution, according to the method proposed by Silva and Queiroz (2002).

Na, K and Ca contents in bone ash were determined by atomic absorption spectrometry and P content was determined by colorimetry. The ash was cooled and added to 10 mL of 6 M hydrochloric acid to dissolve hydroxyapatite crystals and release the minerals. The porcelain crucibles containing the samples were placed on a heating plate and the solution was evaporated in a fume hood until it was completely dry. The precipitate was dissolved in distilled and deionized water and the solution was passed through filter paper into a cylinder, after which point the volume was adjusted to 50 mL. Mineral contents were expressed as the percentage of ash to the weight of dried defatted bone and the Ca:P ratios were obtained by dividing the percentage of Ca by that of P in ash.

The data we gathered were subjected to analysis of variance and the outcome variable was regressed as a function of the levels of added active vitamin D_3 where appropriate, using a significance level of 5%. The statistical analysis was performed using the SAEG software, version 9.1 (UNIVERSIDADE FEDERAL DE VIÇOSA, 2007).

Results and Discussion

The results for weight, length, diameter, Seedor index, and resistance to breakage of dried and defatted tibiotarsus of broilers at 21 days of age, *in natura*, are shown in Table 2. The addition of active vitamin D_3 to feed did not have an effect on these variables. Similar results were obtained by Brusamarelo (2014) who assessed the tibiotarsus of female broilers at 21 days of age and did not observe any effects from the addition of up to 2.0 µg of active vitamin D_3 combined with a 15% reduction in Ca and P in the diets. Similarly, the *in* *natura* weight of dried and defatted bone, as well as the length, diameter, and resistance to breakage at 35 days of age were not affected by the addition of active vitamin D_3 to the diets (**Table 3**).

Table 2. Weight, length, diameter, Seedor index, and resistance of the tibiotarsus of male broilers at 21 days of age fed diets supplemented with *Solanum glaucophyllum.*

Variable	µg of active vitamin D_3/kg of feed						CV (%)	Effect	Means
	0.0	0.50	1.00	1.50	2.00	2.50			
Weight *In Natura* (g)	6.20	6.28	6.47	6.20	6.13	6.19	10.50	NS	6.25
Length (mm)	73.90	73.10	71.50	71.80	73.00	71.60	2.18	NS	72.40
Horizontal diameter (mm)	6.10	6.30	6.30	5.90	6.20	0.61	5.74	NS	6.10
Vertical diameter(mm)	5.20	5.30	5.20	5.00	5.30	5.10	6.21	NS	5.19
Dried and defatted weight (g)	2.25	2.25	1.95	2.05	2.19	2.06	9.78	NS	2.12
Seedor index	84.13	85.91	90.41	86.39	84.00	86.49	10.19	NS	86.22
Bone resistance (kgf)	19.68	19.88	19.76	19.35	19.48	19.84	8.46	NS	19.66

CV = Coefficient of variation; NS = not significant (P,>,0.05).

Table 3. Weight, length, diameter, Seedor index, and resistance of the tibiotarsus of male broilers at 35 days of age fed diets supplemented with *Solanum glaucophyllum.*

Variable	µg of active vitamin D_3/kg of feed						CV (%)	Effect	Means
	0.0	0.50	1.00	1.50	2.00	2.50			
Weight *In Natura* (g)	15.26	14.15	14.59	14.31	13.91	14.56	9.33	NS	14.46
Length (mm)	96.50	98.70	99.80	98.80	98.20	100.27	4.44	NS	99.11
Horizontal diameter (mm)	8.20	8.00	8.30	8.70	8.20	8.10	6.08	NS	82.36
Vertical diameter (mm)	6.70	6.50	6.70	6.80	6.70	6.70	9.54	NS	66.80
Dried and defatted weigth (g)	5.28	5.01	5.11	5.04	4.95	5.14	8.52	NS	5.09
Seedor index	157.87	143.43	146.11	144.68	141.73	142.93	9.22	Q*	146.12
Bone resistance (kgf)	28.85	25.17	25.98	28.90	24.50	24.84	14.96	NS	26.37

CV= Coefficient of variation; NS = not significant ($P > 0.05$); Q* = quadratic effect = $\hat{Y}= 4.8599x^2 - 14.958x + 154.06$ ($R^2 = 0.75$).

Garcia et al. (2013) investigated the addition of vitamin D_3, 25(OH)D_3, 1α(OH)D_3, and 1,25$(OH)_2D_3$ to the diets of broiler chickens and found that diameter, resistance to breakage, ash, Seedor index, and Ca and P content in the tibiotarsi and femora of birds at 7, 21, and 42 days were not affected by different forms of vitamin D. These authors supplied various types of vitamin D_3 in quantities equivalent to the values required by the animals (2000 IU of vitamin D_3 per kg of feed in the initial phase and 1600 IU per kg in the growth phase). They concluded that the tested levels met the requirements for bone tissue development in poultry. Therefore, it is possible that the amount of active vitamin D_3 added assessed in the present study was not sufficient to affect bone development, considering the physiological requirements of poultry.

Bone resistance varied between 19.67 kgf (21 days) and 26.37 kgf (35 days) and was not affected by the addition of active vitamin D_3 to diets. These values were similar to those observed by Reis et al. (2011), who assessed three strains of broilers fed diets containing 200 IU of vitamin

D_3/kg and obtained the following values of bone resistance: 17.44 kgf and 24.05 kgf for tibiae at 21 and 35 days of age, respectively. Bachmann et al. (2013a) studied supplementation of broiler feed with 1,25$(OH)_2D_3$, from dried leaves of *Solanum glaucophyllum* (SG) (10 µg), purified extracts (9.50 µg and 37.9 µg of 1,25$(OH)_2D_3$ per kg of feed), and the synthetic metabolite (2.5 µg and 5.0 µg/kg of feed). Unlike the results from the present study, these authors observed that the values of resistance to breakage and tibiae hardness were higher in treatments that included the addition of metabolite than in the controls. The values obtained with dried SG leaves were similar to those obtained when 5.0 µg of synthetic active vitamin D_3 were used.

The Seedor index is a means to measure bone density, where the higher the index, the higher the density of the bone and vice versa (MURAKAMI et al., 2009). The treatments had a positive quadratic effect on the Seedor index: 142.55 was obtained (at 35 days) with 1.53 µg of added active vitamin D_3 per kg of feed, which is the recommended dose to maintain bone quality.

The percentages of CPs and NCPs in the tibiotarsi of broilers at 21 and 35 days of age are shown in **Table 4**. Organic composition of bone was affected by the addition of the metabolite and NCPs exhibited an increasing linear response to the augmentation in active vitamin D_3 in the bones at 21 days of age.

Table 4. Percentage of collagenous proteins (CP) and non-collagenous proteins (NCP) in the tibiotarsus of male broilers at 21 and 35 days of age fed diets supplemented with *Solanum glaucophyllum.*

Variable	Age (days)	µg of active vitamin D_3/kg of feed						CV (%)	Effect	Means
		0.0	0.50	1.00	1.50	2.00	2.50			
NCP (%)	21	0.62	0.67	0.73	0.74	0.69	0.90	14.59	L*	0.73
NCP (%)	35	0.25	0.21	0.22	0.23	0.20	0.21	14.78	NS	0.22
CP (%)	21	32.81	33.34	34.40	33.63	35.41	33.69	6.30	NS	33.88
CP (%)	35	34.77	34.16	33.98	34.19	33.89	34.42	4.44	Q*	34.24

CV = Coefficient of variation; NS = not significant ($P > 0.05$); L*= linear effect= $\hat{Y}= 0.0825x + 0.6275$ ($r^2=0.67$); Q* = quadratic effect =$\hat{Y}=0.3726x^2 - 1.0655x + 34.719$ ($R^2= 0.79$).

NCP content at 35 days was lower than at 21 days, which similar to the results obtained by Barbosa et al. (2010) and Müller et al. (2012) who reported a reduction in bone metabolic activity with an increase in age. NCPs work as nucleation points for the formation of hydroxyapatite crystals; osteocalcin, which acts as a chemoattractant for osteoclasts, is involved in the process of bone resorption (ROACH, 1994).

Moraes et al. (2010) stated that animals with an elevated incidence of locomotor problems exhibit higher NCP content and that this high concentration is probably associated with bone fragility since it interferes with its complete mineralization. Adequate bone mineralization is essential in broiler production because muscle development depends on a good bone support, which is required for locomotor system function (SCHOULTEN et al., 2003). The relationship between NCP content and bone mineralization can be explained by the increase in Gla-proteins that in turn inhibit mineralization or promote the release of calcium in bones (OLIVEIRA et al., 2006).

The percentage of CP at 21 days of age was not affected by the levels of active vitamin D_3 added. Similar results were obtained by Souza et al. (2012) who assessed the effect of adding up to 5.0 µg of active vitamin D_3 as well as a reducing Ca and P available to male broilers at 21 days. The main constituent of the organic matrix is collagen (80% to 90%), which provides support and contributes to the mineralization process and tensile strength of the tissue by developing structure and orientation for the mineral matrix.

The characteristics of bone are a result of the relative amounts of its three main constituents: water, minerals, and collagen. The relationships between the amounts of these elements affect many biomechanical properties (RATH et al., 2000). However, an adequate measure of collagen allows bones to bend without breaking and to support a greater load, regardless of the rigidity provided by hydroxyapatite crystals (CURREY, 2003).

The addition of 1.42 μg of active vitamin D$_3$ per kg of feed in the diets of broilers at 35 days of age resulted in bone deposition of 33.95%, i.e., the treatment had a quadratic effect ($P < 0.05$) on bone CPs. The CP values obtained confirm the results described by Junqueira and Carneiro (2004) for the organic composition of the bone extracellular matrix.

The results of the assessment of the mineral composition of bones in male broilers at 21 days of age are shown in **Table 5.** Ash, calcium, phosphorus and potassium contents were not affected by the addition of active vitamin D$_3$. However, the vitamin D$_3$ had a significant quadratic effect on the percentage of sodium observed. Indeed, 1.41 μg of 1,25(OH)$_2$D$_3$ added per kg of feed resulted in a deposition of 0.47% of sodium in bone. The mineral composition of bones may vary as it reflects the body's state of chemical equilibrium. Therefore, in cases of severe dysfunction, minerals (Na and K) are mobilized, leading to a change in their concentrations (MÜLLER et al., 2012).

Table 5. Percentage of ash and minerals in the tibiotarsus of male broilers at 21 days of age fed diets supplemented with *Solanum glaucophyllum.*

Variable	μg of active vitamin D$_3$/kg of feed						CV (%)	Effect	Means
	0.0	0.50	1.00	1.50	2.00	2.5			
Ash (%)	45.35	38.67	47.17	46.45	43,.87	45.92	7.82	NS	44.57
Calcium (%)	10.75	10.71	9.42	10.24	9.08	9.98	15.73	NS	10.03
Phosphorus (%)	6.88	7.08	6.72	6.37	6.07	7.73	9.85	NS	6.81
Ca:P ratio	1.58	1.53	1.39	1.60	1.49	1.43	18.08	NS	1.50
Sodium (%)	0.69	0.45	0.55	0.50	0.45	0.59	24.83	Q*	0.54
Potassium (%)	0.32	0.27	0.28	0.30	0.29	0.26	25.38	NS	0.29

CV = Coefficient of variation; NS = not significant ($P > 0.05$); Q* = quadratic effect = $\hat{Y} = 0.095x^2 - 0.2683x + 0.6609$ ($R^2 = 0.57$).

The maximum level of 1,25(OH)$_2$D$_3$ added (2.5 μg/kg) did not affect the percentages of ash or minerals in the initial stage. Sheikhlar and Navid (2009) investigated the characteristics of bones of meat-type quails up to three weeks of age that were fed diets supplemented with 1,25(OH)$_2$D$_3$ and deficient in Ca (0.71%) and P (0.37%). Unlike the present study, they found that 6.5 μg of 1,25(OH)$_2$D$_3$ per kg of feed increased concentrations of ash and Ca in bone.

Ash, Ca, P, Na and K contents in the bones of broilers at 35 days of age are shown in **Table 6.** The inclusion of active vitamin D$_3$ in feed led to a linear increase in the amount of bone ash. The percentage of Ca in the tibiotarsus at 35 days was significantly augmented (34.11%) by the addition of 1.50 μg of the metabolite, when compared with other levels of supplementation. The concentration of P also increased linearly with enhanced levels of vitamin supplementation. Improved absorption and utilization of Ca and P was reported by Cheng et al. (2004), who added 1,25$(OH)_2D_3$ or extracts of the plant *Solanum glaucophyllum* to broiler diets. The increase in bone ash content combined with improved utilization of P may increase bone quality (SOUZA; VIEITES, 2014).

Table 6. Percentage of ash and minerals in the tibiotarsus of male broilers at 35 days of age fed diets supplemented with *Solanum glaucophyllum.*

Variable	µg of active vitamin D_3/kg of feed						CV (%)	Effect	Means
	0.0	0.50	1.00	1.50	2.00	2.5			
Ash (%)	41.13	42.07	43.03	43.37	46.45	45.64	9.88	L*¹	43.61
Calcium (%)	28.56b	29.48b	26.25b	34.11a	29.64b	32.17b	7.27	SIG	30.04
Phosphorus (%)	6.66	7.23	6.94	7.18	9.13	7.88	11.46	L*²	7.34
Ca:P ratio	4.31	4.10	3.85	4.83	3.75	4.13	11.76	NS	4.16
Sodium (%)	0.52	0.48	0.45	0.44	0.43	0.46	14.22	Q*¹	0.46
Potassium (%)	0.29	0.28	0.27	0.27	0.27	0.27	24.66	Q*²	0.28

CV = Coefficient of variation; L*¹ = linear effect = $\hat{Y} = 2.0589x + 41.041 (r^2 = 0,88)$;SIG = means with same letter in row do not differ according to the Dunnett's test at a significance level of 5% ($P < 0.05$); L* = linear effect = $\hat{Y} = 0.5172x + 6.6937$ ($r^2 = 0.74$); NS = not significant ($P > 0.05$); Q*¹ = quadratic effect = $\hat{Y} = 0.0336x^2 - 0.1116x + 0.5298$ ($R^2 = 0.98$); Q*² = quadratic effect = $\hat{Y} = 0.076x2 - 0.0278x + 0.2974$ ($R^2 = 0.96$).

The Ca:P ratio in bones was 1.50:1 at 21 days and 4:1 at 35 days and was not affected by the addition of the vitamin to diets. At 21 days the Ca:P ratio was lower than that recommended by Williams et al. (2000), who suggested that 2.15:1 was an optimal ratio in the tibiotarsus of broilers at 18 days of age; however, these authors reported a variation between 1.82:1 and 3.89:1. The high Ca:P ratio observed in the tibiotarsus at 35 days was the result of enhanced Ca retention; it did not, however, compromise bone quality.

The effect on the levels of Na and K at 35 days was negatively correlated; increasing the metabolite in diets led to a reduction in the percentage of these minerals. The addition of 1.66 µg and 1.82 µg of 1,25$(OH)_2D_3$ per kg of feed resulted in the deposition of 0.43% Na and 0.27% K. According to Müller et al. (2012), under appropriate circumstances a certain amount of Na and K can be mobilized from the skeleton without general dissolution of the crystal's matrix.

Conclusions

We recommend the use of up to 1.50 µg of 1,25$(OH)_2D_3$/kg feed for broilers between 8 and 35 days of age, since the variables that indicate bone quality (Seedor index, proportion of CPs and NCPs, and higher calcium content) were positively affected by this level of supplementation.

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