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1,25-Dihydroxycholecalciferol Glycoside ($1,25(OH)_2D_3$-G) in Broiler Breeder Diets and Its Influence on Broiler Chicken Growth

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ABSTRACT

This study aimed to investigate the effects of 1,25-Dihydroxycholecalciferol glycoside ($1,25(OH)_2D_3$-G) supplementation on performance, carcass yield, carcass cuts, intestinal histomorphometry, bone health, and gene expression in broiler chickens from broiler breeders. A total of 1,152 one-day-old male Ross 308 AP chicks were distributed in a completely randomized design with a 2 x 3 factorial arrangement. One of the experimental factors was the presence or absence of $1,25(OH)_2D_3$-G (0 or 100 mg/kg) in the diets of broiler breeders between 21 and 30 weeks of age. The second experimental factor consisted of three levels of $1,25(OH)_2D_3$-G supplementation (0, 50, and 100 mg/kg) in the broiler chicken diets from day 1 to day 21, followed by a standard commercial diet from day 22 to day 42. The study totaled six treatments with eight replicates and 24 birds per experimental unit. Performance, carcass and cut yields, as well as tibial breaking strength and composition, were evaluated in broiler chickens at 21 and 42 days. Intestinal histomorphometry and gene expression were assessed at 21 days, while tibial dyschondroplasia was analyzed at 42 days. Broiler chickens from broiler breeders supplemented with 100 mg/kg of $1,25(OH)_2D_3$-G showed higher hatch weight, better feed conversion, improved intestinal morphology, and greater carcass yield. However, this supplementation did not enhance calcium and phosphorus deposition in the tibia, resulting in reduced bone strength. It is concluded that broiler chickens from broiler breeders supplemented with 100 mg/kg of $1,25(OH)_2D_3$-G show better growth performance at 21 and 42 days.

Keywords: carcass yield; growth performance; intestinal health; tibial health

INTRODUCTION

1,25-Dihydroxycholecalciferol glycoside derived from the plant *Solanum glaucophyllum* has emerged as a promising alternative in broiler chicken diets as a source of vitamin D_3 (Vieites *et al*., 2016, 2018; Nunes *et al*., 2020). Unlike the fat-soluble form of vitamin D_3, which relies on micelle formation for transport and absorption in the intestine, 1,25-Dihydroxycholecalciferol glycoside, possesses water-soluble properties. This is due to its glycoside linkage. This allows for easy dissolving in intestinal fluids and eliminates the need for micelles for its transport (Souza *et al*., 2020; Asnayanti *et al*., 2024).

Studies indicate that dietary inclusion of 1,25-Dihydroxycholecalciferol glycoside has been associated with an increased rate of muscle tissue deposition and improved feed conversion efficiency, both of which are essential for productive performance (Vieites *et al*., 2016, 2017, 2018; Alves *et al*., 2018). In the immune system, evidence suggests that 1,25-Dihydroxycholecalciferol glycoside can modulate the inflammatory response, promote immune cell differentiation, and enhance the resistance of the birds to health challenges (Nunes *et al*., 2020). Additionally, its role in regulating calcium and phosphorus homeostasis has been shown to be essential for proper bone mineralization, preventing skeletal disorders such as tibial dyschondroplasia, which compromise the structural integrity of the birds (Yavaş *et al*., 2020; Asnayanti *et al*., 2024).

On the other hand, there are no studies on the use of $1,25(OH)_2D_3$-G in broiler breeders. Although, research on vitamin D_3 (cholecalciferol, synthetic) supplementation showed improved nutrient transfer to the eggs, which results in broiler chickens with better bone mineralization and enhanced ability to support weight throughout growth (Wen *et al*., 2019; Adhikari *et al*., 2020; Li *et al*., 2021; Yusuf *et al*., 2023). These findings indicate that supplementation with $1,25(OH)_2D_3$-G in broiler breeders could yield even more favorable results, given the potential for this compound to act more rapidly in the organism. The potential of $1,25(OH)_2D_3$-G underscores

the need for further research to explore its effects and optimize its benefits (Vieites *et al*., 2016, 2018; Nunes *et al*., 2020; Asnayanti *et al*., 2024).

Studying plant-derived $1,25(OH)_2D_3$-G as an alternative to its synthetic counterpart is relevant, particularly due to its lower cost and the potential for more efficient absorption in birds (Souza *et al*., 2020; Kumar *et al*., 2023). However, there is still no consensus on the optimal dosage of $1,25(OH)_2D_3$ -G for broiler chickens (Nunes *et al*., 2020; Wu *et al*., 2022; Setiyaningsih *et al*., 2023), and excessive use may lead to complications such as hypercalcemia and tissue calcification, compromising bird health and welfare (Gili *et al*., 2016; Alves *et al*., 2018; Trautenmüller *et al*., 2021, 2022). Therefore, further studies are needed to establish safe guidelines and assess the actual effectiveness of plant-derived $1,25(OH)_2D_3$-G in poultry production (Hurst *et al*., 2020; Barnkob *et al*., 2020; San *et al*., 2021; Wang *et al*., 2021).

This study thus hypothesizes that breeder supplementation with $1,25(OH)_2D_3$-G enhances bone mineralization in newly hatched broiler chickens, promoting balanced growth and strengthening the immune system. To test this hypothesis, the study evaluated the effects of $1,25(OH)_2D_3$-G supplementation on performance, carcass yield, carcass cuts, intestinal histomorphometry, bone health, and gene expression in broiler chickens from broiler breeders supplemented or not with this metabolite.

MATERIALS AND METHODS

Ethics Committee

The procedures were approved by the Animal Use Ethics Committee of the National Council for Animal Control and Experimentation - UNIOESTE (Protocol No. 01/2021) and were previously sanctioned by the National Council for Animal Control and Experimentation in accordance with Normative No. 37 of February 15, 2018.

Birds, Housing, and Experimental Design

Two breeder houses (G1 and G2) located in the municipality of Pato Branco, Paraná - Brazil, housing 8,000 AP95 (Aviagen) broiler breeders at 21 weeks of age. The houses were identical in terms of physical characteristics, and the management practices were standardized for both. Each house had a dimension of 175 m x 13 m, and was constructed as a metallic structure covered with Brasilit roofing, and the ceiling height was 3 m without insulation. The houses were equipped with bird-proof mesh and white curtains, along with fans to maintain positive pressure ventilation. The nests were mechanical, while the feeding system consisted of automatic chain feeders specifically designed for broiler breeders, and the drinkers were pendular models (Plasson).

The diets provided to the birds in barns G1 and G2 were identical, formulated with corn and soybean meal to meet the nutritional requirements throughout the production period (21 to 30 weeks of age). However, the broiler breeders in house G1 received a mash diet supplemented with $1,25(OH)_2D_3$-G at a dosage of 100 mg/kg starting at 21 weeks of age, while the broiler breeders in house G2 received a mash diet without $1,25(OH)_2D_3$-G supplementation. Both breeder groups were housed under the same environmental conditions, with controlled temperature and humidity following standard management practices for broiler breeders.

At 30 weeks of age, the eggs from each barn were collected separately, identified, selected, and sanitized. The eggs were then transported to the hatchery and incubated in incubators (Jamesway Platinum Series), where they remained for 18 days. Incubation conditions were a temperature of 37.5 °C and a relative humidity of 60%. After the 18-day incubation period, the eggs were transferred to the hatchers, where they remained at 37.2 °C and a relative humidity of 65% until hatching occurred.

After hatching, the chicks were sexed and vaccinated against Marek's disease, Avian Pox, Gumboro disease, and Infectious Bronchitis. Chicks were then identified based on the origin of the parent stock, considering the supplementation or not of $1,25(OH)_2D_3$-G, and subsequently sent to the Poultry Research Center at the State University of Western Paraná, Marechal Cândido Rondon, Paraná, Brazil.

A total of 1,152 one-day-old male Ross 308 AP95 broiler chickens were distributed in a completely randomized design, arranged in a 2 x 3 factorial scheme. One of the factors was the origin of the broiler chickens from broiler breeders fed with or without $1,25(OH)_2D_3$-G (0.0 and 100 mg/kg). The second factor was the diet of the broiler chickens. Broiler chickens were fed with three inclusion levels (0.0, 50, and 100 mg/kg of $1,25(OH)_2D_3$-G) up to 21 days of age. This resulted in 6 treatments, with 8 replicates and 24 birds per experimental unit. Performance, carcass and cut yields, tibia breaking strength and composition, were evaluated in broiler chickens at 21 and 42 days. Intestinal histomorphometry and gene expression were assessed at 21 days. Tibial dyschondroplasia was analyzed at 42 days.

Birds were housed in an experimental poultry house measuring 35 meters in length and 13 meters in width, divided into sections containing multiple experimental units. Each experimental unit had an area of 1.96 m^2 and was equipped with a tubular feeder, nipple drinkers, and a concrete floor. Environmental temperature control was managed by a Smaai IV control panel, which operated the pellet-heating furnace, four exhaust fans, and evaporative cooling panels. The lighting program followed the guidelines of the breed manual. Exhaust fans and evaporative panels ensured environmental cooling and air renewal. Average, minimum, and maximum temperatures and relative humidities were monitored daily and maintained within the thermal comfort range recommended for each growth phase.

Diets and Feeding Management

The experimental broiler chicken diets (Table 1) were isonutritive and isocaloric, based on corn and soybean meal, following the nutritional recommendations

Table 1. Percentual and calculated composition of experimental diets for broiler chickens supplemented or not with $1,25(OH)_2D_3G$

Ingredients (g/kg) as fed	Dietary phases for broiler chickens			
	1 to 7 days	8 to 21 days	22 to 35 days	36 to 42 days
Corn (7.88%)	504.76	525.03	589.79	645.33
Soybean meal (46%)	423.88	402.13	333.75	286.41
Degummed soybean oil	29.70	33.66	39.07	36.54
Dicalcium phosphate	17.86	16.37	15.04	11.34
Limestone	9.39	8.64	8.62	7.16
Salt	4.02	3.68	3.36	3.37
Sodium bicarbonate	1.00	1.50	2.00	2.00
Lysine sulphate (60%)	1.70	1.75	-	-
Lysine sulphate (54.7%)	-	-	2.20	2.43
DL-Methionine (99%)	3.27	3.09	2.58	2.23
L-Threonine (99%)	0.47	0.42	0.31	0.24
Choline chloride (60%)	0.50	0.50	0.50	0.45
Adsorbent	1.00	1.00	1.00	1.00
Vitamin premix	1.30	1.00	1.00	1.00
Mineral premix	0.50	0.50	0.50	0.50
Coccidiostat	0.55	0.55	0.20	-
Enramycin	-	0.08	0.08	-
Inert (kaolin)	0.10	0.10	-	-
Calculated nutrient composition (g/kg) as fed				
ME (kcal/kg)	3000	3050	3150	3200
Crude protein	240.471	231.848	205.060	187.327
Digestible lysine	13.000	12.5	11.000	10.000
dMet+Cys	9.620	9.250	8.140	7.400
Digestible threonine	8.580	8.250	7.260	6.600
Digestible valine	10.000	9.630	8.470	7.700
Digestible tryptophan	2.804	2.688	2.323	2.077
Digestible arginine	15.196	14.573	12.619	11.302
Calcium	9.500	8.780	8.220	6.610
Available phosphorus	4.500	4.190	3.840	3.090
Sodium	2.000	2.000	2.000	2.000
Potassium	9.372	9.039	7.995	7.306

Note: Adsorbent: bentonite. Vitamin supplement, composition per kg of diet in the pre-initial feed: Vitamin A (min) 14.300 IU; Vitamin D3 (min) 5.200 IU; Vitamin E (min) 71.50 IU; Vitamin K3 (min) 3.90 mg; Vitamin B1 (min) 2.99 mg; Vitamin B2 (min) 9.10 mg; Pantothenic acid (min) 15.60 mg; Vitamin B6 (min) 5.20 mg; Vitamin B12 (min) 32.50 mg; Niacin (min) 78.00 mg; Folic acid (min) 2.60 mg; Biotin (min) 0.33 mg; Selenium (min) 0.39 mg. Vitamin supplement, composition per kg of diet initial growth and finishing feeds: Vitamin A 11.000 IU; Vitamin D3 4.000 IU; Vitamin E 55 IU; Vitamin K3 3.00 mg; Thiamine (B1) 2.30 mg; Riboflavin (B2) 7.00 mg; Pyridoxine (B6) 4.00 mg; Cyanocobalamin (B12) 25.00 mg; Pantothenic acid (B5) 12.00 mg; Niacin (B3) 60.00 mg; Folic acid (B9) 2.00 mg; Biotin (B7) 0.25 mg; Selenium 0.30 g. Mineral supplement, composition per kg of diet: Iron (min) 50g; Copper (min) 10g; Manganese (min) 65g; Zinc (min) 65g; Iodine (min) 1.000 mg. Coccidiostat: from 1 to 21 days of age, salinomycin 12% (Coxistac 12%) was used, and from 22 to 35 days of age, salinomycin 24% (Salinocox 24%) was used. Enramycin 8% (Enradin 8%). Inert based on kaolin with the inclusion of 1,25(OH)2D3-G as a weight-for-weight substitution by the inert. ME: Metabolizable energy, dMet+Cys: Digestible Methionine + Cysteine.

of Rostagno *et al*. (2017). The use of $1,25(OH)_2D_3$-G replaced the inert material (kaolin) in the feed, which was used as a non-nutritional component. Kaolin was selected because it is not absorbed by the chickens and does interfere with the nutritional evaluation. The $1,25(OH)_2D_3$-G was provided in the feed only up to 21 days of age. Specifically, the supplementation was included in the diets during the phases of 1 to 7 days, 8 to 14 days, and 15 to 21 days. In the phases of 22 to 35 days and 36 to 42 days, the broiler chickens were fed the non-supplemented control diets. Throughout the experimental period (1 to 42 days), the birds had *ad libitum* access to water and feed.

The supplementation period of 21 days was chosen based on evidence indicating that broiler chickens have a high metabolic demand for calcium and phosphorus during the first 21 days of life. At the same time, the endogenous conversion of vitamin D_3 into its active form is limited (Vieites *et al*., 2016, 2018; Alves *et al*., 2018).

The treatments evaluated for the growth of broiler chickens originating from broiler breeders not supplemented with $1,25(OH)_2D_3$-G included 0.0, 50, and 100 mg/kg of $1,25(OH)_2D_3$-G in the diet. For broiler chickens originating from broiler breeders supplemented with 100 mg/kg of $1,25(OH)_2D_3$-G, the broiler chicken treatments consisted of the dietary addition of 0.0, 50, and 100 mg/kg of $1,25(OH)_2D_3$-G.

The vitamin source used in this study ($1,25(OH)_2D_3$-G) was the product Panbonis® 10, produced from *Solanum glaucophyllum* in powder form, standardized with pre-gelatinized wheat starch and wheat middlings. Its analytical composition

includes 1,25$(OH)_2D_3$-G (minimum of 10 ppm), moisture (maximum of 14%), crude protein (14%-18%), crude fiber (5.25%-8.75%), ether extract (3%-6%), crude ash (3%-6%), sodium (0.0%-0.7%), lysine (0.6%-0.9%), and methionine (0.2%-0.4%).

Performance, Carcass Yield, and Carcass Yield Cuts

Body weight and feed intake of the birds were recorded at 10, 21, and 42 days to evaluate body weight gain, feed intake, and feed conversion ratio. Weight gain was calculated as the difference between the final and initial body weight of the birds during the evaluated period. Average feed intake was determined by dividing the total feed consumed by the number of live birds in the period. The feed conversion ratio was calculated as the total feed intake divided by the weight gain of the birds and was adjusted for mortality, as described by Sakomura and Rostagno (2016).

At 42 days of age, three broiler chickens per experimental unit close to the average weight of each replicate (n= 24), were randomly selected, weighed, identified, and euthanized by electronarcosis followed by bleeding, scalding, plucking, and evisceration. The carcasses were initially weighed, then chilled in ice water for 60 minutes, and subjected to a 10-minute dripping period to remove excess water. Afterward, the chilled carcasses were weighed to calculate the cold carcass yield. The carcasses were then cut into parts, separating the legs (thigh and drumstick), wings, breast fillet, and tenderloin, with each cut individually weighed. Carcass yield was calculated as the carcass weight relative to the live body weight. The cut yield was determined based on the weight of the cuts relative to the cold carcass weight. The liver and abdominal fat (including adipose tissue around the cloaca, gizzard, proventriculus, and adjacent abdominal muscles) were separated and weighed to determine their relative weight to the live body weight.

Intestinal Histomorphometry

At 21 days of age, two broiler chickens per experimental unit, selected to be close to the average weight of each replicate (n= 16), were euthanized by cervical dislocation for the removal of the digestive tract and subsequent evaluation of intestinal histomorphometry (villus height, crypt depth, absorption area, and villus: crypt ratio).

The small intestine was exposed, and the jejunum was isolated for sampling. The segment used was the distal section between the duodenal loop and Meckel's diverticulum. A 2 cm fragment of the jejunum was collected 5 cm before Meckel's diverticulum. This fragment was fixed in 10% buffered formalin, dehydrated in a series of increasing ethanol concentrations, and then embedded in paraffin. Semi-serial sections of 5 μm from each segment were placed on glass slides and stained using the hematoxylin-eosin technique (Luna, 1968).

The measurements were performed using the PROPLUS IMAGE 4.1 imaging system. On each slide, the length and width of the villi, as well as the depth and width of the crypts, were recorded. These morphometric measurements were used to calculate the absorption surface area of the intestinal mucosa (Kisielinski *et al*., 2002). The villus height-to-crypt depth ratio was calculated using the results of villus height and crypt depth measurements.

Tibial Breaking Strength and Composition

The right tibiae from the broiler chickens euthanized at 21 and 42 days of age were used to determine dry matter in an oven with air circulation (105 °C). After this process, they were ashed for 8 hours in a muffle furnace at 600 °C to obtain mineral matter (ash). The calcium and phosphorus contents in the bone were determined as described by Silva and Queiroz (2009). Calcium concentration was measured by flame atomic absorption spectroscopy (FAAS), and phosphorus (P) was measured using ultraviolet-visible (UV-VIS) spectroscopy.

The left tibia was used to determine bone strength using the Brookfield CT3 texture analyzer. This equipment features a base that supports the epiphyseal areas of the bone, applying a force of 5 mm/s with a load of 200 kgf (kilogram-force) to the central region of the bone (diaphysis). During the strength determination, the tibia was always placed on the supports in the same position. The results were expressed in kilogram-force (Ospina-Rojas *et al*., 2018).

Tibial Dyschondroplasia

After bone strength analysis of day 42 tibia, the bone was used to evaluate degrees of tibial dyschondroplasia, following the method described by Edwards (1989). A transverse cut was made at the upper part of the tibia to expose the central portion of the bone for evaluation of the lesion scores according to the degree of cartilage:

0: Normal cartilage, narrow with minor irregularities;
1: Thickened cartilage or with considerable irregularities;
2: Thickened cartilage with evidence of pre-hypertrophic cartilage that is not calcified and has not been invaded by metaphyseal vessels, with deep irregularities in the cartilage being apparent;
3: Large mass of cartilage at the proximal end of the tibia.

Gene Expression

The gene expression of interleukins (IL-10 and IL-1β) was analyzed for their role in the immune response, while CALB-D28K (Calbindin-D28K) was evaluated for its function in calcium metabolism. Since 1,25$(OH)_2D_3$-G regulates both calcium homeostasis and immune modulation, its effects on these genes help us understand its impact on broiler chickens.

At 21 days of age, one bird per experimental unit received stimulation through the intraperitoneal administration of 1 mg of LPS (lipopolysaccharides from *E. coli*, Sigma) per kg body weight. Four hours later, the birds were euthanized by cervical dislocation, and immediately, a fragment of the jejunum was collected and immersed in the stabilizing solution RNAlater™ (Invitrogen, USA), which was subsequently stored in a freezer at -20 °C until RNA extraction.

Total RNA was extracted using *QIAzol Lysis Reagent* (Qiagen GmbH, Germany). Approximately 70 mg of chicken intestine was weighed, minced, and added to a DNase- and RNase-free microtube containing 500 µL of Trizol. The samples were homogenized (vortexed) and incubated at room temperature for 5 minutes. Subsequently, 100 µL of chloroform was added, followed by manual homogenization for 15 seconds. Afterward, the mixture was incubated at room temperature for 3 minutes and centrifuged for 15 minutes at 12,000 × g at 4 °C.

The aqueous phase was transferred into a new tube and 250 µL of isopropanol was added, followed by incubation for 10 minutes (at room temperature) with manual homogenization and centrifugation for 15 minutes at 12,000 × g at 4 °C. The supernatant was discarded, and the pellet was washed with 1 mL of 75% ethanol. The samples were centrifuged once again at 7,500 × g for 5 minutes, and the supernatant was discarded to dry the *pellet* for 15 minutes and then resuspended in DNase- and RNase-free ultrapure water and incubated at 60 °C for 15 minutes.

The RNA concentration for IL-1β, IL-10, CALB-D28K, and β-actin (endogenous control gene) was measured using the NanoDrop™ Lite spectrophotometer (Thermo Fisher Scientific, USA) at a wavelength of 260/280 nm. The integrity of the RNA was assessed on a 1% agarose gel stained with *SYBR Safe™ DNA Gel Stain* (Invitrogen, USA) and visualized using a transilluminator with ultraviolet light.

For cDNA synthesis, the *QuantiTect Reverse Transcription* Kit (Qiagen GmbH, Germany) was used according to the manufacturer's instructions, considering 1 µg of RNA and a final volume of 20 µL. To remove possible genomic DNA residues, each sample was treated with 2 µL of *gDNA Wipeout Buffer* (Qiagen GmbH, Germany) and incubated at 45 °C for 2 minutes. After the removal of genomic DNA, 4 µL of *Quantiscript RT Buffer*, 1 µL of RT Primer Mix, and 1 µL of *Quantiscript Reverse Transcriptase* were added. The reverse transcription reaction was incubated for 15 minutes at 42 °C, followed by 3 minutes at 95 °C, and then immediately placed on ice. The samples were measured using the NanoDrop™ Lite spectrophotometer (Thermo Fisher Scientific, USA) and stored at -20 °C until use.

The *primers*/oligonucleotides used in the reactions were obtained from published works on *Gallus gallus* (Table 2). The primer sequences were aligned using the BLAST (Basic Local Alignment Search Tool) algorithm in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). The *β-actin* gene was considered as a reference/housekeeping gene, and its stability among the treatments was assessed using Statistica® 7.0 software. To assess the amplification efficiency of each *primer*, serial dilutions of the cDNA *pool* containing all treatments were performed using different primer concentrations. For *β-actin*, CALB-D28K, and IL-10, 200 ng of cDNA and 400 nM of primer were used, while for IL-1β, 200 ng of cDNA and 600 nM of *primer* were utilized.

The qRT-PCR analyses were conducted on a Rotor-Gene Q (Qiagen GmbH, Germany) using the *QuantiNova* SYBR Green PCR Kit (Qiagen GmbH, Germany) in duplicates. The total reaction volume was 20 µL. The amplification conditions were 95 °C for 2 minutes, followed by 40 cycles at 95 °C for 5 seconds, and 60 °C for 10 seconds. The dissociation curve of the reaction products was obtained to determine the specificity of the reactions.

The relative gene expression data were recorded as Ct (cycle threshold) values and normalized using the mean Ct values obtained for the reference gene in each sample, for each treatment, and for each target gene, as recommended by Vandesompele *et al*. (2002). The 2-ΔCt method (Livak & Schmittgen, 2001) was used for relative quantification of gene expression (expressed as arbitrary units, AU).

Statistical Procedures

The data were evaluated for residual normality using the Shapiro-Wilk test and variance homogeneity using Levene's test, both conducted through the Univariate procedure. For data with a normal distribution, a two-way analysis of variance (ANOVA) was performed to assess the effects of 1,25$(OH)_2D_3$-G supplementation in broiler breeders and broiler chickens, as well as potential interactions between these factors. When significant effects were detected, treatment means for broiler breeders were compared using the F-test, while treatment means for broiler chickens were compared using the Student-Newman-Keulstest. These analyses were conducted using the GLM procedure. Non-parametric tests were applied to data that did not exhibit a normal distribution (Tibial

Table 2. Genes and primer sequences for gene expression analysis using qPCR

Gene	Primer sequence (5'-> 3')	Amplicon size (bp)	References
β-actina	F: TTCTTTTGGCGCTTGACTCA	88	Proszkowiec-Weglarz *et al*. (2019)
	R: GCGTTCGCTCCAACATGTT		
CALB-D28K	F: TTGGCACTGAAATCCCACTGAA	116	NM_205513.2
	R: CATGCCAAGACCAAGGCTGA		
IL-1β	F: GCTCTACATGTCGTGTGTGATGAG	80	NM_204524.2
	R: TGTCGATGTCCCGCATGA		
IL-10	F: CATGCTGCTGGGCCTGAA	94	NM_001004414.4
	R: CGTCTCCTTGATCTGCTTGATG		

Note: CALB-D28K: Calbindin D28K; IL-1β: Interleukin-1 beta; IL-10: Interleukin 10.

dyschondroplasia). All analyses were conducted using SAS for academic software, with a significance level of 5% (SAS, 2014).

RESULTS

Performance

Performance did not show any interaction between the supplementation of 1,25(OH)$_2$D$_3$-G in the diets of broiler breeders and broiler chickens. However, broiler breeders supplemented with 100 mg/kg of 1,25(OH)$_2$D$_3$-G resulted in heavier broiler chickens ($p<0.003$) at one day of age (Table 3). Moreover, at 21 and 42 days of age, the broiler chickens from broiler breeders supplemented with 1,25(OH)$_2$D$_3$-G exhibited lower feed conversion ($p<0.027$ and $p<0.008$, respectively). Regarding the effect of including 1,25(OH)$_2$D$_3$-G in the growth of broiler chickens, supplementation of 50 g mg/kg resulted in lower feed consumption at 42 days of age.

Carcass Yield and Carcass Cuts

Carcass yield exhibited an interaction ($p<0.021$) between the supplementation of 1,25(OH)$_2$D$_3$-G in the diets of broiler breeders and broiler chickens (Table 4). Supplementation with 100 mg/kg of 1,25(OH)$_2$D$_3$-G in the diets of broiler breeders and broiler chickens resulted in a higher carcass yield ($p<0.001$) in broiler chickens at 42 days of age (Figure 1).

Intestinal Histomorphometry

The intestinal histomorphometry of the jejunum in broiler chickens at 21 days of age did not show any interaction between the supplementation of 1,25(OH)$_2$D$_3$-G in the diets of broiler breeders and broiler chickens. However, broiler chickens from broiler breeders supplemented with 100 mg/kg of 1,25(OH)$_2$D$_3$-G had reduced crypt depth ($p<0.001$) and an increased villus: crypt ratio ($p<0.024$) in the jejunum at 21 days of age (Table 5).

Tibial Breaking Strength and Composition

The tibial breaking strength was lower in broiler chickens at 42 days of age when they were from broiler breeders supplemented with 100 mg/kg of 1,25(OH)$_2$D$_3$-G (Table 6). The calcium concentration ($p<0.032$) in the tibia at 21 days and the phosphorus concentration ($p<0.007$) at 42 days showed a significant interaction between the supplementation

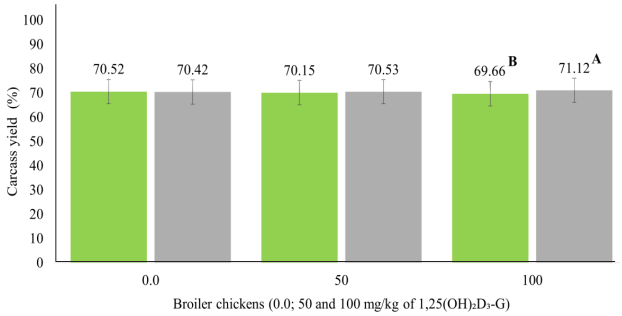

Figure 1. Analysis of the interaction between the supplementation of 1,25-Dihydroxycholecalciferol glycoside (1,25(OH)2D3-G) in the diets of broiler breeders and broiler chickens and its impact on the carcass in the broiler chickens at 42 days of age. [A,B] Means followed by uppercase letters differ by the F-test at 5%. [A,B] Comparisons between broiler chickens supplemented with 100 mg/kg of 1,25(OH)$_2$D$_3$-G from non-supplemented and supplemented broiler breeders with the same additive ($p<0.001$). Broiler breeders (0.0 mg/kg of 1,25(OH)$_2$D$_3$G) (■), Broiler breeders (100 mg/kg of 1,25(OH)$_2$D$_3$G) (■).

Table 3. Performance of broiler chickens at 21 and 42 days of age fed diets supplemented or not with 1,25(OH)$_2$D$_3$-G from broiler breeders supplemented or not with the same additive

1,25(OH)$_2$D$_3$-G levels (mg/kg)	Variables						
	1 to 21 days				42 days		
	IBW (g/bird)	FI (g/bird)	BWG (g/bird)	FCR (g/g)	FI (g/bird)	BWG (g/bird)	FCR (g/g)
1,25(OH)$_2$D$_3$-G in broiler breeders							
0	39.50^{b}	1080	826	1.321^{b}	4327	2884	1.505^{b}
100	39.73^{a}	1093	843	1.297^{a}	4323	2925	1.480^{a}
1,25(OH)$_2$D$_3$-G in broiler chickens							
0	39.59	1092	831	1.314	4361^{A}	2904	1.507
50	39.61	1077	833	1.302	4282^{B}	2891	1.492
100	39.63	1091	841	1.310	4334^{AB}	2918	1.480
SEM	0.35	41.29	31.15	0.04	79.89	78.03	0.03
P value							
1,25(OH)$_2$D$_3$-G in broiler breeders	0.003	0.309	0.117	0.027	0.749	0.067	0.008
1,25(OH)$_2$D$_3$-G in broiler chickens	0.942	0.536	0.440	0.735	0.024	0.573	0.552
Broiler breeders vs Broiler chickens interaction	0.949	0.849	0.969	0.225	0.227	0.248	0.654

Note: 1,25(OH)$_2$D$_3$-G: 1,25-Dihydroxycholecalciferol glycoside; IBW: Initial body weight; FI: Feed intake; BWG: Body weight gain; FCR: Feed conversion ratio; SEM: Standard Error of the Mean; Broiler breeders *vs* Broiler chickens interaction: Broiler breeders supplemented with 0.0 and 100 mg/kg of 1,25(OH)$_2$D$_3$-G *versus* Broiler chickens supplemented with 0.0, 50, and 100 mg/kg of 1,25(OH)$_2$D$_3$-G; [ab]: Means followed by different lowercase letters in the column differ by F test at 5% significance; [A,B]: Means followed by different uppercase letters in the column differ by Student-Newman-Keuls test at 5% significance.

Table 4. Carcass yield, cuts, and relative organ weight of broiler chickens processed at 42 days of age fed diets supplemented or not with 1,25(OH)$_2$D$_3$-G from broiler breeders supplemented or not with the same additive

1,25(OH)$_2$D$_3$-G (mg/kg)	Variables						
	CY (%)	LY (%)	BY (%)	BT (%)	WY (%)	RAF (%)	RLW (%)
1,25(OH)$_2$D$_3$-G in broiler breeders							
0	70.12[b]	31.95	27.45	5.44	9.21	1.11	2.14
100	70.70[a]	32.06	27.50	5.43	9.28	1.04	2.14
1,25(OH)$_2$D$_3$-G in broiler chickens							
0	70.47	31.95	27.6	5.49	9.39	1.09	2.10
50	70.53	31.95	27.44	5.36	9.24	1.09	2.17
100	70.72	32.12	27.39	5.44	9.10	1.04	2.15
SEM	1.36	1.15	1.48	0.40	0.76	0.24	0.24
P value							
1,25(OH)$_2$D$_3$-G in broiler breeders	0.013	0.588	0.857	0.880	0.576	0.079	0.914
1,25(OH)$_2$D$_3$-G in broiler chickens	0.901	0.714	0.775	0.257	0.168	0.524	0.330
Broiler breeders vs Broiler chickens interaction	0.021	0.863	0.290	0.859	0.835	0.094	0.655

Note: 1,25(OH)$_2$D$_3$-G: 1,25-Dihydroxycholecalciferol glycoside; CY: Carcass yield; LY: Leg yield (thigh and drumstick); BY: Breast yield; BT: Breast tenders; WY: wing yield; RLW: Relative liver weight; RAF: Relative abdominal fat weight; SEM: Standard error of the mean; Broiler breeders *vs* Broiler chickens interaction: Broiler breeders supplemented with 0.0 and 100 mg/kg of 1,25(OH)$_2$D$_3$-G *versus* Broiler chickens supplemented with 0.0, 50, and 100 mg/kg of 1,25(OH)$_2$D$_3$-G; [a,b]: Means followed by different lowercase letters in the column differ by F test at 5% significance.

Table 5. Histomorphometry of the jejunum of broiler chickens at 21 days of age fed diets supplemented or not with 1,25(OH)$_2$D$_3$-G from broiler breeders supplemented or not with the same additive

1,25(OH)$_2$D$_3$-G (mg/kg)	Variables			
	VH (μm)	CD (μm)	AA (μm²)	VCR (μm)
1,25(OH)$_2$D$_3$-G in broiler breeders				
0	945.46	132.17[a]	19.22	7.24[b]
100	917.17	111.26[b]	17.64	8.14[a]
1,25(OH)$_2$D$_3$-G in broiler chickens				
0	913.56	130.25	20.17	7.15
50	968.06	119.69	17.43	8.14
100	912.31	115.25	17.7	7.82
SEM	167.48	17.71	3.75	1.35
P value				
1,25(OH)$_2$D$_3$-G in broiler breeders	0.561	0.001	0.15	0.024
1,25(OH)$_2$D$_3$-G in broiler chickens	0.565	0.059	0.086	0.114
Broiler breeders vs Broiler chickens interaction	0.382	0.179	0.938	0.686

Note: 1,25(OH)$_2$D$_3$-G: 1,25-Dihydroxycholecalciferol glycoside; VH: Villus height; CD: Crypt depth; AA: Absorption area; VCR: Villus: Crypt ratio; SEM: Standard error of the mean; Broiler breeders *vs* Broiler chickens interaction: Broiler breeders supplemented with 0.0 and 100 mg/kg of 1,25(OH)$_2$D$_3$-G *versus* Broiler chickens supplemented with 0.0, 50, and 100 mg/kg of 1,25(OH)$_2$D$_3$-G; [a,b]: Means followed by lowercase letters in the column differ from each other by the F test at 5% significance.

of 1,25(OH)$_2$D$_3$-G in the diets of broiler breeders and broiler chickens (Table 7). Supplementation (0, 50, and 100 mg/kg) of 1,25(OH)$_2$D$_3$-G in the diets of broiler chickens from broiler breeders supplemented with 1,25(OH)$_2$D$_3$-G did not increase calcium deposition in the tibia at 21 days of age (Figure 2). Higher calcium concentrations ($p<0.032$) were observed in broiler chickens that did not receive 1,25(OH)$_2$D$_3$-G supplementation and were from non-supplemented broiler breeders ($p<0.027$). Moreover, a higher phosphorus concentration (Figure 3) was observed in the tibia of broiler chickens at 42 days of age when they received supplementation (0 and 50 mg/kg) of 1,25(OH)$_2$D$_3$-G and were from broiler breeders supplemented with 1,25(OH)$_2$D$_3$-G ($p<0.001$).

Tibial Dyschondroplasia

Broiler chickens at 42 days of age did not show significant differences in the results of tibial dyschondroplasia lesions (Table 8).

Gene Expression

The gene expression of calbindin D28K in the jejunum of broiler chickens at 21 days of age did not show any significant interaction between the supplementation of 1,25(OH)$_2$D$_3$-G in the diets of broiler breeders and broiler chickens (Table 9). However, broiler chickens from broiler breeders supplemented with 1,25(OH)$_2$D$_3$-G exhibited higher levels of calbindin D28K ($p<0.019$) compared to those from broiler breeders that did not receive the supplementation of 1,25(OH)$_2$D$_3$-G.

The gene expression of IL-10 and IL 1β showed an interaction between the supplementation of 1,25(OH)$_2$D$_3$-G in the diets of broiler breeders and broiler chickens. Supplementation with 100 mg/kg of 1,25(OH)$_2$D$_3$-G in the diets of broiler breeders ($p<0.046$) and broiler chickens ($p<0.054$) reduced the gene expression of interleukin 10 in the jejunum of broiler chickens at 21 days of age (Figure 4). IL-1β was higher ($p<0.071$) in broiler chickens supplemented with

Table 6. Tibia breaking strength of the tibia expressed in dry matter from broiler chickens fed diets supplemented or not with 1,25(OH)$_2$D$_3$-G from broiler breeders supplemented or not with the same additive

1,25(OH)$_2$D$_3$-G levels (mg/kg)	Breaking strength (kgf)	
	21 days	42 days
1,25(OH)$_2$D$_3$-G in broiler breeders		
0	17.78	42.25^{a}
100	17.76	39.49^{b}
1,25(OH)$_2$D$_3$-G in broiler chickens		
0	17.69	39.62
50	17.60	41.69
100	18.00	41.30
SEM	2.93	4.44
P value		
1,25(OH)$_2$D$_3$-G in broiler breeders	0.994	0.037
1,25(OH)$_2$D$_3$-G in broiler chickens	0.923	0.381
Broiler breeders vs Broiler chickens interaction	0.152	0.746

Note: 1,25(OH)$_2$D$_3$-G: 1,25-Dihydroxycholecalciferol glycoside; SEM: Standard error of the mean; Broiler breeders *vs* Broiler chickens interaction: Broiler breeders supplemented with 0.0 and 100 mg/kg of 1,25(OH)$_2$D$_3$-G *versus* Broiler chickens supplemented with 0.0, 50, and 100 mg/kg of 1,25(OH)$_2$D$_3$-G; ^{a,b}: Means followed by lowercase letters in the column differ by the F-test at 5% significance.

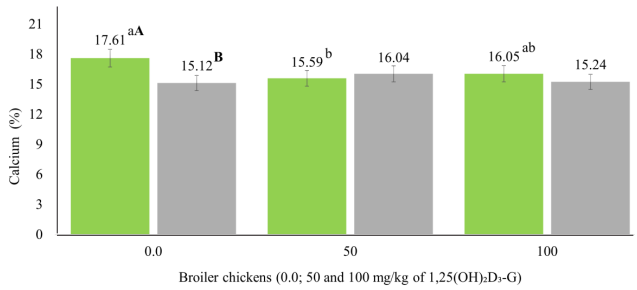

Figure 2. Analysis of the interaction between the supplementation of 1,25-Dihydroxycholecalciferol glycoside (1,25(OH)$_2$D$_3$-G) in the diets of broiler breeders and broiler chickens and its impact on the calcium in the bromatological composition of the tibia of broiler chickens at 21 days of age. ^{a,b} Means followed by different lowercase letters differ by the Student-Newman-Keuls test at 5%. ^{a,b} Broiler chickens supplemented with 0.0, 50, and 100 mg/kg of 1,25(OH)$_2$D$_3$-G, from broiler breeders not supplemented with 100 mg/kg of the additive ($p<0.036$). ^{A,B} Means followed by uppercase letters differ by the F-test at 5%. ^{A,B} Comparisons between broiler chickens not supplemented of 1,25(OH)$_2$D$_3$-G from non-supplemented and supplemented broiler breeders with the same additive ($p<0.001$). Broiler breeders (0.0 mg/kg of 1,25(OH)$_2$D$_3$G) (■), Broiler breeders (100 mg/kg of 1,25(OH)$_2$D$_3$G) (■).

50 mg/kg and when they came from broiler breeders supplemented with 1,25(OH)$_2$D$_3$-G in the diet (Figure 5).

DISCUSSION

Broiler breeders supplemented with 100 mg/kg of 1,25(OH)$_2$D$_3$-G produced heavier broiler chickens at one day of age (Table 3). This probably occurred because 1,25(OH)$_2$D$_3$-G, a source of the active form of vitamin D$_3$, stimulated the production of proteins that facilitate the transport of minerals from the intestinal lumen to the bloodstream (Wang *et al*., 2021; Shojadoost *et al*.,

Table 7. Bromatological composition of the tibia expressed in dry matter from broiler chickens fed diets supplemented or not with 1,25(OH)2D3-G from broiler breeders supplemented or not with the same additive

1,25(OH)$_2$D$_3$-G levels (mg/kg)	21 days		42 days	
	P (%)	Ca (%)	P (%)	Ca (%)
1,25(OH)$_2$D$_3$-G in broiler breeders				
0	8.05	16.36^{a}	5.73^{b}	15.63
100	8.14	15.47^{b}	6.19^{a}	16.04
1,25(OH)$_2$D$_3$-G in broiler chickens				
0	8.34	16.28	6.24	16.19
50	8.10	15.81	5.88	15.71
100	7.84	15.65	5.75	15.62
SEM	0.61	1.46	0.57	1.14
P value				
1,25(OH)$_2$D$_3$-G in broiler breeders	0.607	0.032	0.007	0.231
1,25(OH)$_2$D$_3$-G in broiler chickens	0.086	0.371	0.053	0.329
Broiler breeders vs broiler chickens interaction	0.083	0.027	0.001	0.566

Note: 1,25(OH)$_2$D$_3$-G: 1,25-Dihydroxycholecalciferol glycoside; P: Phosphorus; Ca: Calcium; SEM: Standard error of the mean; Broiler breeders *vs* Broiler chickens interaction: Broiler breeders supplemented with 0.0 and 100 mg/kg of 1,25(OH)$_2$D$_3$-G *versus* broiler chickens supplemented with 0.0, 50, and 100 mg/kg of 1,25(OH)$_2$D$_3$-G; ^{a,b}: Means followed by lowercase letters in the column differ by the F-test at 5% significance.

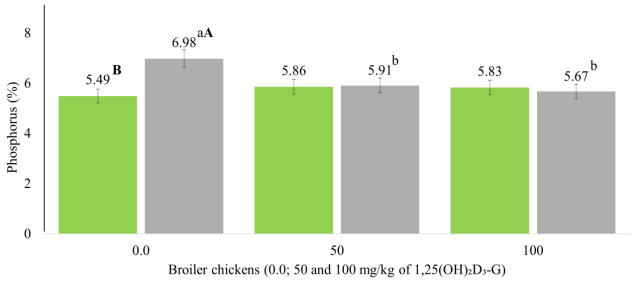

Figure 3. Analysis of the interaction between the supplementation of 1,25-Dihydroxycholecalciferol glycoside (1,25(OH)$_2$D$_3$-G) in the diets of broiler breeders and broiler chickens and its impact on the phosphorus in the bromatological composition of the tibia of broiler chickens at 42 days of age. ^{a,b} Means followed by different lowercase letters differ by the Student-Newman-Keuls test at 5%. ^{a,b} Broiler chickens supplemented with 0.0, 50, and 100 mg/kg of 1,25(OH)$_2$D$_3$-G, from broiler breeders supplemented with 100 mg/kg of the additive ($p<0.001$). ^{A,B} Means followed by uppercase letters differ by the F-test at 5%. ^{A,B} Comparisons between broiler chickens not supplemented of 1,25(OH)$_2$D$_3$-G from non-supplemented and supplemented broiler breeders with the same additive ($p<0.001$). Broiler breeders (0.0 mg/kg of 1,25(OH)$_2$D$_3$G) (■), Broiler breeders (100 mg/kg of 1,25(OH)$_2$D$_3$G) (■).

Table 8. Tibial dyschondroplasia in broiler chickens at 42 days of age that received feed supplemented or not supplemented with $1,25(OH)_2D_3$-G from broiler breeders supplemented or not with the same additive

$1,25(OH)_2D_3$-G levels (mg/kg)	Degree of tibial lesions				
	Average score	Exempt (%)	Mild (%)	Moderate (%)	Severe (%)
$1,25(OH)_2D_3$-G in broiler breeders					
0	0.84	50.72	28.99	5.80	14.49
100	0.93	47.14	27.14	11.43	14.29
$1,25(OH)_2D_3$-G in broiler chickens					
0	1.00	44.44	28.89	8.89	17.78
50	0.78	54.35	28.26	2.17	15.22
100	0.88	47.92	27.08	14.58	10.42
SEM	0.54	-	-	-	-
P value					
$1,25(OH)_2D_3$-G in broiler breeders	0.605	0.672	0.809	0.237	0.972
$1,25(OH)_2D_3$-G in broiler chickens	0.584	0.630	0.980	0.100	0.588
Broiler breeders vs Broiler chickens interaction	0.506	-	-	-	-

Note: $1,25(OH)_2D_3$-G: 1,25-Dihydroxycholecalciferol glycoside; SEM: Standard error of the mean; Broiler breeders vs Broiler chickens interaction: Broiler breeders supplemented with 0.0 and 100 mg/kg of $1,25(OH)_2D_3$-G versus broiler chickens supplemented with 0.0, 50, and 100 mg/kg of $1,25(OH)_2D_3$-G.

Table 9. Jejunum gene expression of 21-day-old broiler chickens fed diets supplemented or not with $1,25(OH)_2D_3$-G from broiler breeders supplemented or not with the same additive

$1,25(OH)_2D_3$-G levels (mg/kg)	Calbindin D28K	Interleukin 10	Interleukin 1β
$1,25(OH)_2D_3$-G in Broiler breeders			
0	0.184^b	1.071	0.560
100	0.293^a	0.923	0.650
$1,25(OH)_2D_3$-G in Broiler chickens			
0	0.214	0.895	0.486
50	0.210	0.960	0.645
100	0.291	1.147	0.688
SEM	0.160	0.55	0.28
P value			
$1,25(OH)_2D_3$-G in Broiler breeders	0.019	0.405	0.372
$1,25(OH)_2D_3$-G in Broiler chickens	0.262	0.613	0.183
Broiler breeders vs Broiler chickens interaction	0.383	0.046	0.022

Note: $1,25(OH)_2D_3$-G: 1,25-Dihydroxycholecalciferol glycoside; SEM: standard error of the mean; Broiler breeders *vs* Broiler chickens interaction: Broiler breeders supplemented with 0.0 and 100 mg/kg of $1,25(OH)_2D_3$-G *versus* Broiler chickens supplemented with 0.0, 50, and 100 mg/kg of $1,25(OH)_2D_3$-G; ^{a,b}: means followed by different lowercase letters in the column differ from each other according to the F-test at 5% significance.

2021; Fatemi *et al*., 2020, 2024, Li *et al*., 2023). As a result, there might be a higher concentration of calcium and phosphorus in the bodies of the broiler breeders (Araujo *et al*., 2019; Setiyaningsih *et al*., 2023). These minerals are essential for the development of bones, both in the broiler breeders and the embryo, as well as for egg mineralization (Barnkob *et al*., 2020; Li *et al*., 2021). During egg formation, minerals are transferred from the broiler breeders to the embryo through the yolk and the shell (Wen *et al*., 2019; Kanaani *et al*., 2022). Calcium, for example, is incorporated into the eggshell, while phosphorus is distributed in the yolk, playing a crucial role in the development of the chicks skeletal system (Li *et al*., 2023; Yusuf *et al*., 2023). When the egg is laid, these nutrients are available to the developing embryo (Fatemi *et al*., 2021, 2022). As the embryo develops, it uses calcium and phosphorus to form its bones, which directly impacts the bone weight at hatch (Shojadoost *et al*., 2021; Li *et al*., 2023).

The reduction in feed conversion ratio observed at 21 and 42 days (Table 3) in broiler chickens from broiler breeders supplemented with 100 mg/kg of $1,25(OH)_2D_3$-G suggests a more efficient use of nutrients (Hsiao *et al*., 2018; Aye-Cho *et al*., 2020). This may be attributed to the modulation of intestinal metabolism, which enhanced the absorption of essential nutrients without increased feed consumption (Wu *et al*., 2022). Similar results were observed in the study by Mathis *et al*. (2016), in which the inclusion of up to 1 g/kg of feed improved the feed conversion ratio, indicating that $1,25(OH)_2D_3$-G may be a viable alternative in broiler diets without compromising feed efficiency (Han *et al*., 2022).

The increase in carcass yield (Table 4) observed at 42 days in broiler chickens from broiler breeders supplemented with 100 mg/kg of $1,25(OH)_2D_3$-G may be related to better nutrient utilization from the early days of life (Fatemi *et al*., 2020). Supplementation with 100 mg/kg of $1,25(OH)_2D_3$-G in the diet of broiler chickens for up to 21 days contributed to the modulation of physiological processes, promoting muscle growth and lean mass deposition (Figure 1), which resulted in increased carcass yield at 42 days of age.

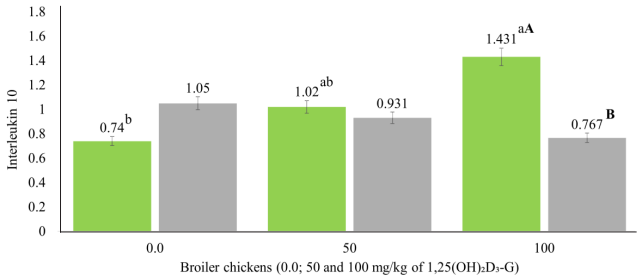

Figure 4. Analysis of the interaction between the supplementation of 1,25-Dihydroxycholecalciferol glycoside ($1,25(OH)_2D_3$-G) in the diets of broiler breeders and broiler chickens and its impact on the gene expression of interleukin 10 in the jejunum of broiler chickens at 21 days of age. [a,b] Means followed by different lowercase letters differ by the Student-Newman-Keuls test at 5%. [a,b] Broiler chickens supplemented with 0.0, 50, and 100 mg/kg of $1,25(OH)_2D_3$-G, from broiler breeders not supplemented with the same additive ($p<0.049$). [A,B] Means followed by uppercase letters differ by the F-test at 5%. [A,B] Comparisons between broiler chickens supplemented with 100 mg/kg of $1,25(OH)_2D_3$-G from non-supplemented and supplemented broiler breeders with the same additive ($p<0.052$). Broiler breeders (0.0 mg/kg of $1,25(OH)_2D_3G$) (■), Broiler breeders (100 mg/kg of $1,25(OH)_2D_3G$) (■).

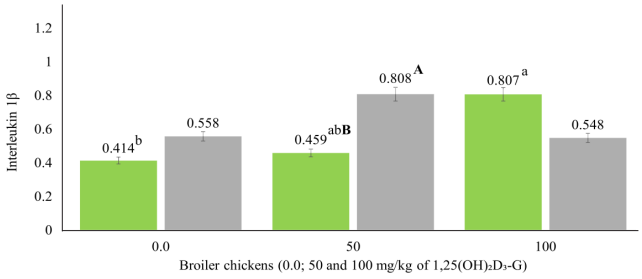

Figure 5. Analysis of the interaction between the supplementation of 1,25-Dihydroxycholecalciferol glycoside ($1,25(OH)_2D_3$-G) in the diets of broiler breeders and broiler chickens and its impact on the gene expression of interleukin 1β in the jejunum of broiler chickens at 42 days of age. [a,b] Means followed by different lowercase letters differ by the Student-Newman-Keuls test at 5%. Broiler chickens supplemented with 0.0, 50, and 100 mg/kg of $1,25(OH)_2D_3$-G, from broiler breeders not supplemented with 100 mg/kg of the additive ($p<0.031$). [A,B] Means followed by uppercase letters differ by the F-test at 5%. [A,B] Comparisons between broiler chickens supplemented with 50 mg/kg of $1,25(OH)_2D_3$-G from non-supplemented and supplemented broiler breeders with the same additive ($p<0.041$). Broiler breeders (0.0 mg/kg of $1,25(OH)_2D_3G$) (■), Broiler breeders (100 mg/kg of $1,25(OH)_2D_3G$) (■).

This is in contrast to other studies that did not observe a significant effect of $1,25(OH)_2D_3$-G supplementation on carcass yield. These studies attributed their results to the proper balance of calcium and phosphorus concentrations in the diet (Vieites *et al*., 2014; Alves *et al*., 2018; Castro *et al*., 2018). However, in the present study, even with normal levels of these minerals, supplementation led to higher carcass yield. This suggests that the observed effect may be related to additional mechanisms of metabolic regulation, possibly involving the modulation of nutrient absorption and utilization (Świątkiewicz *et al*., 2017; Shojadoost *et al*., 2021).

Broiler chickens from broiler breeders supplemented with 100 mg/kg of $1,25(OH)_2D_3$-G exhibited reduced crypt depth and an increased villus-to-crypt ratio in the jejunum at 21 days of age (Table 5). The decrease in crypt depth can be interpreted as an adaptation in the intestinal structure, suggesting a reduced need for cellular renewal. This observation implies greater energy efficiency (Badri *et al*., 2023; Wei *et al*., 2024). It can be hypothesized that it likely reflects the modulation of intestinal stem cell activity by $1,25(OH)_2D_3$-G, leading to a more efficient intestinal architecture with lower energy expenditure for tissue maintenance (Hsiao *et al*., 2018; Shojadoost *et al*., 2021). In addition, the enhanced villus-to-crypt ratio (Table 5) observed at 21 days indicates improved intestinal function. This morphological change, where longer villi and shallower crypts are present, favors efficient nutrient absorption (Nong *et al*., 2023; Gao *et al*., 2024). While no changes in villus height or absorption area were noted, the alteration in the villus-crypt ratio suggests a structural adaptation that optimizes nutrient uptake (Babazadeh *et al*., 2022; Shojadoost *et al*., 2021).

However, it is important to note that no significant improvements were observed in the bone health of broiler chickens at 21 and 42 days of age (Tables 7 and 8). Broiler chickens supplemented with $1,25(OH)_2D_3$-G, even when originating from broiler breeders that received the same supplementation, did not show enhanced bone mineralization. This was evidenced by lower calcium concentration in the tibia at 21 days (Figure 2) and weaker bones at 42 days (Table 6). Similar findings were reported by Vieites *et al*. (2016), who evaluated the inclusion of $1,25(OH)_2D_3$-G in broiler diets at 21 days of age and found no improvements in bone mineralization or strength.

Interestingly, broiler chickens from broiler breeders supplemented with $1,25(OH)_2D_3$-G exhibited higher levels of calbindin D28K in the jejunum at 21 days of age (Table 8), suggesting enhanced intestinal calcium absorption. However, this did not result in greater calcium deposition in the bone, which may have limited mineralization and resulted in reduced fracture resistance of the tibia. One possible explanation for these results is the complex regulation of bone mineralization, which depends not only on mineral availability but also on factors such as bone matrix synthesis, osteoblast and osteoclast activity, and interactions with other essential nutrients (Li *et al*., 2016; Tizziani *et al*., 2019; Lv *et al*., 2022; Han *et al*., 2024).

The higher levels of IL-10 and IL-1β in broiler chickens supplemented with 100 mg/kg of $1,25(OH)_2D_3$-G from non-supplemented broiler breeders (Figures 4 and 5) indicate that the observed immune effect is directly related to continuous supplementation during growth, rather than the nutritional contribution provided by the broiler breeders (Rodriguez-Lecompte *et al*., 2016; Kumar *et al*., 2017; Ismailova & White,

2022). While supplementation of the broiler breeders contributed to bone mineralization in the chicks, it does not appear to have played a significant role in immune modulation (Araujo *et al*., 2019; Setiyaningsih *et al*., 2023).

Direct supplementation of $1,25(OH)_2D_3$-G in broiler chickens during growth influenced immune regulation, particularly through the increase in IL-10 production (Hsiao *et al*., 2018; Nunes *et al*., 2020). This anti-inflammatory cytokine plays a crucial role in modulating the immune system by acting as a regulator of the inflammatory response mediated by IL-1β, a pro-inflammatory cytokine associated with the activation of the innate immune system (Nunes *et al*., 2020; Abascal-Ponciano *et al*., 2022). The increase in IL-10 may have controlled the intensity of the inflammatory response induced by the rise in IL-1β, preventing adverse effects associated with excessive inflammation while maintaining effective immune defense (Nunes *et al*., 2020; Shojadoost *et al*., 2021; Kumar *et al*., 2023).

In broiler chickens from broiler breeders supplemented with $1,25(OH)_2D_3$-G, the baseline immune state may have been less responsive to continuous supplementation during growth due to a lower need for immune compensation (Nunes *et al*., 2020; Chou *et al*., 2021; Wu *et al*., 2022). In contrast, in broiler chickens from non-supplemented broiler breeders the immune system was likely in a less developed initial state, making supplementation during growth crucial to promote the balanced production of cytokines such as IL-10 and IL-1β (Nunes *et al*., 2020; Shojadoost *et al*., 2021; Nong *et al*., 2023).

CONCLUSION

Supplementation with 100 mg/kg of $1,25(OH)_2D_3$-G in the diets of broiler breeders improved the performance of the broiler chickens, resulting in higher birth weight, better feed conversion at 21 and 42 days, and higher carcass yield at 42 days of age. However, the supplementation was not sufficient to improve bone mineralization or tibial break resistance in the broiler chickens. On the other hand, broiler chickens from non-supplemented broiler breeders, when supplemented with 100 mg/kg of $11,25(OH)_2D_3$-G, showed better immune response at 21 days of age.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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