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Effects of *in ovo* injection of $25(OH)D_3$ in combination with Marek's disease vaccine on hatchability, performance and bone health of broilers

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ABSTRACT

1. This study assessed the effects of *in ovo* injection of Marek's disease vaccine (MDV), alone (control) or combined with 1.25 µg of 25-hydroxycholecalciferol ($25(OH)D_3$), on hatchability, chick quality, performance and bone quality in Cobb 500 broilers. At d 19 of embryo development, eggs were injected with 0.05 ml of MDV or MDV plus 1.25 µg of $25(OH)D_3$ into the amniotic fluid following hatchery protocol. Post-hatch, 840, one-day-old male chicks were reared until d 35 of age.
2. Hatchability and general chick quality were not affected by the treatments. However, the male chicks that received MDV + 1.25 µg of $25(OH)D_3$ presented longer length at flock thinning compared to those that received only MDV. At 35 d of age, broiler performance, gait score and leg deformities (valgus and varus) were not affected.
3. Although chicks receiving the MDV + 1.25 µg of $25(OH)D_3$ had lower macroscopic tibial dyschondroplasia (TD) scores at 7 d of age compared to those given MDV, this difference was not sustained as they matured. Tibia weight, thickness and length were similar across treatments at all time points. However, the tibiae from chicks in the MDV + 1.25 µg $25(OH)D_3$ group demonstrated greater strength on d 1 and 7 but not at later times.
4. In conclusion, this study showed that combining $25(OH)D_3$ with MDV does not impact hatchability or overall performance but does enhance early bone strength and growth in broiler chicks.

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25-hydroxycholecalciferol; bone; leg deformities; vitamin D3; hatchability

Introduction

Bone quality is crucial in the livestock production of broiler meat, impacting poultry health and economic outcomes. Optimal bone strength enables broilers to support rapid growth while avoiding fractures and deformities, which can lead to significant economic losses (Lopes et al. 2024). Maintaining bone health through strategic nutrition and management becomes essential for sustainable livestock practices and ensuring high-quality meat production (Trela et al. 2024).

Given the critical role of bone quality in the livestock production of broiler meat, strategic interventions are necessary to enhance skeletal health and prevent economic losses from skeletal issues. One such intervention is *in ovo* injection of vitamin D_3 and its metabolites, including 25-hydroxyvitamin D_3 (25(OH)D3), known as 25-hydroxycholecalciferol and 1,25-dihydroxyvitamin D_3, both of which have been extensively researched in chicks. These studies studied bone development and overall animal performance, but the results were ambiguous (Gonzales et al. 2013; Bello et al. 2013, 2014b, 2014a, 2014c, 2015; Fatemi et al. 2020a, 2020b, 2021a, 2021b; Quadros et al. 2021; Alizadeh et al. 2022; Xu et al. 2023; Fatemi et al. 2024a, 2024b). Based on these results, the most effective *in ovo* form was vitamin D_3 is $25(OH)D_3$, with effective doses ranging from 0.6 to 2.4 µg. However, despite the large number of studies, histological evaluations, particularly related to TD, have been minimally investigated. Furthermore, pre-slaughter assessments of leg problems in birds are lacking, despite their relevance for understanding the practical implications and overall efficacy of *in ovo* vitamin D_3 administration.

The primary benefits of using $25(OH)D_3$ include enhanced embryonic skeletal development, hatchability, hatchling quality, reduced late embryonic mortality, higher yolk dry matter percentages, improved bone formation and mineralisation, increased meat yield and better early performance in post-hatch broilers. This includes improvements in humoral immunity, small intestine histomorphology and upregulated expression of genes associated with vitamin D_3 activity (Gonzales et al. 2013; Bello et al. 2013, 2014b; Fatemi et al. 2020a, 2020b; 2021a, 2021b, 2021c; Xu et al. 2023).

As indicated, vitamin D_3 plays an important role in bone formation and mineralisation and consequently, may play a role in the prevention of leg problems. Leg problems in broiler production are a significant concern, particularly in fast-growing strains (Lopes et al. 2023, 2024). The rapid growth and increased body weight of modern broilers, driven by intensive genetic selection, have led to a higher incidence of skeletal disorders (Çapar Akyüz and Onbaşilar 2020). These disorders not only affect the health and welfare of the broilers and result in decreased meat quality, reduced growth performance and increased mortality (Martins et al. 2020). Mitigating the prevalence of leg disorders and improving overall broiler bone health may be possible with *in ovo* injection of $25(OH)D_3$, which has been shown to

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enhance bone formation and mineralisation (Bello, Hester, et al. 2014b; Fatemi et al. 2020a, 2020b; 2021a, 2021b, 2021c, 2022a).

To optimise the use of *in ovo* injection, it is important to consider the routine practices of hatcheries. The most practical approach would be to combine this with vaccines that are already routinely administered *in ovo* at approximately d 17.5 to 19 of incubation. This combined administration aligns with standard hatchery protocols, ensuring efficiency and integration into existing workflows (Araújo and Lara 2023). However, the combined injection of nutritional substances with vaccines routinely administered in hatcheries worldwide has been relatively unexplored. Therefore, the following study investigated the effects of *in ovo* injection of 1.25 μg 25(OH)D_3 combined with MDV at d 19 of incubation on hatchability, chick quality, performance at 35 d of age and bone quality in Cobb 500 broilers.

Materials and methods

Ethical statement

The current experiment was approved by the Ethical Principles in Animal Experimentation Committee (Protocol number: 014/12) of the Federal University of Goiás. The chicks were reared in an experimental farm, located in Goiânia, Goiás, Brazil (16°35'48.3'S and 49° 17'08.8' W).

Experimental design, treatments and management

A total of 5,160 eggs from a 45-week-old commercial Cobb 500 broiler breeder flock were transported to a hatchery located in Itaberaí, Goiás, Brazil. Eggs were stored for 48 h at 20°C and 65% RH. Before the incubation process, the eggs were gradually warmed at a controlled rate of approximately 1°C per hour, allowing the internal egg temperature to rise steadily until it reached 25°C. This warming process took place over 5 h in a preheating room where the trolleys containing the egg trays were placed. Then, the eggs were randomly distributed across 60 trays with 86 eggs per tray. All trays were placed in one incubation trolley. The trolley with eggs was placed in a CASP ug 62 HT single-stage setter, which has a total capacity of 61 920 eggs and 12 trolleys with 60 trays in each one. The setter was further filled with eggs that were no made part of this experiment. The setter was programmed to maintain an incubation temperature of 37.5°C and a RH of 58.0%. Egg turning (45°) was performed once every hour till d 19 of incubation (ED19).

At ED19, all eggs were candled and 2,752 eggs containing a life embryo were selected. These eggs were distributed over 32 trays with 86 eggs per tray. Half of these eggs (1,376 eggs) were *in ovo* injected in the amniotic fluid with 0.05 ml of the commercial Marek's Disease vaccine (Merial Co., Athens, GA; control group), using a Inovoject System (Embrex Inovoject, Pfizer Poultry Health, U.S.A.) following the hatchery procedures. The other half of these eggs (1,376 eggs) were injected with the same vaccine added with 1.25 μg of 25(OH)D_3 (Rovimix Hy-D 1.25%; DSM Nutritional Products Inc., Parsippany, NJ), using the same *in ovo* system (MDV +1.25 μg 25(OH)D_3). An important highlight was the compatibility of the vaccine with 25(OH)D_3, as tested by Fatemi et al. (2023), which had no adverse effects on application. Consequently, this study employed a combined use of these components for the technical applicability of the *in ovo* injection. The 25(OH)D_3 solution was prepared according to the methods described by Gonzales et al. (2013). This was mixed into 400 ml of a commercial MDV vaccine diluent (Merial Co., Athens, GA), achieving a concentration of 1.25 μg of 25(OH)D_3 per 0.05 ml. Eggs from each treatment were transferred to one of 16 hatching baskets (86 eggs per basket) placed in one hatcher (CASP 108 HR) which were set at a temperature of 36.5°C and a RH of 65.0%. The trial did not include a group without any *in ovo* treatment since the specific goal of this study was to determine whether *in ovo* injection of 25(OH)D_3 may be given concurrently with *in ovo* immunisation and studies to only administer 25(OH)D_3 are conducted more frequently.

Hatchability, hatch window and residual analysis

The hatcher was opened every 4 h during the period between 480 and 516 h of incubation to count the number of chicks that had hatched, allowing the determination of emergence pattern. Hatchability was calculated as the number of chicks divided by the total number of fertile eggs per basket. The hatch window was calculated in hours per basket as the timeframe between the first and last hatching chicken. The unhatched eggs were submitted to residual analysis, as described by Araújo et al. (2016), considering non-pipped eggs, pipped alive embryo, pipped dead embryos and contaminated eggs. Additionally, the number of non-saleable (second grade) chicks was counted, culled chicks which presented splayed legs, unhealed navels and malformations (Molenaar et al. 2008; Tona et al. 2003). For all these variables, the basket was considered as the experimental unit.

Chick physical quality

All hatched chicks were feather-sexed, and a randomised selection of 10 males and 10 females per basket was used for quality evaluation. The selected chicks were assessed for weight at flock thinning, length and overall quality score. Each chick was individually weighed and measured for length, using the method described by Wolanski et al. (2006). The overall quality score of the chicks was evaluated, using the criteria established by Tona et al. (2003).

Broiler chick performance

A total of 840 one-d-old male chicks from the incubation experiment (420 of the MDV +1.25 μg 25(OH)D_3 injection treatment or 420 of the MDV injection only treatment) were randomly selected, weighed and placed in the broiler house. Twenty-four floor pens (experimental units), each measuring 5.25 m^2 and housing 35 chicks, were arranged in two rows of 12 pens, separated by a central 1-m-wide corridor. In both rows, treatments were alternatively assigned to pens. All pens were equipped with two tubular feeders and one pendulum drinker, with wood shavings as litter. Water and feed were provided ad libitum throughout the rearing period (d 1 to 35), under a lighting program of 20 L:4D.

Broilers from both experimental groups were fed identical diets throughout the study period. A two-phase corn and soybean-based diet was formulated to meet the nutritional requirements of Cobb500 (2010). The starter diet (days

1–21) provided 12.9 MJ/kg apparent metabolisable energy, 217.5 g/kg crude protein, 11.7 g/kg digestible lysine and 6.0 g/kg digestible methionine. The grower diet (days 22–35) contained 13.5 MJ/kg apparent metabolisable energy, 200.0 g/kg crude protein, 10.8 g/kg digestible lysine and 5.5 g/kg digestible methionine. Rearing temperature started at 33.0°C and then it was decreased weekly until 20°C (Cobb-Vantress, 2021), Throughout the experimental period, the average temperature in the broiler house was 27.5°C ±4.2°C. Body weight (BW), feed intake and mortality were determined at weekly basis. Feed conversion ratio (FCR; feed intake/BW gain) was calculated for each week and over the total trial period after correction for mortality.

Bone health

At 35 d of rearing, chicks were tested for walking ability. Birds were placed on a wooden platform fixed in the floor and encouraged to walk using hand claps and their gait score was scored, using the scale of Garner et al. (2002): Score 0 indicated normal gait; Score 1 slight and undefined defect; Score 2 unilateral defect or asymmetrical legs; Score 3 was 5 to 10 steps and sat down when touched; Score 4, was 1 or 2 steps; Score 5, the chickens could no longer stand up.

After gait scoring, each leg was scored for *valgus-varus*, using the method described by Leterrier and Nys (1992). Evaluation was performed with the femoro-tibiotarsal joint fixing. Legs were classified as: normal without deformity); *valgus* deformity when the angulation showed a lateral (outward) deviation or *varus* deformity when the angulation showed a medial (inward) deviation.

Broiler chick bone analysis

On d 7, 14, 21, 28 and 35 of age, one chick per pen (12 chicks per treatment per day) was euthanised by cervical dislocation to remove the left and right tibiae. The feathers, skin, soft and connective tissue was manually removed from all tibiae using surgical tweezers and scissors (Castro et al. 2018). The sampled bones were put in coded bags and send to the laboratory of Animal Nutrition of Federal University of Goiás.

Half of the left tibiae (6/treatment/day) were dried at 550°C for 72 h, after which their dry weight, length and mid-shaft thickness were measured using an analytical scale and calliper (Universal analogic calliper, Cat. No. 539–104, Mitutoyo, Tokyo, Japan). After this, biophysical tests were performed to evaluate bone strength. This variable was assessed using a 3-point bending test with a support span of 70 mm, a 500 kg load cell and a static testing speed of 2 mm/min until break bone. The tests were conducted using a universal testing machine (Instron, model 3367, Grove City, U.S.A.).

Half of the right tibiae (6/treatment/day evaluated) were used to access macroscopic TD score. The TD occurrences were determined *via* a longitudinal cut across the right tibia according to the method described by Edwards and Veltmann (1983). The confirmation of TD was characterised by abnormal white, opaque, unmineralised and unvascularised cartilage at the proximal end of the tibia. Longitudinal cuts of the tibiae were made and the lesions were measured for thickness using a digital calliper, as described by Almeida Paz et al. (2005). Bone lesions were scored from 1 (mild) to 3 (severe) depending on the cartilage plug abnormality size. The tibiae were scored according to the white cartilage plug abnormality (0 = normal; 1 = mild; 2 = intermediate; and 3 = severe) according to Thorp et al. (1997).

The other half of right tibiae were used for histopathological analysis (one slide per tibia), using the methods described by Almeida Paz et al. (2005) and Lopes et al. (2024). The slides were evaluated by optical microscopy and photographed with a Spot Colour Insight digital camera coupled to a light microscope (BX-40, Olympus, Tokyo, Japan) and images were captured, using the Image Pro Plus® version 4.6 program. Slides were histologically characterised by the severity of TD and the growth plate was divided into proliferative zone, pre-hypertrophic zone and hypertrophic zone and scored from 0 to 3 according to Almeida Paz et al. (2005).

The other half of left tibiae from d 14, 21, 28 and 35 of age were used for radiographic and densitometric analysis. Tibiae were X-rayed alongside a 12-step aluminium scale (alloy 6063, ABTN) with varying thicknesses used as a densitometric standard. The radiographic plate was digitised, using an HP Scanjet 4C scanner with a transparency adapter. Using the software CROMOX1®, the middle third of the tibiae was selected for optical density analysis, expressed in greyscale, which was then converted into equivalent mm of aluminium. Subsequently, with the digitised image, the length, average thickness, distal epiphysis area and total area of each tibia were analysed using software (Image-Pro Plus, Media Cybernetics, version 4.1) calibrated for bone mineral density. Bone mineral density was calculated on the diaphysis, using a digital scanner (Scanion A3). Values were expressed as mm of aluminium (mmAl).

Statistical analysis

Data were analysed using the following fixed effect model:

$$Y_{ij} = \mu + T_i + e_{ij},$$

where Y was the response variable, μ the overall mean, T_i the fixed effect of treatment (i = MDV +1.25 μg 25(OH)D_3 or MDV) and e the residual error.

The data was submitted to tests of normality and homoscedasticity was verified by the Shapiro–Wilk test at $p \leq 0.05$. The LS means ± pooled SEM were then subjected to analysis of variance (ANOVA), using R Software (R, 2010). Data that deviated from normality were evaluated using non-parametric statistics and analysed using the Mann–Whitney test at $p \leq 0.05$.

Results

Treatments did not affect hatch results, physical chick quality (Table 1) or hatching pattern (Figure 1).

However, the length of male chicks at flock thinning was significantly greater ($p < 0.05$) in the 25(OH)D_3 treatment compared to control treatment (17.5 *vs*. 17.1 cm, respectively). Treatment did not affect broiler chick performance at 35 d of age (Table 2) or the gait score and prevalence of leg deformities (Table 3).

Table 1. Effect of *in ovo* injection of 25(OH)D_3 in combination with Marek's disease vaccine at day 19 of incubation on hatch results and old-chick quality at flock thinning.

	25(OH)D_3			
Hatch results*	0.00 µg	1.25 µg	SEM	*P-value*
Hatchability, %	86.2	86.8	1.77	0.344
Culled chicks[1], %	3.19	2.92	0.96	0.721
Non-pipped eggs, %	3.90	3.91	0.22	0.981
Pipped alive embryos, %	3.98	2.98	0.78	0.780
Pipped dead embryos, %	2.87	2.80	0.88	0.811
Contaminated eggs, %	0.56	0.56	0.05	1.000
Hatch window, h	29.5	31.5	2.50	0.561
Chick quality[#]				
Male chick weight, g	45.0	45.1	1.33	0.132
Female chick weight, g	46.3	44.8	2.05	0.222
Male chick length, cm	17.0^{b}	17.4^{a}	1.09	0.021
Female chick length, cm	17.1	17.1	1.22	0.507
Male quality chick[2]	92.9	95.1	4.51	0.334
Female quality chick[2]	92.6	92.1	3.91	0.585

Note: [1]Culled chicks were defined as chicks with splayed legs, unhealed navels, malformations alive (Molenaar et al. 2008; Tona et al. 2003). [2]According to Tona et al. (2003). [a-b] LSMeans within a row lacking a common superscript differ ($p \le 0.05$). SEM: standard error of the mean. *$n = 16$ per treatment; [#]$n = 10$ per treatment.

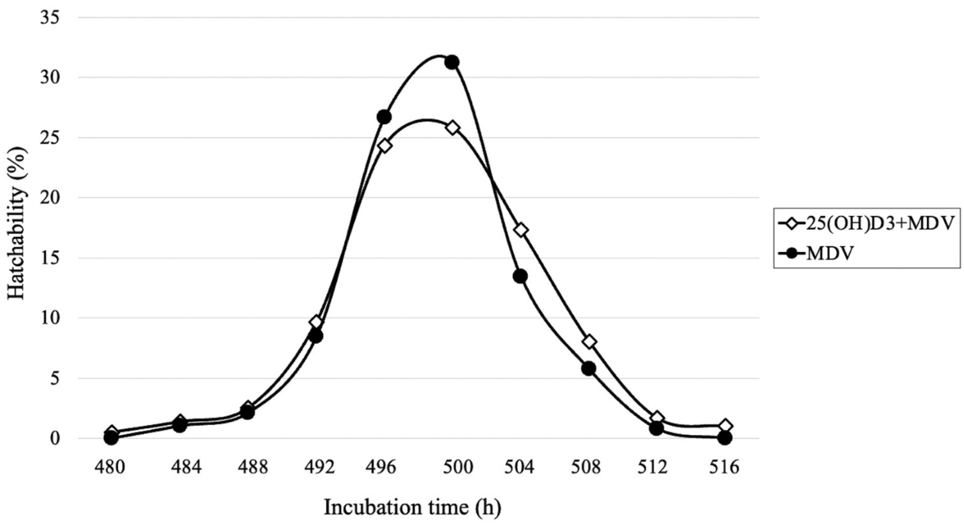

Figure 1. Hatching pattern of embryos from eggs injected with Marek's disease vaccine (MDV) at day 19 d of incubation in combination with or without 1.25 µg 25(OH)D_3. Treatments did not significantly affect the hatching pattern (P > 0.05).

Table 2. Effect of *in ovo* injection of 25(OH)D_3 in combination with Marek's disease vaccine at day 19 of incubation on performance from 1 to 35 days of age.

	25(OH)D_3			
Performance	0.00 µg	1.25 µg	SEM	*P-value*
Initial weight, g	44.1	44.3	0.56	0.265
Weight final day 35, g	2,396	2,359	89.5	0.754
Total feed intake, g	3,507	3,501	80.4	0.811
Overall feed conversion ratio, g:g	1.49	1.51	0.35	0.380
Overall mortality, %*	6.14	5.19	1.22	0.341

Note: *Mann-Whitney test ($p \le 0.05$). SEM: Standard Error of the Mean. $n = 12$.

Table 3. Effect of *in ovo* injection of 25(OH)D_3 in combination with Marek's disease vaccine at day 19 of incubation on gait score and *valgus-varus* on 35 days of age.

	25(OH)D_3		SEM	*P-value*
Legs deformities	0.00 µg	1.25 µg		
Gait score[1]	2.00	1.50	0.90	0.179
Normal legs, %	58.7	50.9	5.89	0.569
Valgus legs deformities, %	38.4	38.1	3.30	0.795
Varus legs deformities, %	6.81	10.9	4.95	0.091

Note: [1]Score 0 indicates a normal chick; Score 1, shows a slight and undefined defect; Score 2, shows a unilateral defect or asymmetrical legs; Score 3, chick can take 5 to 10 steps and sits down when touched; Score 4, chick can take 1 or 2 steps; Score 5, chick can no longer stand up according to Garner et al. (2002). SEM: Standard Error of the Mean. $n = 788$ and 796 birds respectively.

Table 4. Effect of *in ovo* injection of 25(OH)D3 in combination with Marek's disease vaccine at day 19 of incubation on macroscopic and microscopic analysis of growth plate lesion thickening in the proximal portion of the tibia at 7, 14, 21, 28 and 35 days of age.

	$25(OH)D_3$		SEM	*P-value*
Lesion thickening (mm)	0.00 µg	1.25 µg		
7 days	4.70[b]	2.76[a]	0.21	0.017
14 days	3.09	2.78	0.44	0.761
21 days	4.13	5.58	0.96	0.448
28 days	4.03	4.05	0.35	0.981
35 days	5.86	3.85	1.21	0.274
	$25(OH)D_3$		SEM	*P-value*
Macroscopic lesion score[1,*]	0.00 µg	1.25 µg		
7 days	2.09[b]	0.66[a]	0.25	0.015
14 days	1.08	0.75	0.55	0.463
21 days	1.08	1.66	0.46	0.371
28 days	1.58	1.33	0.70	0.757
35 days	1.33	0.75	0.55	0.409
	$25(OH)D_3$		SEM	*P-value*
Microscopic lesion score[2,*]	0.00 µg	1.25 µg		
7 days	0.00	0.00	–	–
14 days	0.50	0.27	0.55	0.609
21 days	0.67	0.67	0.90	0.945
28 days	1.00	0.50	0.55	0.323
35 days	1.25	0.83	0.68	0.522

Note: [a-b] LSMeans within a row lacking a common superscript differ according ($p \le 0.05$). [1]The tibiae were scored according to the white cartilage plug abnormality (0 = normal; 1 = mild; 2 = intermediate; and 3 = severe) according to Thorp et al. (1997). [2]Scored from 0 to 3 according to Almeida Paz et al. (2005). *Mann–Whitney test ($p \le 0.05$). SEM: Standard Error of the Mean. $n = 6$.

Chicks from the MDV +1.25 µg $25(OH)D_3$ treatment showed decreased lesion thickness and macroscopic TD score at 7 d of age compared to the control treatment ($p < 0.05$). However, this difference did not continue in subsequent weeks. Furthermore, the microscopic TD analysis was unaffected by the treatments (Table 4).

Treatment did not affect tibia weight, thickness, or length at 7, 14, 21, 28 and 35 d of age (Table 5). However, the tibiae from chicks in the 1.25 µg $25(OH)D_3$ group showed greater strength on d 1 and 7 of evaluation ($p < 0.05$).

No differences were observed in tibial strength between treatments on the other days evaluated. Additionally, the treatments did not affect the mineral bone density of the tibiae in any of the weeks evaluated (Table 6).

Discussion

No significant consistent differences were seen for gait scores or macroscopic bone lesion analysis over time. However, the significant reduction in microscopic TD at 7 d of age in chicks treated with $25(OH)D_3$ suggested a potential early benefit on bone health. *In ovo* injection of MDV +1.25 µg $25(OH)D_3$ did not affect hatchability, the percentage of culled chicks, unhatched eggs or the hatch window. These findings were consistent with previous studies (Fatemi et al. 2020a; Fatemi et al. 2024a; Gonzales et al. 2013; Quadros et al. 2021), who observed no effect on hatchability after *in ovo* injection with various doses of $25(OH)D_3$, although these studies did not include MDV vaccine. In contrast, Bello et al. (2013)reported higher hatchability (93%) in broiler chick injected with 0.6 µg of $25(OH)D_3$ at ED18 compared to controls injected with MDV vaccine diluent (86%). Additionally, Xu et al. (2023) reported higher hatchability (96 and 98%), after *in ovo* injection of 0.8 and 1.6 µg $25(OH)D_3$ diluted with sterile water at ED17.5. These authors used a 0.1 ml injection volume, while the current study employed a smaller volume of 0.05 ml containing 1.25 µg of $25(OH)D_3$ and MDV vaccine. However, variations in the timing of the injection, diluent, volume administered and dosage may have been responsible for the differences in hatchability outcomes between these studies.

Bone and cartilage growth, as well as cell differentiation-related physiological processes, have been shown to be accelerated by $25(OH)D_3$ (Bello et al. 2013; Fatemi et al. 2024a), which can impact embryonic development. This has been demonstrated by the activation of genes linked to embryonic development following an *in ovo* vitamin D3 injection (Fatemi et al. 2022b). Additionally, the higher initial body weight in broiler chicks has been linked to enhanced 1α-hydroxylase expression (Fatemi et al. 2024a).

The lack of effect of an *in ovo* injection of 1.25 µg of $25(OH)D_3$ into amniotic fluid at ED19 on newly hatched chicks performance indicated limited influence. Male chicks that received 1.25 µg of $25(OH)D_3$ were longer at flock thinning, which might affect the final broiler performance (Molenaar et al. 2008). This was not found in the current study at 35 d of age, which agreed with Fatemi, Alqhtani, et al. (2021a), but conflicted with the results from Xu et al. (2023). These authors used *in ovo* injections of 0.8 and 1.6 µg of $25(OH)D_3$ diluted in sterilised water and reported higher body weight of broilers from 1 to 28 d of age compared to non-injected embryos and those injected with only sterilised water. Similarly, Gonzales et al. (2013) reported higher body weight at 10 d of age in broiler chicks injected with 1.2 µg of $25(OH)D_3$. Fatemi, Alqhtani, et al. (2021a) studied the effects of *in ovo* injection of 2.4 µg of $25(OH)D_3$ on broiler performance at 40 d and found no effect compared to broilers injected with MDV vaccine diluent. Based on the results of the current study and literature, it appeared that *in ovo* injection of $25(OH)D_3$ for broiler embryos may affect body

Table 5. Effect of *in ovo* injection of 25(OH)D_3 in combination with Marek's disease vaccine at day 19 of incubation on broiler tibial characteristics at 1, 7, 14, 21, 28 and 35 days of age.

25(OH)D_3	Tibia weight (g)	Bone strength (kgf)	Tibia length (mm)	Tibia thickness (mm)
1 day				
0.00 µg	0.08	12.9^{b}	26.4	1.72
1.25 µg	0.09	14.6^{a}	26.1	1.71
SEM	0.05	0.70	1.35	0.88
P-value	0.863	0.021	0.375	0.750
7 days				
0.00 µg	0.31	64.8^{b}	37.1	2.73
1.25 µg	0.32	78.4^{a}	36.7	2.78
SEM	0.23	5.36	4.54	0.91
P-value	0.952	0.011	0.605	0.559
14 days				
0.00 µg	1.17	194	47.4	4.34
1.25 µg	1.22	232	48.8	4.42
SEM	0.60	28.03	1.73	1.06
P-value	0.434	0.135	0.145	0.617
21 days				
0.00 µg	2.51	259	59.9	5.72
1.25 µg	2.55	254	61.0	5.92
SEM	0.87	25.29	2.82	1.52
P-value	0.688	0.841	0.111	0.395
28 days				
0.00 µg	4.26	225	72.4	6.86
1.25 µg	4.36	275	72.9	7.18
SEM	1.10	48.94	3.79	2.16
P-value	0.703	0.335	0.689	0.281
35 days				
0.00 µg	6.13	273	84.2	8.55
1.25 µg	6.49	276	84.5	7.81
SEM	2.51	28.15	3.30	2.32
P-value	0.501	0.882	0.873	0.091

Note: $^{a\text{-}b}$ LSMeans within a row lacking a common superscript differ ($p \leq 0.05$). SEM: Standard Error of the Mean. $n = 6$.

Table 6. Effect of *in ovo injection* of 25(OH)D_3 in combination with Marek's disease vaccine at day 19 of incubation on tibia bone mineral density of post-hatch chicks at 14, 21, 28 and 35 days of age.

	25(OH)D_3		SEM	*P-value*
Mineral bone density (mmAl)	0.00 µg	1.25 µg		
14 days	1.32	1.33	0.46	0.855
21 days	1.78	1.83	0.40	0.539
28 days	1.86	1.81	0.55	0.721
35 days	1.91	1.84	0.61	0.428

Note: SEM: Standard Error of the Mean. $n = 6$.

weight development only during the early stages of broiler development.

In the current investigation, broiler bone indices were not significantly impacted long term by MDV +1.25 µg 25(OH)D_3. However, there were early life-stage effects on the prevalence of TD and tibial strength. The gait score for broilers in the MDV +1.25 µg 25(OH)D_3 and control treatments was 2.0 and 1.5 at 35 days of age, respectively, both falling below the threshold of 3.0 required by the Bristol Gait Score, which is an indicator of acceptable locomotory function (Martins et al. 2020). This result aligned with previous findings whereby a significant proportion of broilers exhibited gait abnormalities even in the absence of severe bone pathologies (Oviedo-Rondón et al. 2008).

A higher variation in *valgus-varus* deformities was reported by Van Den Brand et al. (2022). This variation was influenced by the method used for determination. In the current study, *valgus* deformities observed at 35 d were 56.4% in the control and 48.1% in the MDV +1.25 µg 25(OH)D_3 treatment. *Valgus* deformities have been frequently reported in broilers, as documented by Leterrier and Nys (1992), Shim et al. (2012), González-Cerón et al. (2015) and Martins et al. (2020). These have been attributed to mechanical stress on rapidly growing bones, a condition exacerbated by genetic selection for faster growth rates.

One of the main conclusions of this study was that, at 7 d of age, the MDV +1.25 µg 25(OH)D_3 injection reduced macroscopic lesions related to TD; however, this effect did not last at later ages. Early interventions using 25(OH)D_3 appear to positively influence early bone development. This was consistent with the findings of Xu et al. (2023), who reported higher tibial weight, strength and mineral content in response to 25(OH)D_3 *in ovo* injection. Yair et al. (2013, 2015) demonstrated that *in ovo* injection with vitamin D_3 affected structural and mechanical properties of both tibia and femur bones, influencing skeletal development in young chicks.

Despite these early improvements in bone strength, no differences in mineral density were observed in the current study, which was in agreement with Bello, Bricka, et al. (2014a). However, these authors observed higher bone strength in male broilers at 28 d post-hatch after *in ovo* injection of 25(OH)D_3. In the current study, the lack of a sustained effect on BMD suggested that, while mechanical properties of bone, such as strength, may improve with *in ovo* vitamin D_3

injections, these effects were not necessarily reflected in BMD measurements.

Additionally, there were no significant differences in tibial weight, thickness, or length at 7, 14, 21, 28, or 35 d of age. This finding aligned with the results from Quadros et al. (2021), who studied various doses of *in ovo* injection of 25(OH)D_3 and found no significant effects for bone characteristics. These results suggested that MDV +1.25 µg 25(OH)D_3, like the doses tested by Quadros et al. (2021), may not be sufficient to induce significant changes in bone health, even though transient improvements in bone strength have been observed in younger birds.

The *in ovo* injection of 25(OH)D_3 boosted tibial strength at various time periods, especially at 14 and 28 d of age (Xu et al. 2023). Higher concentrations of 25(OH)D_3 increased tibial ash, calcium and phosphorus. In contrast, lower concentrations of 25(OH)D_3 resulted in diminished mineral content. This result was partially consistent with the current findings, where there were early improvements in tibial strength, but not mineral density. It is possible that the current dosage used was insufficient to sustain long-term benefits, as other studies have reported responses to 25 (OH)D_3 supplementation (Quadros et al. 2021).

The complex interaction between immune activation and bone metabolism has been reported. For instance, in poultry, vitamin D plays a key role in bone health and in modulating immune function (Shojadoost et al. 2021; Xu et al. 2023). The dual role of vitamin D could mean that its skeletal benefits are partially negated by its involvement in the immune response triggered by the MDV vaccine. Moreover, the inclusion of MDV may have further attenuated the potential benefits of 25(OH)D_3 on bone health by triggering an immune response that diverted resources away from skeletal development (Fatemi et al. 2024a, 2024b). Addressing skeletal health in broilers requires a multifactorial approach that considers the timing and dosage of vitamin D_3 supplementation, as well as potential interactions with vaccinations, genetic selection and management practices, to support sustained improvements in bone integrity.

Conclusions

Injecting 1.25 µg of 25(OH)D_3 combined with MDV *in ovo* at ED19 may enhance early tibial strength and reduce the incidence of TD in Cobb 500 broilers. Importantly, this treatment does not affect hatchability or overall broiler performance at 35 d of age.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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