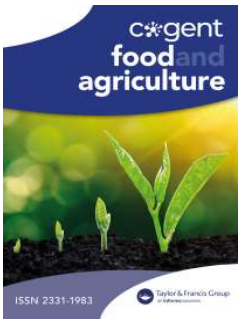

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25-Hydroxyvitamin D3 supplementation in broiler chickens: Integrating nutritional, physiological, and management factors under controlled environmental conditions

Ahmed A. Saleh[a], Whad Fayed[b], Fuad Saleh[b], Abdul Rahman A. Alkhamisi[a], Khairy Amber[a], Neamat Badwi[a], Ahmed El Hadi[c], Mahmoud M. Aboud[a] and Mohammed A. Kamal[d]

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ABSTRACT

This experiment evaluated the effects of dietary 25-hydroxyvitamin D3 (25-OH-D3) supplementation in combination with vitamin D3 on broilers from 1 to 32 days of age. Four hundred one-day-old male broiler chicks were randomly allocated to four treatment groups with ten replicates of ten birds each ($n = 10$). Treatment groups included: (1) control containing vitamin D3 at 5000 IU/kg feed (125 mcg/kg feed); (2) diet with 2500 IU/kg (62.5 mcg/kg feed) vitamin D3 plus 2500 IU/kg (62.5 mcg/kg feed) 25-hydroxycholecalciferol; (3) control diet supplemented with 1250 IU/kg(31.25 mcg/kg feed) 25-OH-D3; and (4) control diet supplemented with 2500 IU/kg (62.5 mcg/kg feed) 25-OH-D3. Results demonstrated that supplementation with 25-OH-D3 (1250 or 2500 IU/kg) (31.25 or 62.5 mcg/kg feed) combined with vitamin D3 (5000 IU/kg) (125 mcg/kg feed); significantly improved body weight and feed conversion efficiency compared to the control group. Enhanced digestibility of protein, calcium, and phosphorus was also observed. Blood concentrations of calcium, phosphorus, vitamin D, Newcastle Disease (ND) antibody titers, and influenza virus (H9N1) titers were significantly elevated ($p < 0.05$). Hepatic malondialdehyde concentrations decreased while glutathione peroxidase activity increased ($p < 0.05$) with 25-OH-D3 supplementation. Tibial ash, calcium, and phosphorus content were substantially enhanced in supplemented birds. Gene expression analysis revealed increased expression of occludin and CaBP-D28k tight junction proteins ($p < 0.05$) in the jejunum and elevated interleukin-17 cytokine (IL-17) expression in the duodenum, while IL-10 expression was reduced in all treatment groups compared to controls. These findings indicate that 25-OH-D3 supplementation with vitamin D3 enhances broiler performance, antioxidant capacity, immune function, nutrient digestibility, bone mineralization, and calcium metabolism-related gene expression.

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Introduction

The global poultry industry has experienced remarkable expansion over recent decades, driven primarily by increasing worldwide demand and elevated consumption patterns (Erdaw & Beyene, 2022). Consequently, poultry nutritionists have intensified their research efforts on feed ingredients to ensure optimal growth performance in broiler chickens. Achieving ideal vitamin concentrations in poultry diets enables birds to realize their maximum genetic potential (Adhikari et al., 2020).

Vitamin D3 (cholecalciferol) represents the inactive precursor of vitamin D that can be synthesized endogenously in animal skin following ultraviolet light exposure. The conversion of vitamin D3 to its biologically active form requires a two-step hydroxylation process involving two critical enzymes: 25-hydroxylase and 1α-hydroxylase. Initial hydroxylation occurs in the liver via 25-hydroxylase, which adds a hydroxyl group

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at the 25-carbon position to form 25-hydroxycholecalciferol (Norman, 2008). Vitamin D3 sources enhance calcium absorption through increased calbindin activity (Bikle & Munson, 1985).

Recent advances have established 25-hydroxycholecalciferol (25-OH-D3) as increasingly popular due to commercial availability and superior bioavailability. Unlike vitamin D3, dietary 25-OH-D3 bypasses the hepatic 25-hydroxylation step due to its existing hydroxyl group, making it immediately available for biological utilization (Zhang & Piao, 2021). Supplementing with 25-hydroxycholecalciferol rather than cholecalciferol increases local active vitamin D availability (Morris et al., 2014). Research has demonstrated that 25-hydroxycholecalciferol exhibits greater potency than cholecalciferol, as evidenced by increased tibial ash content in broiler chickens (Boris et al., 1977).

Both vitamin D3 and its metabolite 25-OH-D3 influence small intestinal morphology and humoral and inflammatory responses in broiler chickens (Chou et al., 2009; Morris et al., 2015). These compounds function as multifaceted nutrients involved in calcium and phosphorus absorption (Bar et al., 1980), bone metabolism and mineralization (Fritts & Waldroup, 2003), muscle development (Vignale et al., 2015), immune function (Chou et al., 2009; Morris et al., 2015; Morris & Selvaraj, 2014), and intestinal morphology (Chou et al., 2009; Ding et al., 2011).

Previous studies have shown that 25-hydroxycholecalciferol supplementation improves body weight gain and reduces tibial dyschondroplasia incidence compared to cholecalciferol alone (Atencio et al., 2005). Recent research by Liu et al. (2023) has reported that broiler chickens receiving various concentrations of 25-OH-D3 (3.125, 6.25, 12.5, 25, and 50 µg/kg) at 21 days of age demonstrated improved body weight gain, feed intake, feed conversion ratio, femur development, and intestinal calcium transporter gene expression compared to controls. Prokoski et al. (2021) found that birds supplemented with 25-OH-D3 (2760 IU or 69 mcg) combined with 3000 IU vitamin D3/kg showed significantly increased ($p < 0.05$) plasma 25-OH-D3 concentrations compared to non-supplemented birds. Moreover, Bassi et al. (2024) reported that 25-hydroxycholecalciferol (25-OH-D3) supplementation improves digestibility in broilers, particularly for calcium (Ca) and phosphorus (P). It leads to higher feed intake, greater body weight gain, and a lower feed conversion ratio (FCR). The bio activated form of vitamin D3, 25-OH-D3, is more efficiently absorbed and utilized by the birds, resulting in better overall performance and mineral absorption. Also, it can improve liver function in broilers, primarily by reducing inflammation, mitigating hepatic steatosis (fatty liver), and ameliorating related pathological conditions, particularly in birds under stress or with metabolic challenges (Lin et al., 2019).

This study was conducted to test the hypothesis that 25-OH-D3 combined with vitamin D3 enhances growth performance, nutrient digestibility, blood parameters, antioxidant status, and immune responses in broiler chickens.

2. Materials and methods

2.1. Birds and experimental design

Four hundred one-day-old male Ross 308 broiler chicks purchased from broiler commercial company with an average initial body weight of 40.50 g were randomly distributed among four treatments, each containing ten replicates of ten chickens. The experimental design is detailed in Table 1. Treatment groups were formulated as follows: (1) control diet containing vitamin D3 at 5000 IU/kg feed(125 mcg/kg feed); (2) diet with 2500 IU/kg (62.5 mcg/kg feed)vitamin D3 plus 2500 IU/kg(62.5 mcg/kg feed) 25-hydroxycholecalciferol; (3) control diet supplemented with 1250 IU/kg 25-OH-D3(31.25 mcg/kg feed); and (4) control diet supplemented with 2500 IU/kg 25-OH-D3 (62.5 mcg/kg feed).

Table 1. The feeding protocol of the broilers during the experiment.

Treatment	Vitamin D3 (cholecalciferol)	25-hydroxycholecalciferol[a]
A, Control (VD3 100%)	5000 IU/kg feed	–
B, (VD3 50% + HC 50%)	2500 IU/kg feed	2500 IU/kg feed (62.5 mcg/kg feed)
C, (VD3 100% + HC 25%)	5000 IU/kg feed	1250 IU/kg feed (31.25 mcg/kg feed
D, (VD3 100% + HC 50%)	5000 IU/kg feed	2500 IU/kg feed (62.5 mcg/kg feed)

[a]1 kg Smart Hydroxy D = 10 Million IU VIT D/kg (0.025% concentration).

Basal diets were formulated according to Ross 308 nutritional requirements and fed in two phases: starter (1–18 days) and grower (19–32 days) the experiment was started from April to May 2025. Table 2 presents the chemical composition and analysis of experimental diets for each growth phase. The 25-hydroxyvitamin D3 used was obtained from Devenish Smart Hydroxy D with concentration 0.025% (250 mcg/gm) (96 Duncrue St, Belfast BT3 9AR, United Kingdom).

Birds were housed in a temperature-controlled facility with an 18-hour light and 6-hour dark photoperiod. Initial temperature was set at 32 ± 1 °C and gradually reduced by 1 °C every three days until reaching 24 ± 1 °C, which was maintained throughout the experiment. Relative humidity was maintained between 50–70%. Daily health assessments and mortality records were maintained throughout the trial period.

2.2. Growth performance and organ weights

Body weights were recorded weekly, while feed intake was monitored daily throughout the experimental period. Feed conversion ratio was calculated from these parameters according to standard procedures (Aviagen, 2019). At experiment completion, six birds per treatment with similar body weights (one bird per replicate) were selected for carcass evaluation following established protocols (Cobb-Vantress, 2018). Weights of breast, thigh, liver, gizzard, heart, spleen, and abdominal fat were measured following slaughter and dissection according to standard carcass evaluation procedures (Barbut, 2015). All organs were weighed and expressed as percentages of total body weight as described by Lesson and Summers (2001).

2.3. Nutrient digestibility

During the final three days of the feeding trial, excreta from six birds per treatment (one bird per replicate) were collected, weighed, and stored frozen until analysis following established procedures (Hill & Anderson, 1958). Samples were dried at 60 °C for 24 h, homogenized, and ground for analysis according to standard methods (AOAC, 1990). Nitrogen, calcium, and phosphorus content in both feed and excreta were determined using AOAC methods (AOAC, 1990). The total collection method was used as described by Sibbald (1976). Nutrient digestibility was calculated using the formula established by McDonald et al. (2010):

$$\text{Nutrient digestibility}(\%) = (\text{total nutrient intake} - \text{total nutrient excreted}) / \text{total nutrient intake} \times 100$$

Table 2. Composition of the control starter and grower diets.

Ingredients, g/kg	Starter (1–18 days)	Grower (19–32 days)
Yellow corn	545	575
Soybean meal, 46%	339	322
Corn gluten meal, 62%	41	20
Soya oil	19	27
Dicalcium phosphate	16.2	15.2
Wheat bran	11	13
DL-Methionine, 99%	2.55	2.75
L-Lysine, 98%	3.5	2.4
L-Threonine	0.125	0.125
Limestone	14.375	13.475
Salt	3	3
Premix[a]	3	3
Sodium bicarbonate	1.75	1.75
Calcium carbonate	0.5	1.3
Total, kg	1000	1000
Chemical analysis		
Crude protein%	22.96	20.93
ME, kcal/kg	3000	3050
Calcium,%	0.97	0.92
Available P%	0.45	0.42
L-Lysine, 98%	1.45	1.35
DL-Methionine, 99%	0.67	0.63

[a]Mineral and vitamin premix provided the following (per kg of diet): Mn, 88 mg; Fe, 62.5 mg; Zn, 81.3 mg; Cu, 12.5 mg; I, 1.25 mg; Se, 0.375 mg; vit. A, 9,375 IU; vit. D_3, 2,375 IU; vit. E, 35 IU; vit. B_1, 2.5 mg; vit. B_6, 3.5 mg; vit. B_5, 12.5 mg; vit. B_7, 0.088 mg; vit. K_3,1.88 mg; folic acid, 0.875 mg; nicotinic acid, 37.5 mg; vit. B_{12}, 0.015 mg.

2.4. Serum biochemical parameters and hepatic lipid peroxidation

Blood samples were collected from ten birds per group (one per replicate) via wing vein puncture prior to slaughter following established procedures for poultry blood sampling (Campbell & Ellis, 2007). Samples were placed in heparinized tubes and immediately centrifuged at 3000 rpm for 20 minutes at 5 °C for plasma separation according to standard protocols (Sturkie, 2000). Plasma was stored at −20 °C until analysis following recommended storage procedures (Kaneko et al., 2008).

Blood parameters including total cholesterol, albumin, globulin, glutamic oxaloacetic transaminase (GOT), glutamate pyruvate transaminase (GPT), glucose, total protein, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol were measured colorimetrically using Diamond Diagnostics reagent kits (Egypt) following manufacturer instructions and established clinical chemistry procedures (Thrall et al., 2012).

Hepatic malondialdehyde (MDA), catalase (CAT), and glutathione peroxidase (GPX) activities were determined according to established methods described by Marklund and Marklund (1974) for antioxidant enzyme analysis. Lipid peroxidation was assessed by measuring MDA concentration using the thiobarbituric acid reactive substances (TBARS) method as described by Buege and Aust (1978).

2.5. Tibial bone mineralization

Left tibiae were collected from ten broilers per treatment, defatted in hexane for 48 h, dried at 100 °C to constant weight, and ashed in a muffle furnace at 550 °C. Ash percentage and calcium and phosphorus content were measured according to AOAC (1995) procedures.

2.6. Real-time polymerase chain reaction (PCR)

Total RNA was extracted from intestinal mucosa using RNeasy Mini Kit (Catalogue no. 74104, Metabion, Germany). RNA concentration was analyzed spectrophotometrically, and purity was evaluated by OD260/280 measurements (acceptable range: 1.8–2.0). Reverse transcription was performed using a reverse transcription reagent kit (Takara, Dalian, LN, China).

Primers for glyceraldehyde 3-phosphate dehydrogenase, tight junction occludin, CaBP-D28k, IL-10, and IL-17 were synthesized by Sangon Biotech Co., Ltd (Shanghai, China; Table 3). Real-time PCR amplifications were conducted using the Roche Lightcycler 480 Real-time PCR system (Roche Ltd., Basel, Switzerland) and SYBR premix PCR kit (Takara, Dalian, LN, China; Table 4).

2.7. Statistical analysis

Using SPSS Statistics 17.0 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL, USA, released August 23, 2008), the differences between the experimental treatments and the control were examined using a General Linear model. While all of the chosen broilers from each replicate served as the experimental unit for blood analyses and nutrient digestibility, the pen served as the experimental unit for performance data. The significance between treatments was determined using Tukey's multiple comparison test, with a significance level of $p < 0.05$.

Table 3. Primer sequences used for real-time PCR.

Gene	Primer sequence (5′–3′)	References
IL10	CATGCTGCTGGGCCTGAA CGTCTCCTTGATCTGCTTGATG	Suzuki et al. (2009)
IL17	CAGATGCTGGATGCCTAACC CTTTAAGCCTGGTGCTGGAT	Yoo et al. (2008)
Occludin	GAGCCCAGACTACCAAAGCAA GCTTGATGTGGAAGAGCTTGTTG	Chen et al. (2015)
CaBP-D28k	AGATCTGGCACCACTACGAC TGAGCAAGCTCAACGATTCCT	Liu et al. (2023)
β-actin	CCACCGCAAATGCTTCTAAAC AAGACTGCTGCTGACACCTTC	Yuan et al. (2007)

Table 4. Cycling conditions for real-time PCR.

Gene	Reverse transcription	Initial denaturation	Amplification (40 cycles) Secondary denaturation	Annealing	Extension	Dissociation curve (1 cycle) Secondary denaturation	Annealing	Final denaturation
IL10	50 °C/30 min	95 °C/10 min	95 °C/15 sec	60 °C/30 sec	72 °C/30 sec	95 °C/15 sec	60 °C/1 min	95 °C/15 sec
IL17	50 °C/30 min	95 °C/10 min	95 °C/15 sec	60 °C/30 sec	72 °C/30 sec	95 °C/15 sec	60 °C/1 min	95 °C/15 sec
Tight junction (Occludin)	50 °C/30 min	95 °C/10 min	95 °C/15 sec	60 °C/30 sec	72 °C/30 sec	95 °C/15 sec	60 °C/1 min	95 °C/15 sec
CaBP-D28k	50 °C/30 min	95 °C/10 min	95 °C/15 sec	60 °C/30 sec	72 °C/30 sec	95 °C/15 sec	60 °C/1 min	95 °C/15 sec
β-actin	50 °C/30 min	95 °C/10 min	95 °C/15 sec	51 °C/30 sec	72 °C/30 sec	95 °C/15 sec	51 °C/1 min	95 °C/15 sec

PCR conditions included initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s (51 °C for β-actin), and extension at 72 °C for 30 s. A dissociation curve analysis was performed from 60 °C to 95 °C with increments of 0.5 °C every 5 s to verify amplification specificity. Gene expression variations were calculated using the ΔΔCt method (Livak & Schmittgen, 2001) with the formula: fold change = 2^(−ΔΔCt).
Whereas $\Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{target}$.
$\Delta Ct_{target} = Ct_{control} - Ct_{treatment}$ and $\Delta Ct_{reference} = Ct_{control} - Ct_{treatment}$.

Table 5. Effects of dietary 25-hydroxyvitamin D3 supplementation on growth performance and organ weights.

Treatment	Control, 100% V.D3	50% V.D3 + 50% 25-hydroxyvitamin	100 % V.D3 + 50% 25-hydroxyvitamin	100 % V.D3 + 100% 25-hydroxyvitamin	P-Value
Initial body weight, g	40.25 ± 0.16	40.97 ± 0.29	40.95 ± 0.23	40.64 ± 0.22	0.113
Final body weight,32 day, g	1914.8 ± 29.64^{c}	2082.9 ± 24.88^{b}	2167.2 ± 19.37^{a}	2165.2 ± 33.49^{a}	0.009
Body weight gain, g	1874.6 ± 29.62^{c}	2042 ± 24.92^{b}	2126.3 ± 19.39^{a}	2124.6 ± 33.45^{a}	0.011
Feed intake, 32 day, g	2630 ± 31.58^{c}	2766 ± 25.95^{b}	2827 ± 27.90^{b}	2905 ± 14.73^{a}	0.023
Feed conversion ratio (FCR)	1.373 ± 0.030^{a}	1.328 ± 0.020^{b}	1.305 ± 0.010^{c}	1.342 ± 0.020^{ab}	0.041
Nitrogen digestibility,%	77.70 ± 4.10^{b}	76.00 ± 3.60^{b}	80.60 ± 3.70^{a}	82.50 ± 2.30^{a}	0.043
Calcium digestibility, %	52.70 ± 3.90^{b}	53.80 ± 4.60^{b}	62.30 ± 4.10^{a}	68.30 ± 3.30^{a}	0.037
Phosphorus digestibility, %	51.10 ± 7.20^{b}	52.30 ± 4.50^{b}	59.30 ± 2.80^{a}	60.60 ± 4.60^{a}	0.045
Organ weights (%), body weight					
Carcass, %	71.03 ± 0.45	73.13 ± 1.02	73.19 ± 1.08	71.89 ± 0.7	0.126
Breast muscle, %	24.76 ± 0.37	25.58 ± 0.24	23.61 ± 1.55	24.11 ± 0.8	0.332
Thigh muscle, %	15.97 ± 0.76	14.99 ± 0.86	15.31 ± 0.75	15.17 ± 0.56	0.794
Gizzard, %	0.95 ± 0.03	0.94 ± 0.1	1.08 ± 0.08	1.15 ± 0.04	0.074
Liver, %	2.43 ± 0.13	2.28 ± 0.12	2.26 ± 0.17	2.35 ± 0.05	0.779
Spleen, %	0.12 ± 0.003	0.11 ± 0.01	0.12 ± 0.01	0.09 ± 0.001	0.091
Heart, %	0.62 ± 0.06	0.52 ± 0.02	0.49 ± 0.01	0.50 ± 0.01	0.087
Abdominal fat, %	1.01 ± 0.09	0.94 ± 0.05	1.08 ± 0.03	0.85 ± 0.07	0.061

^{a,b,c}Mean values with different letters in the same row differ significantly at $p < 0.05$; values are expressed as means ± standard error.

3. Results

3.1. Growth performance and organ weights

Results presented in Table 5 demonstrate that dietary supplementation with 25-OH-D3 (1250 or 2500 IU/kg) (31.25 or 62.5 mcg/kg feed) combined with vitamin D3 significantly influenced body weight, body weight gain, feed consumption, and feed conversion ratio ($p \leq 0.05$). Broilers receiving vitamin D3 (5000 IU/kg) (125 mcg/kg feed) with 25-OH-D3 (1250 or 2500 IU/kg) (31.25 or 62.5 mcg/kg feed) exhibited superior growth performance compared to controls, including higher body weight, weight gain, and feed intake, while achieving improved feed conversion efficiency. Additionally, these birds demonstrated enhanced digestibility of protein, calcium, and phosphorus compared to controls. However, no significant effects ($p > 0.05$) of vitamin D3 and 25-OH-D3 supplementation on carcass characteristics were observed at 32 days of age.

3.2. Biochemical parameters

Table 6 results indicate that dietary supplementation with 25-OH-D3 (1250 or 2500 IU/kg) (31.25 or 62.5 mcg/kg feed) combined with vitamin D3 significantly affected blood concentrations of calcium, phosphorus, vitamin D3, Newcastle disease titers, and avian influenza titers (H9N1) ($p \leq 0.05$). However, total protein, albumin, globulin, creatinine, uric acid, and liver enzymes (GPT and GOT) remained unaffected.

3.3. Hepatic oxidative status

Supplementation with 25-OH-D3 (1250 or 2500 IU/kg) (31.25 or 62.5 mcg/kg feed) combined with vitamin D3 significantly ($p \leq 0.05$) affected hepatic glutathione peroxidase (GPX) activity and malondialdehyde

(MDA) concentrations. Broilers fed 25-OH-D3 with vitamin D3 showed notable increases in GPX activity compared to controls (Figure 1). All dietary treatments significantly decreased ($p \leq 0.05$) MDA levels compared to the control group.

3.4. Bone mineralization

As shown in Table 7, ash, calcium, and phosphorus content in tibiae were significantly increased ($p < 0.05$) in birds receiving 25-OH-D3 (1250 or 2500 IU/kg) (31.25 or 62.5 mcg/kg feed) with vitamin D3 compared to controls.

3.5. Gene expression in intestines

Results of jejunal and duodenal gene expression analysis at day 32 are presented in Table 8. Tight junction occludin and CaBP-D28k expression increased ($p < 0.05$) in the jejunum as a function of 25-OH-D3

Table 6. Effects of dietary 25-hydroxyvitamin D3 supplementation on broiler plasma parameters.

Item	Control, 100% V.D3	50% V.D3 + 50% 25-hydroxyvitamin	100 % V.D3 + 50% 25-hydroxyvitamin	100 % V.D3 + 100% 25-hydroxyvitamin	*p* value
GPT, (I/U)	3.88 ± 0.39	3.29 ± 0.26	2.93 ± 0.35	3.01 ± 0.40	0.268
GOT, (I/U)	243 ± 11.14	224 ± 5.24	211 ± 9.85	213 ± 12.20	0.132
Creatinine, (mg/dL)	0.15 ± 0.01	0.15 ± 0.02	0.13 ± 0.02	0.13 ± 0.02	0.484
Uric acid,(mg/dL)	3.2 ± 0.27	2.96 ± 0.06	2.89 ± 0.27	2.56 ± 0.27	0.345
Total protein, (mg/dl)	2.12 ± 0.012	2.13 ± 0.01	2.23 ± 0.04	2.28 ± 0.09	0.143
Albumin, (mg/dl)	1.38 ± 0.09	1.50 ± 0.13	1.30 ± 0.10	1.35 ± 0.07	0.606
Globulin, (mg/dl)	0.74 ± 0.08	0.63 ± 0.13	0.93 ± 0.08	0.92 ± 0.14	0.238
Calcium, mmol/L	1.94 ± 0.02^{c}	2.7 ± 0.03^{bc}	2.88 ± 0.04^{ab}	3.64 ± 0.03^{a}	0.004
25 (OH) D_3, mg/L	0.307 ± 0.006^{c}	0.329 ± 0.005^{b}	0.363 ± 0.003^{a}	0.328 ± 0.004^{b}	0.023
Phosphorus, mmol/L	6.48 ± 0.22^{c}	8.44 ± 0.13^{ab}	7.95 ± 0.48^{b}	9.09 ± 0.40^{a}	0.017
ND titer	3.80 ± 0.06^{c}	4.00 ± 0.02^{bc}	5.20 ± 0.07^{a}	4.80 ± 0.06^{ab}	0.009
H9 titer	4.80 ± 0.07^{b}	5.60 ± 0.04^{a}	6.20 ± 0.07^{a}	5.80 ± 0.08^{a}	0.013

GPT = Glutamic Pyruvic Transaminase, GOT = Glutamic-Oxaloacetic Transaminase, ND = Newcastle disease, H9 = Avian influenza.
^{a,b,c}Mean values with different letters in the same row differ significantly at $p < 0.05$; values are expressed as means ± standard error.

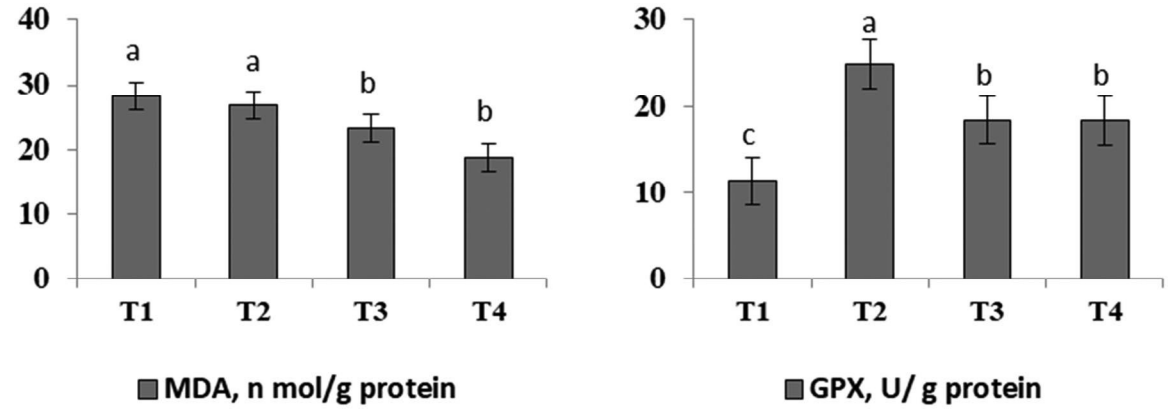

Figure 1. Effects of dietary 25-hydroxyvitamin D3 supplementation on liver malondialdehyde (MDA) and glutathione peroxidase (GPX) T1: Vitamin D3 (5000 IU/kg feed) T2: 2500 IU/kg of Vitamin D3 plus 2500 IU/kg of 25-Hydroxycholecalciferol T3: 5000 IU/kg of Vitamin D3 plus 1250 IU/kg of 25-Hydroxycholecalciferol T4: 5000 IU/kg of Vitamin D3 plus 2500 IU/kg of 25-Hydroxycholecalciferol.

Table 7. Effects of dietary 25-hydroxyvitamin D3 supplementation on bone minerals.

Item	Control, 100% V.D3	50% V.D3 + 50% 25-hydroxyvitamin	100 % V.D3 + 50% 25-hydroxyvitamin	100 % V.D3 + 100% 25-hydroxyvitamin	*p* value
Ash,%	37.66 ± 1.96^{c}	42.16 ± 0.80^{b}	46.72 ± 1.02^{a}	46.3 ± 1.18^{a}	0.023
Calcium, g/100g ash	28.88 ± 0.72^{d}	34.6 ± 3.76^{c}	37.5 ± 1.76^{b}	38.2 ± 2.31^{a}	0.001
Phosphorus, g/100g ash	12.88 ± 1.10^{d}	13.86 ± 1.57^{c}	15.5 ± 1.58^{b}	16.24 ± 0.89^{a}	0.002

^{a,b,c,d}Mean values with different letters in the same row differ significantly at $p < 0.05$; values are expressed as means ± standard error.

Table 8. Effects of dietary 25-hydroxyvitamin D3 supplementation on gene expression.

Item	Control, 100% V.D3	50% V.D3 + 50% 25-hydroxyvitamin	100 % V.D3 + 50% 25-hydroxyvitamin	100 % V.D3 + 100% 25-hydroxyvitamin	*p* value
			Jejunum		
Tight junction occludin	1.00 ± 0.00^{d}	3.68 ± 0.19^{c}	7.41 ± 0.28^{a}	5.59 ± 0.27^{b}	0.001
CaBP-D28k	1.00 ± 0.00^{d}	4.73 ± 0.33^{c}	9.36 ± 0.39^{a}	7.87 ± 0.23^{b}	0.002
			Duodenum		
IL-10	1.00 ± 0.00^{a}	0.822 ± 0.019^{b}	0.317 ± 0.019^{d}	0.509 ± 0.02^{c}	0.001
IL-17	1.00 ± 0.00^{d}	3.153 ± 0.13^{c}	5.746 ± 0.228^{a}	4.101 ± 0.105^{b}	0.001

[a,b,c,d]Mean values with different letters in the same row differ significantly at $p < 0.05$; values are expressed as means ± standard error.

and vitamin D3 supplementation, with highest expression observed in birds receiving treatment 3 (5000 IU/kg vitamin D3 plus 1250 IU/kg 25-OH-D3) (125 mcg/kg vitamin D3 plus 31.25 mcg/kg feed). IL-17 expression increased in the duodenum with supplementation, while IL-10 expression decreased in all treatment groups compared to controls.

4. Discussion

4.1. Growth performance and organ weights

The current findings demonstrate that 25-OH-D3 supplementation combined with vitamin D3 substantially improves broiler growth performance parameters. In our study, birds receiving 25-OH-D3 (1250 or 2500 IU/kg) (31.25 or 62.5 mcg/kg feed) with vitamin D3 showed significantly higher final body weight (2167.2 and 2165.2 g vs. 1914.8 g in controls, $p < 0.05$) and improved feed conversion ratio (1.305 and 1.342 vs. 1.373 in controls). These results align with recent studies showing enhanced broiler performance with 25-OH-D3 supplementation (Fatemi et al., 2021; Vazquez et al., 2018; Zhang et al., 2020). According to Fatemi et al. (2022), body weight gain in coccidiosis-challenged birds has been shown to increase when fed 25-OH-D3 compared to vitamin D3 alone. Fritts and Waldroup (2003) reported that 125 IU/kg of 25-OH-D3 enhanced body weight gain to levels comparable to birds receiving 1000 IU/kg of vitamin D3.

Our findings also revealed significantly improved nutrient digestibility, with calcium digestibility increasing from 52.70% in controls to 62.30% and 68.30% in supplemented groups ($p < 0.05$). Similarly, phosphorus digestibility improved from 51.10% to 59.30% and 60.60% respectively. Leyva-Jimenez et al. (2019) found that replacing 50% of dietary vitamin D3 with 25-OH-D3 increased body weight, tibial breaking strength, and plasma 25-OH-D3 concentrations in broilers challenged with a coccidiosis vaccine. Supplementing different vitamin D metabolites in combination reduces energy expenditure associated with vitamin D metabolism (Prokoski et al., 2021). Supplementing vitamin D3 or its metabolite 25-OH-D3 to broiler diets can improve growth performance (Gómez et al., 2013).

The improved performance with 25-OH-D3 supplementation can be attributed to its immediate bioavailability, bypassing the hepatic 25-hydroxylation step required for vitamin D3 activation. 25-OH-D3 is an active metabolite of vitamin D3 and plays a significant role in calcium absorption in the intestine, calcium utilization for bone growth and calcium fixation (Applegate et al., 2003; Fritts & Waldroup, 2005; Gómez et al., 2013; Ledwaba & Roberson, 2003). This enhanced bioavailability allows for more efficient calcium and phosphorus utilization, leading to improved bone development and overall growth performance. The improvement may also be due to increased villus and crypt morphology, which may have increased nutrient absorption, 25-OH-D3 supplementation may improves growth performance by increasing the bioavailability of vitamin D, which enhances calcium and phosphorus absorption for better bone health, while also boosting the immune system and antioxidant capacity. As a pre-metabolized form of vitamin D3, 25-OH-D3 can be more effectively absorbed and used by the body, leading to improved weight gain and overall development (Fatemi et al., 2021, 2022).

4.2. Biochemical parameters

The physiological and immunological state of the body can be reflected by blood biochemical indicators, which are related to the activity of certain enzymes and blood protein levels (Dawood et al., 2020; Kurtz & Travlos, 2017). Our results demonstrated significant improvements in key blood parameters, with

plasma calcium concentrations increasing from 7.94 mmol/L in controls to 9.64 mmol/L in the highest supplemented group ($p < 0.05$). Vitamin D levels rose from 30.70 ng/mL to 36.33 ng/mL, while phosphorus increased from 6.48 to 9.09 mmol/L. Serum 25-OH-D or vitamin D3 concentration serves as an important indicator of vitamin D status (Li et al., 2021). This study's results showing increased 25-OH-D concentrations with supplementation agree with previous findings (Chen et al., 2020; Wang et al., 2020; Zhang et al., 2020). The partial substitution of vitamin D3 with 25-OH-D3 increased serum calcium, phosphorus, and vitamin D concentrations (Leyva-Jimenez et al., 2019; Prokoski et al., 2021).

Notably, our study showed enhanced immune responses, with Newcastle disease titers increasing from 3.80 in controls to 5.20 in the best-performing group, and H9N1 titers improving from 4.80 to 6.20 ($p < 0.05$). Enhanced Newcastle disease and H9 titers in supplemented broilers indicate improved immunity and disease resistance. These results support previous findings by Vazquez et al. (2018), where 25-OH-D3 supplementation at 69 µg/kg improved cellular immune responses and bone mineral deposition in broilers. Vitamin D modulates immune responses by selectively suppressing proinflammatory cytokine production and leukocyte infiltration (Bahar-Shany et al., 2010; Helming et al., 2005; Jadhav et al., 2018).

The metabolite 1,25-(OH)2-D3 stimulates hematopoiesis when bound to vitamin D3 receptors located on hematopoietic cell membranes, including B and T lymphocytes (Capiati et al., 2002; Enioutina et al., 2009; Gómez et al., 2013; Han et al., 2009), thereby enhancing immune function and antibody production against viral challenges such as Newcastle disease and avian influenza. Previous data by Khan et al. (2010) shows that commercial levels of vitamin D3 in chicken diets are effective at inducing robust antibody responses against NDV vaccination.

4.3. Hepatic oxidative status

Our investigation of hepatic antioxidant status revealed that 25-OH-D3 supplementation significantly enhanced glutathione peroxidase (GPX) activity while reducing malondialdehyde (MDA) levels compared to controls ($p < 0.05$). These findings support research by Sardar et al. (1996) and Garcion et al. (1999) indicating that vitamin D3 reduces oxidative stress by upregulating antioxidant defense systems including glutathione, glutathione peroxidase, and superoxide dismutase. The prevention of MDA level increases suggests that 25-OH-D3 supplementation can prevent lipid peroxidation (Zhang et al., 2020).

In the poultry industry, broilers inevitably face oxidative stress associated with bacterial challenges or their products, which could impair health status (Zhang et al., 2020). The inclusion of 25-OH-D3 with vitamin D3 in broiler diets raised hepatic GPX activity, indicating enhanced antioxidant capacity and improved cellular protection against oxidative damage. Dietary 25-OH-D3 supplementation prevented the increase of MDA levels in serum of broilers, suggesting that lipid peroxidation could be prevented by dietary 25-OH-D3 supplementation moreover; 25-OH-D3 supplementation reduces lipid peroxidation in broilers primarily by enhancing the activity of the body's natural antioxidant enzyme systems and by modulating the inflammatory response. This dual action helps to restore the balance between the production of reactive oxygen species (ROS) and the body's ability to neutralize them, thereby preventing oxidative damage to lipids (Zhang et al., 2020).

4.4. Bone mineralization

Our bone analysis revealed substantial improvements in tibial mineralization, with ash content increasing from 22.66% in controls to 32.3% in the highest supplemented group ($p < 0.05$). More remarkably, calcium content increased dramatically from 24.88% to 201.2%, while phosphorus rose from 12.88% to 47.24%. These results align with studies showing increased tibial ash in broilers receiving 25-OH-D3 supplementation (Boris et al., 1977). Sakkas et al. observed improved femur and tibia mineralization when 3000 IU/kg of 25-OH-D3 was added to diets. Vazquez et al. (2018) reported enhanced mineral deposition in bones of 1–21 day old broilers supplemented with 69 µg/kg of 25-OH-D3.

The enhanced bone mineralization observed in this study can be attributed to improved calcium and phosphorus absorption and utilization. The active form of vitamin D, 1,25-(OH)2-D3, directly regulates calcium-binding protein synthesis in the intestine, facilitating more efficient mineral absorption and subsequent deposition in bone tissue.

4.5. Gene expression in intestines

Our molecular analysis revealed significant upregulation of intestinal genes involved in calcium metabolism and barrier function. In the jejunum, tight junction occludin expression increased 7.41-fold and CaBP-D28k expression increased 9.36-fold in the best-performing treatment compared to controls ($p<0.05$). In the duodenum, IL-17 expression increased 5.75-fold while IL-10 expression decreased to 0.317-fold relative to controls. Tight junctions, primarily formed by claudins and occludin proteins, are fundamental for maintaining intestinal epithelial integrity and barrier function, allowing selective nutrient absorption (García et al., 2021). The observed increases in occludin expression support improved intestinal barrier function and enhanced nutrient absorption capacity.

CaBP-D28k facilitates calcium transport from apical to basolateral membranes of intestinal cells (Wu et al., 2022). Previous research has shown that 1,25-(OH)2-D3 injection increased CaBP-D9k mRNA expression in mouse duodenum (Benn et al., 2008; Khuituan et al., 2012; Okano et al., 2004) and CaBP-D28k expression in chicken small intestine (Clemens et al., 1988; Hall & Norman, 1990; Sechman et al., 1996; Wu et al., 2022; Yang et al., 2019).

The upregulation of CaBP-D28k expression in the jejunum demonstrates enhanced calcium transport capacity, which directly correlates with the improved calcium digestibility (68.30% vs. 52.70% in controls) and bone mineralization observed in this study. This molecular mechanism explains the superior calcium utilization in birds receiving 25-OH-D3 supplementation.

25-OH cholecalciferol with vitamin D3 supplementation increased IL-17 mRNA amounts in the duodenum, while IL-10 mRNA decreased. IL-10 inhibits cellular immunity, suppresses the production of proinflammatory cytokines, prevents the differentiation of monocytes into tissue macrophages and apoptosis, and enhances the production of IL-2 and IFN-γ (Ouyang & O'Garra, 2019). Also, IL-10 is limiting the host's ability to mount an effective immune response (Rasheed et al., 2020).

The decreased IL-10 expression observed in supplemented birds suggests reduced anti-inflammatory activity, which may enhance the birds' ability to mount effective immune responses against pathogens. Conversely, the increased IL-17 expression indicates enhanced pro-inflammatory responses and improved cellular immunity, contributing to the elevated antibody titers (Newcastle disease: 5.20 vs. 3.80; H9N1: 6.20 vs. 4.80) observed in this study.

5. Conclusion

Based on the findings from this study, we can confidently say that incorporating commercial amounts of vitamin D3 into broiler chicken diets, alongside 25-OH-D3 supplementation, is an effective strategy for enhancing growth performance. This approach also positively influences liver antioxidant status, bolsters immunity, promotes bone mineralization, and modulates gene expression related to calcium metabolism in broilers aged from 1 to 32 days. The results demonstrate that the combination of 25-OH-D3 and vitamin D3 significantly improves growth performance, liver antioxidant levels, and overall health aspects connected to bone and immune function in these chickens.

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Author contributions

CRediT: **Ahmed A. Saleh**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing; **Whad Fayed**: Conceptualization, Data curation, Funding acquisition, Software; **Fuad Saleh**: Conceptualization, Data curation, Funding acquisition, Software; **Abdul Rahman A. Alkhamisi**: Data curation,

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Informed consent statement

Not applicable.

Ethical statement

All practices and managemental procedures were authorized by the Ethics Committee of Local Experimental Animals Care of Kafrelsheikh University, Egypt, The animal ethics approval number (KFSIACUC/235/2025). Also, these procedures of the experiment were carried out in accordance with applicable veterinary guidelines, such as the American Veterinary Medical Association.

ARRIVE guidelines statement

The research adherent the ARRIVE guidelines.

Disclosure statement

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Data availability statement

The data presented in this study are available from the corresponding authors upon reasonable request.

References

Adhikari, R., White, D., House, J. D., & Kim, W. K. (2020). Effects of additional dosage of vitamin D3, vitamin D2, and 25-hydroxyvitamin D3 on calcium and phosphorus utilization, egg quality and bone mineralization in laying hens. *Poultry Science*, *99*(1), 364–373. https://doi.org/10.3382/ps/pez502

AOAC. (1990). *Official methods of analysis* (15th ed.). Association of Official Analytical Chemists.

AOAC. (1995). *Official methods of analysis* (16th ed.). Association of Official Analytical Chemists.

Applegate, T. J., Angel, R., & Classen, H. L. (2003). Effect of dietary calcium, 25-hydroxycholecalciferol, or bird strain on small intestinal phytase activity in broiler chickens. *Poultry Science*, *82*(7), 1140–1148. https://doi.org/10.1093/ps/82.7.1140

Atencio, A., Pesti, G. M., & Edwards, H. M.Jr. (2005). Twenty-five hydroxycholecalciferol as a cholecalciferol substitute in broiler breeder hen diets and its effect on the performance and general health of the progeny. *Poultry Science*, *84*(8), 1277–1285. https://doi.org/10.1093/ps/84.8.1277

Aviagen. (2019). *Ross 308 broiler nutrition specifications*. Aviagen Ltd.

Bahar-Shany, K., Ravid, A., & Koren, R. (2010). Upregulation of MMP-9 production by TNFalpha in keratinocytes and its attenuation by vitamin D. *Journal of Cellular Physiology*, *222*(3), 729–737. https://doi.org/10.1002/jcp.21991

Bar, A., Wasserman, R. H., & Taylor, A. N. (1980). The role of vitamin D and its metabolites in the maintenance of calcium homeostasis and eggshell formation in the laying hen. *Comparative Biochemistry & Physiology Part A: Physiology*, *66*, 119–125. https://doi.org/10.1016/0300-9629(80)90373-3

Barbut, S. (2015). *The science of poultry and meat processing*. University of Guelph.

Bassi, L. S., Moreno, F. A., Martins, C. C. S., Sens, R. F., Lozano-Poveda, C. A., & Maiorka, A. (2024). Effect of 25-hydroxycholecalciferol supplementation with different dietary available phosphorus levels for broilers. *British Poultry Science*, *65*(1), 71–78. https://doi.org/10.1080/00071668.2023.2278474

Benn, B. S., Ajibade, D., Porta, A., Dhawan, P., Hediger, M., Peng, J. B., Jiang, Y., Oh, G. T., Jeung, E. B., Lieben, L., Bouillon, R., Carmeliet, G., & Christakos, S. (2008). Active intestinal calcium transport in the absence of transient receptor potential vanilloid type 6 and calbindin-D9k. *Endocrinology*, *149*(6), 3196–3205. https://doi.org/10.1210/en.2007-1655

Bikle, D. D., & Munson, S. (1985). 1,25-Dihydroxyvitamin D increases calmodulin binding to specific proteins in the chick duodenal brush border membrane. *Journal of Clinical Investigation*, *76*(6), 2312–2316. https://doi.org/10.1172/JCI112234

Boris, A., Hurley, J. F., & Trmal, T. (1977). Relative activities of some metabolites and analogs of cholecalciferol in stimulation of tibia ash weight in chicks otherwise deprived of vitamin D. *Journal of Nutrition*, *107*(2), 194–198. https://doi.org/10.1093/jn/107.2.194

Buege, J. A., & Aust, S. D. (1978). Microsomal lipid peroxidation. *Methods in Enzymology*, *52*, 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6

Campbell, T. W., & Ellis, C. K. (2007). *Avian and exotic animal hematology and cytology* (3rd ed.). Blackwell Publishing.

Capiati, D., Benassati, S., & Boland, R. L. (2002). 1,25(OH)2-vitamin D3 induces translocation of the vitamin D receptor (VDR) to the plasma membrane in skeletal muscle cells. *Journal of Cellular Biochemistry*, *86*(1), 128–135. https://doi.org/10.1002/jcb.10191

Chen, C., Turner, B., Applegate, T. J., Litta, G., & Kim, W. K. (2020). Role of long-term supplementation of 25-hydroxyvitamin D3 on laying hen bone 3-dimensional structural development. *Poultry Science*, *99*(11), 5771–5782. https://doi.org/10.1016/j.psj.2020.06.080

Chen, C., Zhang, J., Xu, H., Zheng, P., Yu, B., & He, J. (2015). Molecular cloning, tissue distribution and ontogenetic expression of claudin-1, claudin-4 and occludin genes in the intestine of weaning piglets. *Journal of Animal Science & Biotechnology*, *6*, 1–11. https://doi.org/10.1186/s40104-015-0003-2

Chou, S. H., Chung, T. K., & Yu, B. (2009). Effects of supplemental 25-hydroxycholecalciferol on growth performance, small intestinal morphology, and immune response of broiler chickens. *Poultry Science*, *88*(11), 2333–2341. https://doi.org/10.3382/ps.2009-00283

Clemens, T. L., McGlade, S. A., Garrett, K. P., Horiuchi, N., & Hendy, G. N. (1988). Tissue-specific regulation of avian vitamin D-dependent calcium-binding protein 28-kDa mRNA by 1,25-dihydroxyvitamin D3. *Journal of Biological Chemistry*, *263*(26), 13112–13116. https://doi.org/10.1016/S0021-9258(18)37682-5

Cobb-Vantress. (2018). *Cobb500 broiler performance & nutrition supplement*. Cobb-Vantress Inc.

Dawood, M. A. O., Zommara, M., Eweedah, N. M., & Helal, A. I. (2020). Synergistic effects of selenium nanoparticles and vitamin E on growth, immune-related gene expression, and regulation of antioxidant status of Nile tilapia (*Oreochromis niloticus*). *Biological Trace Element Research*, *195*(2), 624–635. https://doi.org/10.1007/s12011-019-01857-6

Ding, X., Li, D., Bai, S., Wang, J., Zeng, Q., Su, Z., Xuan, Y., & Zhang, K. (2011). Effect of dietary xylooligosaccharides on intestinal characteristics, gut microflora, cecal short-chain fatty acids, and plasma immune parameters of laying hens. *Poultry Science*, *90*, 1650–1658. https://doi.org/10.3382/ps.2011-01484

Enioutina, E. Y., Bareyan, D., & Daynes, R. A. (2009). TLR-induced local metabolism of vitamin D3 plays an important role in the diversification of adaptive immune responses. *Journal of Immunology (Baltimore, Md.: 1950)*, *182*(7), 4296–4305. https://doi.org/10.4049/jimmunol.0804005

Erdaw, M. M., & Beyene, W. T. (2022). Trends, prospects and the socio-economic contribution of poultry production in sub-Saharan Africa: A review. *World's Poultry Science Journal*, *78*(3), 835–852. https://doi.org/10.1080/00439339.2022.2069314

Fatemi, S. A., Alqhtani, A. H., Elliott, K. E. C., Bello, A., Levy, A. W., & Peebles, E. D. (2021). Improvement in the performance and inflammatory reaction of Ross 708 broilers in response to the in ovo injection of 25-hydroxyvitamin D3. *Poultry Science*, *100*(1), 138–146. https://doi.org/10.1016/j.psj.2020.09.079

Fatemi, S. A., Alqahtani, A. H., Bello, A., Crossley, J. R., Elliott, K. E. C., Holliday, N. J., Zhang, H. J., & Peebles, E. D. (2022). Effects of the in ovo injection of 25-hydroxyvitamin D3 on the broiler immune response to a coccidiosis vaccine and Eimeria challenge. *Poultry Science*, *101*, 101651. https://doi.org/10.1016/j.psj.2021.101651

Fritts, C. A., & Waldroup, P. W. (2003). Effect of source and level of vitamin D on live performance and bone development in growing broilers. *Journal of Applied Poultry Research*, *12*(1), 45–52. https://doi.org/10.1093/japr/12.1.45

Fritts, C. A., & Waldroup, P. W. (2005). Comparison of cholecalciferol and 25-hydroxycholecalciferol in broiler diets designed to minimize phosphorus excretion. *Journal of Applied Poultry Research*, *14*(1), 156–166. https://doi.org/10.1093/japr/14.1.156

García, A. F., Murakami, A. E., Duarte, C. R., Rojas, I. C., Picoli, K. P., & Puzotti, M. M. (2021). Use of vitamin D3 and its metabolites in broiler chicken feed on performance, bone parameters and meat quality. *Asian-Australasian Journal of Animal Sciences*, *26*, 408–415. https://doi.org/10.5713/ajas.2012.12739

Garcion, E., Wion-Barbot, N., Montero-Menei, C. N., Berger, F., & Wion, D. (1999). New clues about vitamin D functions in the nervous system. *Trends in Endocrinology & Metabolism*, *10*, 25–32. https://doi.org/10.1016/S1043-2760(98)00099-X

Gómez, V. G., Morales, L. R., & Avila, G. E. (2013). Use of 25-hydroxycholecalciferol in diets of broiler chickens: Effects on growth performance, immunity and bone calcification. *Journal of Poultry Science*, *50*, 60–64. https://doi.org/10.2141/jpsa.0110142

Hall, A. K., & Norman, A. W. (1990). Regulation of calbindin-D28k gene expression in the chick intestine: Effects of serum calcium status and 1,25-dihydroxyvitamin D3. *Journal of Bone & Mineral Research: The Official Journal of the American Society for Bone & Mineral Research*, *5*(4), 331–336. https://doi.org/10.1002/jbmr.5650050404

Han, J. C., Yang, X. D., Zhang, T., Li, H., Li, W. L., Zhang, Z. Y., & Yao, J. H. (2009). Effects of 1alpha-hydroxycholecalciferol on growth performance, parameters of tibia and plasma, meat quality, and type IIb sodium phosphate cotransporter gene expression of one-to twenty-one-day-old broilers. *Poultry Science*, *88*(2), 323–329. https://doi.org/10.3382/ps.2008-00252

Helming, L., Böse, J., Ehrchen, J., Schiebe, S., Frahm, T., Geffers, R., Probst-Kepper, M., Balling, R., & Lengeling, A. (2005). 1alpha,25-Dihydroxyvitamin D3 is a potent suppressor of interferon gamma-mediated macrophage activation. *Blood*, *106*(13), 4351–4358. https://doi.org/10.1182/blood-2005-03-1029

Hill, F. W., & Anderson, D. L. (1958). Comparison of metabolizable energy and productive energy determinations with growing chicks. *Journal of Nutrition*, *64*(4), 587–603. https://doi.org/10.1093/jn/64.4.587

Jadhav, N. J., Gokhale, S., Seervi, M., Patil, P. S., & Alagarasu, K. (2018). Immunomodulatory effect of 1,25 dihydroxy vitamin D3 on the expression of RNA sensing pattern recognition receptor genes and cytokine response in dengue virus infected U937-DC-SIGN cells and THP-1 macrophages. *International Immunopharmacology*, *62*, 237–243. https://doi.org/10.1016/j.intimp.2018.07.016

Kaneko, J. J., Harvey, J. W., & Bruss, M. L. (2008). *Clinical biochemistry of domestic animals* (6th ed.). Academic Press.

Khan, S. H., Shahid, R., Mian, A. A., Sardar, R., & Anjum, M. A. (2010). Effect of the level of cholecalciferol supplementation of broiler diets on the performance and tibial dyschondroplasia. *Journal of Animal Physiology & Animal Nutrition*, *94*(5), 584–593. https://doi.org/10.1111/j.1439-0396.2009.00955.x

Khuituan, P., Teerapornpuntakit, J., Wongdee, K., Suntornsaratoon, P., Konthapakdee, N., Sangsaksri, J., Sripong, C., Krishnamra, N., & Charoenphandhu, N. (2012). Fibroblast growth factor-23 abolishes 1,25-dihydroxyvitamin D3-enhanced duodenal calcium transport in male mice. *American Journal of Physiology. Endocrinology & Metabolism*, *302*(8), E903–E913. https://doi.org/10.1152/ajpendo.00585.2011

Kurtz, D. M., & Travlos, G. S. (2017). *The clinical chemistry of laboratory animals*. CRC Press.

Ledwaba, M. F., & Roberson, K. D. (2003). Effectiveness of twenty-five-hydroxycholecalciferol in the prevention of tibial dyschondroplasia in Ross cockerels depends on dietary calcium level. *Poultry Science*, *82*(11), 1769–1777. https://doi.org/10.1093/ps/82.11.1769

Lesson, S., & Summers, J. D. (2001). *Scott's nutrition of the chicken* (4th ed.). University Books.

Leyva-Jimenez, H., Gardner, K., Al-Jumaa, Y., Padgett, J. C., & Bailey, C. A. (2019). Partial replacement of dietary cholecalciferol with 25-hydroxycholecalciferol on broiler chickens subjected to a coccidiosis vaccine challenge. *Journal of Applied Poultry Research*, *28*(3), 743–754. https://doi.org/10.3382/japr/pfz020

Li, X., Zhang, D., & Bryden, W. L. (2021). Calcium and phosphorus metabolism and nutrition of poultry: Are current diets formulated correctly? *Animal Feed Science & Technology*, *271*, 114731. https://doi.org/10.1016/j.anifeedsci.2020.114731

Lin, H. Y., Chou, P. C., Chen, Y. H., Lai, L. S., Chung, T. K., Walzem, R. L., Huang, S. Y., & Chen, S. E. (2019). Dietary Supplementation of 25-hydroxycholecalciferol improves livability in broiler Breeder Hens-amelioration of cardiac

pathogenesis and hepatopathology. *Animals: An Open Access Journal from MDPI*, *9*(10), 770. https://doi.org/10.3390/ani9100770

Liu, M. Y., Wu, L. H., Lv, X. L., He, L., Hao, J. F., Ma, B. B., Xi, L., Qiao, Y. Y., Tang, F., & Han, J. C. (2023). 25-hydroxycholecalciferol affects growth performance, bone calcium content and intestinal calcium transporter gene expression in broiler chickens. *Journal of Animal & Feed Sciences*, *32*(2), 138–145. https://doi.org/10.22358/jafs/150845/2023

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods (San Diego, Calif.)*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Marklund, S., & Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry*, *47*(3), 469–474. https://doi.org/10.1111/j.1432-1033.1974.tb03714.x

McDonald, P., Edwards, R. A., Greenhalgh, J. F. D., Morgan, C. A., Sinclair, L. A., & Wilkinson, R. G. (2010). *Animal nutrition* (7th ed.). Prentice Hall.

Morris, A., & Selvaraj, R. K. (2014). In vitro 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 modulates developmental stage specific immune responses in broiler chickens. *Domestic Animal Endocrinology*, *49*, 108–115. https://doi.org/10.1016/j.domaniend.2014.07.001

Morris, A., Shanmugasundaram, R., Lilburn, M. S., & Selvaraj, R. K. (2014). 25-Hydroxycholecalciferol supplementation improves growth performance and decreases inflammation during an experimental lipopolysaccharide injection. *Poultry Science*, *93*(8), 1951–1956. https://doi.org/10.3382/ps.2014-03939

Morris, A., Shanmugasundaram, R., McDonald, J., & Selvaraj, R. K. (2015). Effect of in vitro and in vivo 25-hydroxyvitamin D treatment on macrophages, T cells, and layer chickens during a coccidia challenge. *Journal of Animal Science*, *93*(6), 2894–2903. https://doi.org/10.2527/jas.2014-8866

Norman, A. W. (2008). From vitamin D to hormone D: Fundamentals of the vitamin D endocrine system essential for good health. *American Journal of Clinical Nutrition*, *88*(2), 491S–499S. https://doi.org/10.1093/ajcn/88.2.491S

Okano, T., Tsugawa, N., Morishita, A., & Kato, S. (2004). Regulation of gene expression of epithelial calcium channels in intestine and kidney of mice by 1α,25-dihydroxyvitamin D3. *The Journal of Steroid Biochemistry & Molecular Biology*, *89–90*(1–5), 335–338. https://doi.org/10.1016/j.jsbmb.2004.03.025

Ouyang, W., & O'Garra, A. (2019). IL-10 family cytokines IL-10 and IL-22: From basic science to clinical translation. *Immunity*, *50*(4), 871–891. https://doi.org/10.1016/j.immuni.2019.03.020

Prokoski, K., Bittencourt, L. C., Teixeira, L. V., Bortoluzzi, C., Vanroo, E., Palma, S., & Fernandes, J. I. (2021). Classic and non-classic effects of the duration of supplementation of 25-hydroxicholecalciferol in broiler chicken diets. *Animals: An Open Access Journal from MDPI*, *11*(10), 2971. https://doi.org/10.3390/ani11102971

Rasheed, M. A., Tiwari, U. P., Oelschlager, M. L., Smith, B. N., Jespersen, J. C., Escobar, J., & Dilger, R. N. (2020). Dietary supplementation with anti-IL-10 antibody during a severe Eimeria challenge in broiler chickens. *Poultry Science*, *99*(12), 6493–6502. https://doi.org/10.1016/j.psj.2020.08.070

Sardar, S., Chakraborty, A., & Chatterjee, M. (1996). Comparative effectiveness of vitamin D3 and dietary vitamin E on peroxidation of lipids and enzymes of the hepatic antioxidant system in Sprague-Dawley rats. *International Journal for Vitamin and Nutrition Research. Internationale Zeitschrift Fur Vitamin-Und Ernahrungsforschung. Journal International de Vitaminologie et de Nutrition*, *66*(1), 39–45.

Sechman, A., Shimada, K., Saito, N., Ieda, T., & Ono, T. (1996). Triiodothyronine (T3) enhances the stimulatory effect of 1,25-dihydroxyvitamin D3 on calbindin-D28k mRNA expression in the kidney and intestine but not in cerebellum of the chick. *Asian-Australasian Journal of Animal Sciences*, *9*(1), 37–44. https://doi.org/10.5713/ajas.1996.37

Sibbald, I. R. (1976). A bioassay for true metabolizable energy in feedingstuffs. *Poultry Science*, *55*(1), 303–308. https://doi.org/10.3382/ps.0550303

Sturkie, P. D. (2000). *Avian physiology* (5th ed.). Academic Press.

Suzuki, T., Yoshinaga, N., & Tanabe, S. (2009). Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. *Journal of Biological Chemistry*, *284*(10), 6519–6527. https://doi.org/10.1074/jbc.M802161200

Thrall, M. A., Weiser, G., Allison, R. W., & Campbell, T. W. (2012). *Veterinary hematology and clinical chemistry* (2nd ed.). Wiley-Blackwell.

Vazquez, J. R., Gómez, G. V., López, C. C., Cortés, A. C., Díaz, A. C., Fernández, S. R. T., Rosales, E. M., & Avila, A. G. (2018). Effects of 25-hydroxycholecalciferol with two D3 vitamin levels on production and immunity parameters in broiler chickens. *Journal of Animal Physiology & Animal Nutrition*, *102*(1), e493–e497. https://doi.org/10.1111/jpn.12696

Vignale, K., Caldas, J. V., England, J. A., Boonsinchai, N., Sodsee, P., Putsakum, M., Coon, C. N., & Coto, C. (2015). The effect of 25-hydroxycholecalciferol (OHD3) as compared to cholecalciferol (vitamin D3) on pectoralis major muscle development and myopathy incidence in Ross 708 male broilers. *Poultry Science*, *94*(10), 2371–2378. https://doi.org/10.3382/ps/pev195

Wang, J., Qiu, L., Gong, H., Celi, P., Yan, L., Ding, X., Bai, S., Zeng, Q., Mao, X., Xu, S., Zhang, K., & Yin, J. (2020). Effect of dietary 25-hydroxycholecalciferol supplementation and high stocking density on performance, egg quality, and tibia quality in laying hens. *Poultry Science*, *99*(5), 2608–2615. https://doi.org/10.1016/j.psj.2019.12.054

Wu, L., Wang, X., Lv, X., He, L., Qu, H., Shi, C., Zhang, L., Zhang, J., Wang, Z., & Han, J. (2022). 1,25-Dihydroxycholecalciferol improved the growth performance and up-regulated the calcium transporter gene expression levels in the small intestine of broiler chickens. *Journal of Poultry Science*, *59*(2), 129–136. https://doi.org/10.2141/jpsa.0210125

Yang, X., Wang, X. N., Zhang, N., Qu, H. X., Shi, C. X., Zhang, J. L., Lv, X. L., & Han, J. C. (2019). Differentially expressed genes in vitamin D regulating calcium and phosphorus absorption in duodenum of broiler chickens using RNA-sequencing. *Chinese Journal of Animal Nutrition*, *31*, 5202–5213.

Yoo, J., Yi, H., Kang, C., Kim, Y., Kim, B., Kim, H., Lee, H. S., & Lillehoj, H. S. (2008). Role of interleukin-17 in disease resistance and growth performance of commercial broiler chickens. *Veterinary Immunology and Immunopathology*, *123*(1–2), 149–158. https://doi.org/10.1016/j.vetimm.2008.01.014

Yuan, J. S., Reed, A., Chen, F., & Stewart, C. N.Jr. (2007). Statistical analysis of real-time PCR data. *BMC Bioinformatics*, *8*(1), 85. https://doi.org/10.1186/1471-2105-8-85

Zhang, H. Y., Zeng, Q. F., Bai, S. P., Wang, J. P., Ding, X. M., Xuan, Y., Su, Z. W., Fraley, G. S., Yao, B., & Zhang, K. Y. (2020). Effect of dietary 25-hydroxycholecalciferol on the sternal mass of meat ducks under different vitamin regimens. *Poultry Science*, *99*(3), 1241–1253. https://doi.org/10.1016/j.psj.2019.10.063

Zhang, L., & Piao, X. (2021). Use of 25-hydroxyvitamin D3 in diets for sows: A review. *Animal Nutrition (Zhongguo xu mu Shou yi Xue Hui)*, *7*(3), 728–736. https://doi.org/10.1016/j.aninu.2021.01.006

Zhang, L. H., He, T. F., Hu, J. X., Li, M., & Piao, X. S. (2020). Effects of normal and low calcium and phosphorus levels and 25-hydroxycholecalciferol supplementation on performance, serum antioxidant status, meat quality, and bone properties of broilers. *Poultry Science*, *99*(11), 5663–5672. https://doi.org/10.1016/j.psj.2020.07.025