Original Article

Effects of 25-Hydroxyvitamin D_3 supplementation as a partial replacement for vitamin D_3 on laying performance, egg quality, and bone traits in late-stage laying hens

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Abstract

The objective of this study was to assess the impact of dietary 25-hydroxy vitamin D_3 ($25OHD_3$) supplementation during the late laying hens on egg quality, hen performance, and bone computerized tomography (CT) scanning parameters. In total, 24,862 Hy-Line Brownmax hens 61 weeks old were randomly divided into two groups: the control group (n = 12,368) received a diet containing vitamin D_3 at 3,000 IU/kg, and the treatment group (n = 12,494) received a diet containing vitamin D3 at 1,500 IU/kg and $25OHD_3$ at 1,500 IU/kg. The dietary intervention from 61 weeks of age to 75 weeks of age. At the start of the supplementation period, 2,400 hens were randomly selected (1,200 from each group) for individual body weight measurements, which were repeated after 15 weeks of dietary treatment. The three hens per group were randomly euthanized at 72 weeks of age. The femoral bones were harvested and analyzed using histomorphometry and micro-CT scanning. A significantly reduced feed intake was observed for hens in the treatment group compared with the control group (120.0 vs. 125.4 g/day, $P < 0.001$). No effects of $25OHD_3$ supplementation on body weight, body weight gain, or FCR were found ($P > 0.05$). Eggshell thickness was higher in the treatment group compared with the control group (0.390 vs. 0.380, $P = 0.030$). The egg mass in the treatment group was significantly lower than in the control group (53.3 ± 0.4 g vs. 56.2 ± 0.4 g, $P < 0.001$). Furthermore, in the treatment group, bone volume, bone surface, bone surface density, and connectivity density were higher in the treatment group compared to the control group ($P < 0.05$). However, the trabecular bone or the trabecular area was not affected ($P > 0.05$). In conclusion, $25OHD_3$ supplementation affected egg quality and bone traits of late-production laying hens.

Keywords: 25-hydroxy vitamin D_3, bone quality, egg, late production, laying hen

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Introduction

Egg-laying hens require high levels of calcium for eggshell formation, often facing challenges like osteoporosis during the late egg-laying phase (Whitehead, 2004). The dissolution of calcium from bones during this phase leads to significant economic losses and impacts the overall health of hens (Whitehead, 2004; Kim *et al.*, 2012). Medullary bones play a key role in calcium storage for eggshell formation, experiencing calcium depletion at rates up to 10–15 times higher than cortical bones (Hurwitz, 1965).

Vitamin D is critical for maintaining bone health and regulating calcium homeostasis. Its active form, 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$), enhances calcium absorption in the intestines and promotes its deposition in bones, which directly influences bone health, egg production, and eggshell quality (Rodriguez-Lecompte *et al.*, 2016; Swiatkiewicz *et al.*, 2017). Animals typically obtain vitamin D from dietary cholecalciferol (vitamin D_3), which undergoes hydroxylation in the liver to form 25-hydroxyvitamin D_3 ($25OHD_3$) and is further converted into $1,25(OH)2D_3$ in the kidneys. Dietary supplementation with vitamin D or its metabolites plays a critical role in sustaining egg production in laying hens, particularly during prolonged laying periods when consistent nutrient intake is essential (Chen *et al.*, 2020a). Various forms of vitamin D are currently used in poultry nutrition, each with distinct properties. For instance, vitamin D_3 (cholecalciferol) is stable in feed and widely utilized, yet increasing its dietary levels beyond standard recommendations has shown limited effects on egg production and bone quality (Keshavarz, 2003; Mattila *et al.*, 2004; Persia *et al.*, 2013). In contrast, $25OHD_3$, a bioactive metabolite of vitamin D_3, bypasses hepatic hydroxylation and exhibits higher bioavailability, allowing for more efficient calcium absorption, enhanced bone mineralization, and improved eggshell quality (Keshavarz, 2003; Swiatkiewicz *et al.*, 2017; Chen *et al.*, 2020a). Many studies have demonstrated the beneficial effects of $25OHD_3$ on skeletal integrity and eggshell strength, even under suboptimal nutritional conditions (Adhikari *et al.*, 2020; Li *et al.*, 2023). However, findings on its effectiveness have been somewhat inconsistent, potentially due to variations in supplementation timing, duration, and dosage across studies (Frost *et al.*, 1990; Kappeli *et al.*, 2011; Mattila *et al.*, 2011; do Nascimento *et al.*, 2014). Despite this, the inclusion of $25OHD_3$ remains a promising strategy to mitigate bone mineral loss and maintain egg production performance in aging hens. In this context, combining $25OHD_3$ at 1,500 IU/kg with an equal dose of vitamin D_3 represents a physiologically balanced approach that avoids reliance on high-dose cholecalciferol while enhancing overall vitamin D status. This dual-supplementation strategy has shown potential in supporting bone health, maintaining eggshell quality, and improving the overall performance of laying hens during late production phases (Chen *et al.*, 2020a; Adhikari *et al.*, 2020).

To assess bone changes, especially in small-sized bones such as those of laying hens, micro-computerized tomography (micro-CT) is a standard tool that offers high-resolution and rapid imaging capabilities. Micro-CT has been used to analyze bones in humans, mice, and various other species, enabling the precise detection of subtle changes in bone structure (Ritman, 2007; Bouxsein *et al.*, 2010). This study hypothesizes that $25OHD_3$ supplementation during the late production phase of laying hens will enhance bone health and egg quality more effectively than standard vitamin D_3. The present study investigates the combined use of $25OHD_3$ and vitamin D_3 at moderate levels during the late laying period—a critical phase often characterized by deteriorating shell quality and bone health. Therefore, the objective of this study was to evaluate the effect of $25OHD_3$ supplementation during the late production phase of laying hens on bone computerized tomography (CT) scanning parameters and laying hen performance.

Materials and Methods

Ethics Statement: The experiment was reviewed and approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Science, Chulalongkorn University (2231009).

Animals, Diets, and Experimental Design: The study was conducted at a commercial laying hen farm located in Thailand, involving a total of 24,862 Hy-Line Brownmax hens, which were 61 weeks of age (2 groups × 1 repetition × 8 hens per cage). The hens were housed in an evaporative cooling system. All hens were randomly divided into two groups: a control group (n = 12,368) and a treatment group (n = 12,494) housed in wire cages. The cages measured 90 cm in length, 46 cm in width, and 38 cm in height. Different dietary regimens were administered: in the control group, the hens received vitamin D_3 at a concentration of 3,000 IU/kg feed. In the treatment group, the hens received a combination of vitamin D_3 at 1,500 IU/kg feed and 25-hydroxyvitamin D_3 (Bio D®, Huvepharma, Bangkok, Thailand; 69.7 mg/kg) at 1,500 IU/kg feed from 61 weeks of age to 75 weeks of age (a 15-week intervention period). The feed formulation during the experimental period was based on the Hy-Line Brownmax nutritional guide (2016) and is described in Table 1. Diet samples were analyzed to measure crude protein (AOAC, 2016; method 2001.11), crude fat (AOAC, 2016; method 920.39), and crude fiber (AOAC, 2016; method 978.10), respectively. Water and feed were provided ad libitum throughout the study. All hens were subjected to a continuous lighting program consisting of 16 hrs of light followed by 8 hrs of darkness.

Determination of performance in lay: In total, 2,400 hens were individually weighed weekly (1,200 hens × 2 groups × 15 weeks). The percentage of hen day production (HDP), which was defined as the total number of eggs laid by a group of hens during a specific week divided by the original number of hens at the beginning of the experiment, and feed intake was recorded weekly and calculated throughout the study period. Additionally, parameters such as body weight gain and feed conversion ratio (FCR) (feed intake/egg

mass from 61 to 75 weeks of age) were calculated based on the recorded data. Egg production was also monitored and recorded daily to assess the overall productivity of the laying hens.

Table 1 Diet formulation and calculated nutrient composition for the late production phase of laying hens.

Feed formulation	**%**
Ingredients, %	
Corn	64.69
Soybean meal-48%	21.45
Soybean oil	1.89
Limestone	9.28
Mono dicalcium phosphate-21%	1.66
Sodium bicarbonate	0.55
Salt	0.16
DL-Methionine	0.07
Premix†	0.25
Nutrient composition‡, %	
Metabolizable energy (kcal/kg)	2,800
Crude fat	4.6
Crude fiber	2.9
Crude protein	15.0
Lysine	0.75
Methionine	0.32
Methionine + Cysteine	0.59
Threonine	0.57
Total Ca	3.89
Available P	0.39
Sodium	0.29
Chloride	0.14
Potassium	0.66

†Supplied per kg/diet: Vitamin A, 10,000 IU; Vitamin D_3, 2,500 IU; Vitamin E, 15 IU; Vitamin K 3 mg; Biotin, 0.025 mg; Folic acid, 0.5 mg; Niacin, 30 mg; Pantothenic acid, 8 mg; Riboflavin B2, 4 mg; Thiamin B1, 1mg; Pyridoxine B6, 3 mg; Vitamin B12, 15 microgram; Choline, 890.72; Copper, 5 mg; Iodine, 0.5 mg; Iron, 25 mg; Manganese, 100 mg; Selenium, 0.2 mg; Zinc, 60 mg

‡Analyzed for crude protein (AOAC, 2016; method 2001.11), crude fat (AOAC, 2016; method 920.39), and crude fiber (AOAC, 2016; method 978.10), respectively.

Egg Quality: During the entire laying period from 61 to 75 weeks of age, egg quality was assessed weekly throughout the experimental period. In total, 900 eggs were randomly collected (30 eggs × 2 groups × 15 weeks). Measurements of egg quality, including Haugh unit, shell strength, shell thickness, egg weight, and egg mass, were measured using a digital egg tester (DET-6500, Nabel®, Atscience Trading Co., Ltd., Chiang Mai, Thailand). Various additional parameters, such as grade egg, undergrade egg, and cumulative egg production, were recorded to comprehensively evaluate egg quality throughout the study.

Bone Collection: At 72 weeks of age, three hens per group were randomly selected and euthanized humanely. Both femurs were harvested, the soft tissue was removed, and the bone quality was analyzed. The right femoral bone was prepared for histomorphometry, while the left femoral bone was used to assess bone mineralization using micro-CT scanning.

Histomorphometry: The right femur was fixed and decalcified using 10% neutral buffered formalin mixed with 3.5% nitric acid (v/v) for 14 days, following the method of Thorat *et al.* (2011) with minor modifications. Subsequently, the bone was sectioned beneath the femoral head and processed for routine histological analysis. In brief, a 4 µm section of tissue was fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. The sections were examined under a light microscope. Three regions of interest (ROIs) were evaluated at 400× magnification. ImageJ software (National Institutes of Health, Maryland, USA) was employed to analyze various parameters, including osteoid area and trabecular separation (Abulmeaty, 2017).

Micro-CT Scanning: The left femur was fixed in 10% neutral buffered formalin for 24 hours, dried, and then preserved for micro-CT scanning. In brief, the bone was placed into a micro-CT scanner (Skyscan 1173; Bruker micro-CT, Kontich, Belgium) at the same location for the histopathologic evaluation. Prior to the CT evaluation, the femur sample was placed on the sample holder, and the bone was scanned using a micro-CT scanner (Skyscan 1173; Bruker micro-CT, Belgium) with the following scanning parameters: random movement with 360° scanning; 70 kVp; 114 mA; exposure time, 250 ms; 0.4° of rotation; three average frames; scan resolution at 1120 × 1120; and pixel size of 44.86 µm. A 1.0 mm aluminum filter was added to reduce the beam hardening. After scanning, the appropriate alignment and mathematical method correction (20% beam-hardening correction, NA for smoothing, and 7% ring artifact reduction) was applied to all samples, followed by reconstruction using NRecon software (version 1.6.9.18, Bruker micro-CT, Belgium). The dynamic range was set at 0.0024. The 3D model of bone was analyzed using CTan software (version 1.14.10.07, Bruker micro-CT, Belgium). The threshold was set between 106 and 255 for all samples, with the ROI drawn to fit the shape of the bone. As the phantom was not applied for the calibration of the hydroxyapatite, the samples were compared between groups.

Various parameters were analyzed, including bone volume, percentage bone volume, bone surface, bone surface density, trabecular separation, number of closed pores, closed porosity (%), volume of open pore space, open porosity (%), total volume of pore space, total porosity (%), and connectivity density.

Statistical Analysis: Statistical analysis of all parameters was performed using the SAS program (SAS 9.6, SAS Institute, Cary, NC) to conduct multiple analyses of variance using a generalized linear model. The dependence variable included feed intake, FCR, egg production, egg quality (including Haugh unit, shell strength, shell thickness, egg weight, egg mass, grade egg, undergrade egg, and cumulative egg production), trabecular bone, trabecular area, and micro-CT parameters. The fixed effects in the model included the groups (control and treatment). A *P* value of < 0.05 was considered statistically significant.

Result

General Performance: The present study did not reveal any significant effect of the treatment group on body weight, body weight gain, or FCR ($P > 0.05$). However, laying hens in the treatment group exhibited a notable reduction in feed intake compared with the control group and a tendency toward lower FCG (Table 2).

Egg-Laying Performance: There was no significant effect of the treatment group on cumulative egg production ($P > 0.05$). However, hens in the treatment group exhibited lower HDP, egg weight, and egg mass compared with those in the control group ($P < 0.05$) (Table 3).

Egg Quality: In the treatment group, the egg grade and Haugh index were lower than in the control group ($P < 0.05$) (Table 4). However, the eggshell thickness was greater than in the control group ($P = 0.030$). Bone quality as Assessed Using Histomorphometry. There was no significant effect of the treatment group on the trabecular area and the trabecular bone ($P > 0.05$) (Figure 1A and 1B). The osteoid area and trabecular separation are presented in Figures 1C and 1D.

Bone Quality as Assessed Using Micro-CT: The bone volume, bone surface, bone surface density, and connectivity density were significantly higher in the treatment group than in the control group ($P < 0.05$) (Table 5). However, there was no significant effect of the treatment group on the number of closed pores, closed porosity (%), volume of open pore space, and total volume of pore space ($P > 0.05$). The micro-CT scans of the control and treatment groups are presented in Figure 2.

Table 2 Effect of 25-hydroxyvitamin D_3 supplementation on growth performance during the late production phase in laying hens from the control (n = 1,200) and treatment (n = 1,200) groups (least squares mean ± standard error of the mean).

Parameters	Control	Treatment	*P*-value
Body weight, kg	2.00 ± 0.01	2.01 ± 0.01	0.326
Body weight gain, g/day	4.22 ± 0.07	4.23 ± 0.07	0.921
Feed intake, g/day	125.4 ± 0.8	120.0 ± 0.8*	<0.001
FCR†	2.23 ± 0.02	2.25 ± 0.02	0.346
FCG‡	29.83 ± 0.47	28.48 ± 0.47	0.051

*Significant difference between groups at $P < 0.05$
†Feed Conversion Ratio
‡Feed Conversion per Gain

Table 3 Effect of 25-hydroxyvitamin D_3 supplementation on egg-laying performance during the late production phase in laying hens from the control (n = 12,368 hens; 450 eggs) and treatment (n = 12,494 hens; 450 eggs) groups (least squares mean ± standard error of the mean).

Parameters	Control	Treatment	*P*-value
Hen day production, %	87.0 ± 0.5	84.7 ± 0.5*	0.005
Egg weight†, g	64.6 ± 0.1	62.9 ± 0.1*	<0.001
Egg mass‡, g	56.2 ± 0.4	53.3 ± 0.4*	<0.001

*Significant difference between group $P < 0.05$
†Egg weight = mean average egg weight.
‡Egg Mass = Average Egg Weight × Total Number of Eggs

Table 4 Effect of 25-hydroxyvitamin D_3 supplementation on egg quality during the late production phase in laying hens from the control (n = 450) and treatment (n = 450) groups (least squares mean ± standard error of the mean).

Parameters	Control	Treatment	*P*-value
Egg grade†, %	92.4 ± 0.3	90.8 ± 0.3*	0.001
Undergrade egg, %	7.6 ± 0.3	9.2 ± 0.3*	0.001
Haugh Unit	95.5 ± 1.0	92.1 ± 1.0*	0.020
Shell strength, kg force	4.2 ± 0.1	4.2 ± 0.1	0.769
Shell thickness, mm	0.380 ± 0.004	0.390 ± 0.004*	0.030

*Significant difference between groups at $P < 0.05$
†Egg grade = Percentage of standardized egg.

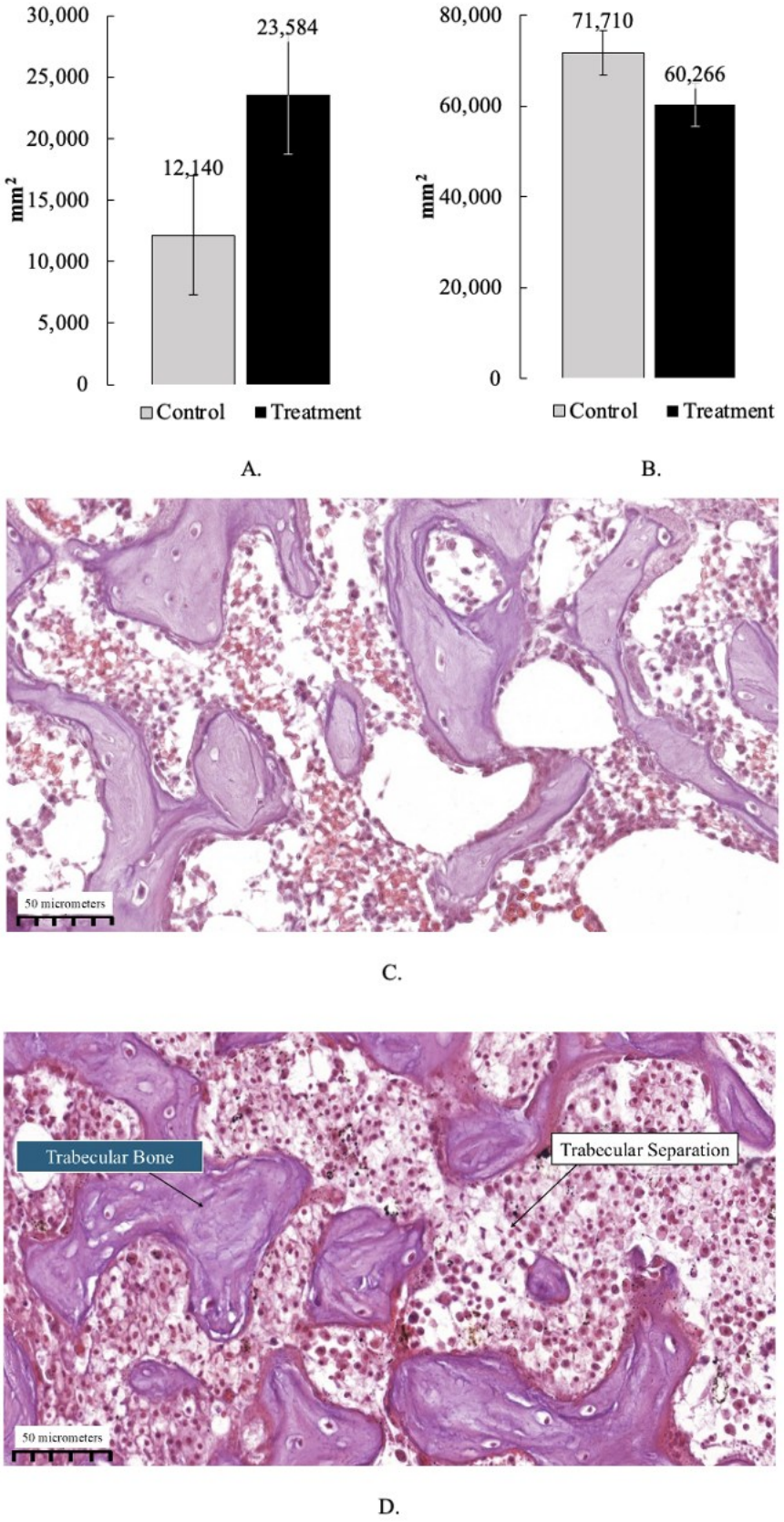

Figure 1 Histomorphometric differences in A) trabecular bone and B) trabecular area, and histomorphometry of the trabecular area and trabecular separation in C) the control and D) treatment groups at 72 weeks of age (n = 3 per group).

Table 5 Effect of 25-hydroxyvitamin D_3 supplementation on bone micro-CT parameters during the late production phase in laying hens (least squares mean ± standard error of the mean).

Parameters	Control	Treatment	*P* value
n	3	3	
Bone volume, mm^3	264.60 ± 18.96	332.90 ± 18.96*	0.044
Percent bone volume, %	32.94 ± 3.11	42.37 ± 3.11	0.076
Bone surface, mm^2	1371.85 ± 73.76	1756.45 ± 73.76*	0.010
Bone surface density, 1/mm	1.70 ± 0.08	2.22 ± 0.08*	0.005
Trabecular separation, mm	33.19 ± 0.41	31.91 ± 0.41	0.072
Number of closed pores, n	242.00 ± 36.33	227.75 ± 36.33	0.791
Closed porosity, %	0.79 ± 0.22	0.55 ± 0.22	0.456
Volume of open pore space, mm^3	538.60 ± 38.31	457.04 ± 38.31	0.183
Open porosity, %	66.80 ± 3.14	57.38 ± 3.14	0.078
Total volume of pore space, mm^3	540.68 ± 38.12	458.93 ± 38.12	0.180
Total porosity, %	67.06 ± 3.11	57.63 ± 3.11	0.076
Connectivity density, $1/mm^3$	0.54 ± 0.31	1.87 ± 0.31*	0.025

*Significant difference between groups at $P < 0.05$

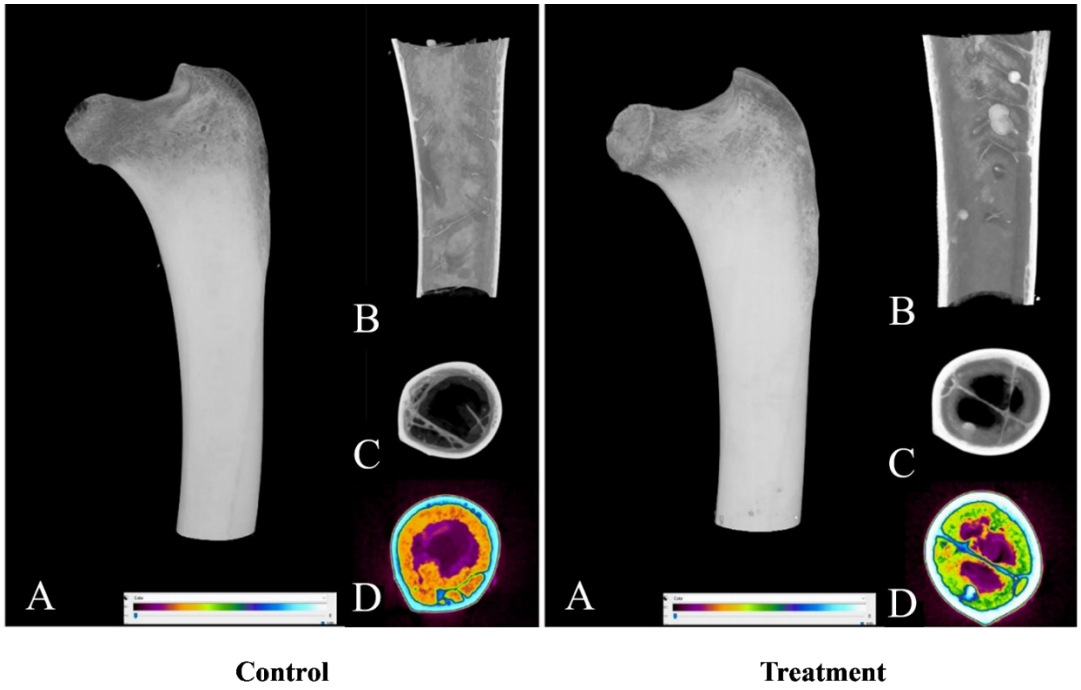

Figure 2 Three-dimensional micro-computed tomographic images of the left femur from hens in the 1) control group (n = 3) and 2) treatment group (n = 3), showing osteolytic lesions at the femoral head (A) and a thin bone cortex (B–D).

Discussion

Dietary supplementation of $25OHD_3$ for laying hens during various stages of their life cycle has been shown to impact various aspects of nutrition and egg production. In this study, significant changes were observed in several variables related to performance and bone quality as assessed using micro-CT in response to continuous $25OHD_3$ supplementation, even for a short period of application. Hens receiving $25OHD_3$ had notably lower feed intake compared with the control group, a finding consistent with previous studies (Li *et al.*, 2023). However, no significant difference in body weight gain was observed. The reduction in feed intake observed in the treatment group may be associated with the higher bio-efficacy and metabolic availability of $25OHD_3$, which is more readily absorbed and utilized compared to native cholecalciferol (vitamin D_3) (Keshavarz, 2003; Swiatkiewicz *et al.*, 2017). Because $25OHD_3$ bypasses the initial hepatic hydroxylation step, it may enhance calcium and phosphorus homeostasis more efficiently, thereby reducing the hens' physiological demand for additional nutrient intake (Chen *et al.*, 2020; Adhikari *et al.*, 2020). Furthermore, it has been suggested that vitamin D metabolites influenced hypothalamic signaling related to appetite regulation, though the exact mechanism in poultry remains unclear and warrants further investigation (Rodriguez-Lecompte *et al.*, 2016). This reduction in feed intake benefits poultry farmers by reducing production costs associated with feed expenses, which could provide economic advantages by reducing feed costs, though this did not translate into improved overall productivity metrics like egg production or feed conversion ratio.

Furthermore, eggshells were thicker in hens supplemented with $25OHD_3$ in the present study. This is possibly due to an enhanced mineral accumulation in the bones resulting from the $25OHD_3$ supplementation, which in turn increased the mineral availability for eggshell formation (Frost *et al.*, 1990). The $25OHD_3$ supplementation also led to a tendency to accumulate osteoid tissue in the medullary bones of the leg. Older laying hens often experience bone loss,

making their bones more fragile and prone to fracture. The increase in osteoid tissue and the reduction in trabecular area in the bones of laying hens supplemented with $25OHD_3$ suggest the bones were strengthened through the increase in bone mass via an alternative pathway (Li *et al.*, 2023).

Recent research highlights the essential role of vitamin D in eggshell formation, particularly through its influence on calcium transport and metabolism in the shell gland. The presence of vitamin D receptors (VDR) and metabolic enzymes such as 1α-hydroxylase facilitates local synthesis and regulation of active vitamin D metabolites, which are critical for calcium binding and transport proteins like CaBP-D28K during eggshell calcification (Hrabia *et al.*, 2023; Ohira *et al.*, 1998). These mechanisms are crucial for efficient calcium deposition in eggshells, especially in older hens with diminished vitamin D metabolism (Bar *et al.*, 1999). In the present study, the supplementation of $25OHD_3$ resulted in increased shell thickness. Previous studies have reported elevated plasma calcium levels in laying hens supplemented with $25OHD_3$ (Jing *et al.*, 2022; Gao *et al.*, 2024). Moreover, $25OHD_3$ bypasses the hepatic 25-hydroxylation required for cholecalciferol activation, allowing for more efficient systemic utilization of calcium (Käppeli *et al.*, 2011). This metabolic advantage likely contributes to the improved mineralization and structural integrity of the eggshells observed in the present study, particularly during the late laying period when calcium metabolism tends to decline. However, the trade-offs observed, such as reduced Haugh unit scores and increased undergrade eggs, may indicate an imbalance in calcium homeostasis, aligning with evidence that excessive vitamin D may disrupt hen reproductive performance (Bar, 2008).

This observation was supported by micro-CT scans of bone microstructures, which demonstrated increased bone volume and surface area in the treatment group supplemented with $25OHD_3$ compared to the control group (Chen *et al.*, 2020). The increase in bone volume and surface area observed in this study aligns with previous research that highlighted the anabolic effects of vitamin D on bone microstructure (Chen *et al.,* 2020b). Micro-CT analysis revealed significantly higher bone density and improved structural integrity in the treatment group in the present study, consistent with findings by Martineau *et al.* (2020), who demonstrated that $25OHD_3$ enhanced bone volume through its positive impact on bone mineral density. The increased surface area observed in this study is also similar to Calvi (2020), which emphasized the role of vitamin D analogs in promoting effective bone remodeling and mineralization. These effects are mediated by the stimulation of osteoblast activity, leading to bone formation and the suppression of osteoclast-mediated resorption (Sun *et al.*, 2016; Calvi, 2020). Additionally, $25OHD_3$ supplementation maintained calcium homeostasis without inducing hypercalcemia, which is essential for the observed improvements in bone health (Martineau *et al.*, 2020). However, the results underscore the importance of careful dose optimization to maximize bone health benefits while minimizing potential risks, such as increased osteoclast activity and bone loss associated with excessive vitamin D supplementation (Sun *et al.*, 2016). Although a decrease in internal pore space within non-mineralized bone tissue was suggested by trabecular separation measurements, micro-CT results revealed increased mineral accumulation, indicating that the bones of hens receiving $25OHD_3$ supplementation were denser and contained more minerals than those of the control group.

The limitation of the present study lies in the relatively small number of hens used for bone assessment via micro-computed tomography (micro-CT), which may affect the robustness and generalizability of the skeletal findings. Additionally, the experiment was conducted under commercial field conditions, where environmental and management factors may have influenced the results. Future studies should consider evaluating the long-term impact of combined vitamin D_3 and $25OHD_3$ supplementation on skeletal integrity and production performance throughout the entire laying cycle to strengthen the evidence base.

In conclusion, dietary supplementation with $25OHD_3$ supplementation affected egg quality and bone traits of late-production laying hens. However, due to the limited sample size in bone analysis, these findings should be interpreted with caution and warrant further investigation in larger populations.

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