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Dietary 25-hydroxy-cholecalciferol and additional vitamin E improve bone development and antioxidant capacity in high-density stocking broilers

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Abstract

This study aimed to investigate the effects of diets supplemented with 25-hydroxycholecalciferol [**25-(OH)D3**] and additional vitamin E on growth performance, antioxidant capacity, bone development, and carcass characteristics at different stocking densities on commercial broiler farms. A total of 118,800 one-day-old Arbor Acres broilers were assigned to a 2 × 2 factorial treatment consisting of two dietary vitamin levels (5,500 IU vitamin D3 and 60 IU vitamin E: normal diet, using half 25-(OH)D3 as a source of vitamin D3 and an additional 60 IU of vitamin E: 25-(OH)D3+VE diet) and two stocking densities (high density of 20 chickens/m^2: HD and 16 chickens/m^2: LD). The experiment lasted for 42 d. The results showed that high-density stocking negatively affected the growth performance of broilers during the first four weeks, whereas the vitamin diet treatment significantly improved the feed conversion ratios (**FCR**) during the last 2 wk. Vitamin diets increased catalase at 14 and 42 d, and the glutathione peroxidase (**GSH-px**) levels at 42 d in high-density-stocked broilers. The interaction showed that serum vitamin E levels were significantly improved at 28 d of age in high-density-stocked broilers as a result of the vitamin diets. Stocking density and dietary treatments were found to significantly affect bone development, with the vitamin diet significantly increasing metatarsal length and femoral bone strength in broilers from high-density stocking density at 28 d of age. High stocking density increased the proportion of leg muscles and meat yield per square meter. In general, 25-(OH)D3 and additional vitamin E suppressed oxidative stress and ameliorated the negative effects of high-density stocking on bone development in a commercial chicken farm setting. Vitamin diets improved the FCR of broilers, while high-density stocking resulted in better economic outcomes.

Lay Summary

High-density stocking is often associated with animal welfare risks in broilers, mainly in terms of oxidative stress and bone development. Nevertheless, farming at too low a density remains for the most part economically unviable. Modulation of antioxidant capacity and bone development by nutritional strategies in high-density-farmed broilers has proven an effective tool in developing countries. Therefore, the present study investigated the effects of applying diets with a higher biological potency of vitamin D3 25-hydroxycholecalciferol [25-(OH)D3] and a higher concentration of vitamin E on broiler production performance, antioxidant capacity and meat production performance at different densities of stocking under commercial farming conditions. The results indicated that the vitamin dietary treatments suppressed oxidative stress and ameliorated the negative effects of high-density farming on bone development.

Key words: 25-hydroxy-cholecalciferol, broiler, oxidative stress, stocking density, vitamin E

Abbreviations: 25-(OH)D3, 25-hydroxycholecalciferol; ADFI, average daily feed intake; ADG, average daily gain; CAT, catalase; FCR, feed conversion ratio; GSH-px, glutathione peroxidase; HD, high stocking density; LD, low stocking density; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase

Introduction

Stocking density is inextricably linked to animal welfare and broiler production. In developed countries or regions, the welfare of broilers is regulated through two strategies: the development of animal welfare regulations and the marketing of market-specific labels at a premium (Oviedo-Rondón, 2022; Sandøe et al., 2022). For instance, the legal limit for stocking density of broilers in the EU has been 42 kg/m^2 since 2007. In contrast, broilers in organic production systems are stocked as low as 20 kg/m^2 (Marchewka et al., 2022). The objective of high-density broiler farming is to reduce energy and labor costs, yet this may adversely affect poultry health and ultimately broiler performance and chicken meat quality (Nasr et al., 2021; Wang et al., 2022). Researchers have identified farming density as an important cause of stress in broilers. Since birds are covered in feathers, increased stocking density can reduce the bird's ability to dissipate heat, indirectly inducing heat stress (Stamp Dawkins et al., 2004).

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As such, this suggests that broiler feeding behavior is significantly restricted as stocking density increases. Heat stress, disease, and starvation caused by high-density rearing can also directly contribute to broiler oxidative stress, which is a result of a disruption in the balance of pro-oxidants and antioxidants in the body (Rahal et al., 2014). A range of antioxidant enzymes makes up a complex antioxidant defense system, including glutathione peroxidase (**GSH-px**), superoxide dismutase (**SOD**), and catalase (**CAT**) (Kong et al., 2022b). These antioxidant enzymes scavenge already-formed reactive oxygen species (**ROS**) during the onset of oxidative stress and prevent an increase in the levels of harmful products such as malondialdehyde (**MDA**). In contrast, acute or prolonged oxidative stress also promotes an inflammatory response that can cause intestinal disease and even death in poultry (Lin et al., 2006). It has also been argued that environmental differences provided by broiler producers cause a greater impact on animal welfare than the stocking density itself, indicating that other factors in the production system amplify the negative effects (Kumar et al., 2022). Therefore, how to evaluate a reasonable stocking density has been a controversial topic, but there is no doubt that broiler antioxidant enzyme levels, growth performance, and mortality can reflect the health status and stress intensity of broilers raised at different densities.

Owing to the low cost and extensive research, the use of nutritional strategies to mitigate oxidative stress in broilers is commonplace. Probiotics, prebiotics, polyphenolic compounds, medium-chain fatty acids, and vitamin E have been shown to protect cells from oxidative damage by increasing the activity of antioxidant enzymes, subsequently avoiding various pathologies and impairing animal welfare in broilers (Mishra and Jha, 2019). Vitamin E consists of forms belonging to two groups: tocopherols and tocotrienols, which are concentrated in the membrane-rich fractions of the body, such as the mitochondria and microsomes, whereby they are able to protect cell membranes from lipid peroxidation (Surai et al., 2019). The α-tocopherol form is the most biologically active form of vitamin E, reducing lipid peroxide radicals in biological membranes to lipid hydrogen peroxide; reducing free radical levels, which is known to play a pivotal role in preventing oxidative damage (Sahin et al., 2001). The supplementation of vitamin E in diets has been shown to promote higher plasma concentrations of vitamin E and to maintain growth performance and antioxidant capacity in broilers (Niu et al., 2009).

Vitamin D3 is an essential vitamin for poultry growth, promoting calcium and phosphorus absorption and metabolism as well as maintaining normal bone development (Jiang et al., 2015). Studies have shown that the addition of appropriate levels of vitamin D3 to diets can improve growth performance and increase ash content in the tibiae of broilers (Atencio et al., 2005). 25-hydroxycholecalciferol [**25-(OH)D3**] is the bioactivated form of vitamin D3. Given that it is able to enter the portal vein directly without needing to be absorbed with fatty acids, 25-(OH)D3 has a higher absorption efficiency than its inactive counterpart (Ong et al., 2021). Numerous studies on 25-(OH)D3 in feed have demonstrated improved body weight gain, reduced incidence of leg disease, and increased calcium and phosphorus utilization in broilers (Liem, 2009; Garcia et al., 2013). At high stocking densities, fewer chickens are able to maintain a normal gait, which limits broilers' free access to feeders (Dozier 3rd et al., 2005).

With increasing stocking density, the strength of broiler tibial bones decreases, which may be due to reduced bird activity in crowded spaces (Ma et al., 2020). Moreover, due to the cramped living space, broilers adopt a sitting posture more frequently, which causes greater compression of the leg bones (Buijs et al., 2012).

It is generally accepted that the application of vitamin E and 25-(OH)D3 in diets can improve the antioxidant capacity and skeletal development of broilers, but little research has been conducted on the potential implications of vitamin E and 25-(OH)D3 under different stocking densities. Research performed on commercial farms is perfectly suited to industry production but is underutilized due to the high costs and complex processes involved. On this basis, the aim of this study was to investigate the effects of supplementation with these two vitamins on broiler performance, antioxidant capacity, bone development, and meat production efficiency under different-density farming environments.

Materials and Methods

Experimental design and animal management

All experimental procedures were approved by the Ethics Committee of Shandong Agricultural University (No. SDAUA-2022-50) and conducted in accordance with the Ministry of Science and Technology (Beijing, People's Republic of China) Guide for Laboratory Animals. In all cases, feeding and euthanasia procedures were carried out with due consideration for animal welfare.

A total of 118,800 one-day-old Arbor Acres (**AA**) broilers of mixed sex were obtained from a commercial hatchery (Luanping Huadu Company, China) and transferred to six standardized commercial farming chicken houses of 19,800 birds each. The experimental treatments followed a 2 × 2 factorial design and consisted of two dietary vitamin levels (a standard diet (normal diet: 60 IU/kg vitamin E and 5,500 IU/kg vitamin D3) and a vitamin treatment [25-(OH)D3+VE diet]: an additional 60 IU/kg vitamin E and half 25-(OH)D3 [69 μg/kg, 1 μg 25-(OH)D3 is equivalent to 40 IU of vitamin D3] as a source of vitamin D3 and two stocking densities (high density of 20 chickens/m^2 [HD] and low density of 16 chickens/m^2 [LD]). Each treatment consisted of six replicates (n = 6), with all treatments included in each chicken house. All birds were randomly allocated to metal cages (70 cm × 70 cm × 42 cm, the area of each cage was 0.49 m^2) with automatic feeders and nipple drinkers. In accordance with the area of each cage, there were 10 birds per cage for the HD group and 8 birds per cage for the LD group. All six identical chicken houses covered all four treatments, with each treatment including 550 cages.

The base diet for the experiment was pelleted, and the feed composition and nutrient concentrations are shown in Table 1. The vitamin premix was processed by DSM Vitamins Co. Ltd. (Chengdu, China), and the concentrations of vitamins were detected using the high-performance liquid chromatography method. The results were presented in the same table as the feed composition. The basal diet was formulated according to the National Research Council recommendations for broilers, and normal vitamin levels were developed with reference to the nutritional standards of the breeding company (Aviagen). Depending on the characteristics of the automatic feeding system, birds on different diets were distributed according to the location of the automatic feeder, but

Table 1. Composition and nutritional levels of the experimental diet[1,2,3]

Items	1 to 21 d	22 to 42 d
Ingredient (%)		
Corn	60	65.33
Soybean meal (43%)	28.8	27.81
Corn protein flour (60%)	5.3	2.7
Salt	0.16	0.18
Baking soda	0.2	0.20
Limestone	1.3	1.1
Dicalcium phosphate	0.75	1.2
Soybean oil	2.2	4.10
Vitamin premix[2]	0.03	0.03
Mineral premix[3]	0.2	0.2
Choline chloride (50%)	0.1	0.1
Methionine	0.23	0.15
Lysine (70%)	0.58	0.70
Threonine (98.5%)	0.134	0.20
Phytase (20,000 U)	0.02	0.02
Total	100	100
Nutritional level		
Metabolizable energy	2,850 (kcal/kg)	3,050 (kcal/kg)
Crude protein	21.5	19.5
Lysine	1.26	1.19
Methionine	0.57	0.55
Calcium	0.60	0.72
Total phosphorus	0.60	0.56
Available phosphorus	0.40	0.36

[1]The vitamin diet was added to a bespoke vitamin premix. The 25-hydroxycholecalciferol content was 69 μg/kg.
[2]Provided per kilogram of compound diet: vitamin A, 12,000 IU; vitamin D3, 5,500 IU; vitamin E, 60 mg; vitamin K, 3.2 mg; vitamin B1, 3.2 mg; vitamin B2, 8.6 mg; nicotinic acid, 65 mg; pantothenic acid, 20 mg; vitamin B6, 4.3 mg; biotin, 0.22 mg; folic acid, 2.2 mg; vitamin B12, 0.017 mg. Nutrition levels shown are calculated values.
[3]Provided per kilogram of compound diet: I, 1.25 mg; Fe, 20 mg; Mn, 120 mg; Se, 0.3 mg; Zn, 110 mg. Nutrition levels shown are calculated values.

we ensured that each treatment group was in a substantially similar environment and that the two diets were stored in separate tanks to ensure that the trials were carried out properly. In particular, the first four of the eight rows of cages were selected to receive the normal diet and the last four rows to receive 25-(OH)D3+VE diet. Half of the birds in each row were assigned to the HD group, and the remaining birds to the LD group. The broilers were housed in environmentally controlled rooms where feed and water were freely available. On the first day, the temperature was 34 °C, and it gradually decreased to 26 °C by the 21st day, after which it remained constant. A relative humidity of 70% was maintained in the chicken house for the first three days and between 55% and 65% for the remainder of the time. Chickens were exposed to 23 h of light and 1 hour of darkness for the first week, gradually progressing to 20 h of light and 4 h of darkness. Vaccinations were administered by drinking water with Newcastle disease and infectious bronchitis vaccines on day 6. Antibiotics were strictly prohibited throughout the feeding process.

Sample collection

On days 14, 28, and 42, two birds of near average body weight were selected for sample collection (after 12 h of fasting) in each replicate. Approximately 5 mL of blood were collected from the wing vein using a sterile syringe into vacuum tubes without anticoagulant, left for 30 min, and then centrifuged for 10 min at 3,000 rpm and 4 °C to obtain serum, which was stored in a −20 °C freezer for further analysis. Broilers were slaughtered using a cervical dislocation, and the femur, tibia, and metatarsus on the left side of each chicken were carefully removed from the soft tissue and stored at −20 °C for testing bone quality and metrics.

The pectoralis and leg muscles on the same side were removed intact, bagged, and labeled before being promptly transferred to a 4 °C environment for further meat quality analysis. On the other side of the body, 2 to 3 g muscle samples were collected from the pectoralis and leg muscles at the same position each time and stored at −20 °C for indexing.

Daily feed consumption was obtained automatically from the coop feeding control system. Feed consumption was counted for each replicate, and average daily feed intake (**ADFI**) was calculated on days 14, 28, and 42. On days 14 and 28, 20% of the cages were evenly sampled according to spatial distribution for each replicate before weight was measured and average daily gain (**ADG**) was calculated. The feed conversion ratio (**FCR**) was defined as ADFI:ADG and dead birds were weighed and used to correct for FCR. The experiment was completed on day 42 when all broilers were weighed and counted.

Carcass characteristics

Upon sample collection at 42 d of age, broilers with similar weights were weighed, slaughtered, and subsequently soaked in hot water at 60 °C for 60 s to facilitate the removal of feathers. After stripping the feathers, each bird was dissected, and the digestive tract, abdominal fat, and internal organs were removed as part of the carcass weight. The abdominal fat, pectoralis and leg muscles on the same side were stripped intact, weighed, and calculated as a percentage of live weight before slaughter before being statistically analyzed. The weight of carcasses obtained per square meter of farmed area was used as an indicator (meat yield efficiency) to assess the efficiency of meat production in the different treatments.

Analysis of antioxidant capacity

The levels of CAT, GSH-px, MDA, and SOD in the serum were measured using commercial kits to assess the antioxidant capacity. The kits (CAT, ref no.: A007-1; GSH-px, ref no.: A005-1; MDA, ref no.: A003-1; SOD, ref no.: A001-1) were provided by Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). All assay procedures were carried out in strict accordance with the manufacturer's instructions. The CV of the data was <5%, and the interbatch CV was <8%.

Bone properties

Femur, tibia, and metatarsal samples placed in sealed bags were thawed at room temperature, and the length of the fully thawed bone samples was measured and recorded using Vernier calipers. The length of the bone was defined as the straight-line distance along the bone shaft between the two crests. The weight of the bones was obtained by weighing on an analytical balance. The mechanical properties of the bones were examined using a physical properties tester (EZ-LX HS, Shimadzu, Japan). To measure bone strength, two fulcrums 30 mm apart supported the ends of the bone, and pressure was applied at a constant rate of 10 mm/min until the bone fractured.

Bone content analysis

The numbered femur, tibia, and metatarsal samples were placed in glass containers, soaked in ether for 96 h to remove the fat, dried to a constant weight at 105 °C and then reduced to ash in a muffle furnace (550 to 600 °C for 24 h). The ash content was measured and expressed using the defatted dry weight, and the ashes were used in the next step to analyze the calcium and phosphorus content, the process for which followed the procedures of the Association of Official Analytical Chemistry.

Serum content analysis

Serum levels of calcium and phosphorus were determined to assess the contribution of the diet to calcium and phosphorus absorption. Calcium (ref no.: C004-2) and phosphorus (ref no.: C006-1) levels were determined using kits from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Vitamin E (ref no.: A008-1) levels in serum were also measured using kits from the same company. All assay procedures were carried out in strict accordance with the manufacturer's instructions. The CV of the data was <5%, and the interbatch CV was <8%.

Statistical analysis

All data are expressed as the means and SEM. Data were counted and analyzed in replicates using SPSS 22.0 software (SPSS. Inc., Chicago, USA). The Shapiro–Wilk test (95% confidence interval) was applied, followed by a two-way ANOVA using a general linear model. Tukey's post hoc test was used to determine differences between groups. Data were considered to be significantly different when $P < 0.05$.

RESULTS

Growth performance

The effects of different diets and stocking density treatments on growth performance for the three ages are shown in Table 2. No interactive effects of stocking density and dietary vitamin treatments on growth performance were observed ($P > 0.05$). High-density stocking significantly increased FCR of birds both in 1 to 14 d ($P = 0.001$) and 15 to 28 d ($P = 0.015$), decreased ADG ($P = 0.009$) of 1 to 14 d. In the last 2 wk (29 to 42 d), the dietary treatment significantly reduced FCR ($P = 0.025$). Throughout the animal experiment, stocking density and vitamin diet treatments did not affect changes in ADFI and mortality ($P > 0.05$).

Antioxidant capacity

Serum indicators at three ages were used to assess antioxidant capacity and are shown in Table 3. At 14 and 42 d of age, a significant interaction between dietary vitamins and stocking density was observed in serum CAT and GSH-px activities. The vitamin-treated group at high stocking density had significantly higher CAT at 14 and 42 d of age compared with normal diet group, and the GSH-px activity showed the same trend at 42 d of age. The main effect showed that high-density stocking significantly reduced serum CAT activity in broilers on the 42-d diet ($P < 0.001$), whereas the vitamin diet reduced serum MDA levels in broilers at 14 d of age ($P = 0.033$) and 42 d of age ($P = 0.028$) and increased CAT activity at 28 d of age ($P = 0.004$).

Serum mineral and vitamin E levels

Table 4 shows the results for serum calcium, phosphorus, and vitamin E contents under different diets and stocking density treatments. Significant interactions between dietary vitamin supplementation and stocking density were observed in the serum vitamin E levels of broiler chickens at 14 d of age ($P = 0.002$) and 28 d of age ($P = 0.047$). Vitamin supplementation increased serum vitamin E concentration in broilers reared at low density at 14 d of age, while at 28 d of age, vitamin supplementation showed significant benefits in both stocking density groups compared with normal diet treatment at high stocking density. Serum calcium levels in 14-d-old broilers significantly increased due to vitamin supplementation ($P = 0.01$), and a similar trend was observed in serum vitamin E levels at 28 d of age ($P < 0.001$) and 42 d of age ($P < 0.001$). Additionally, high stocking density decreased serum vitamin E levels in 14-d-old broilers ($P = 0.004$), as well as serum calcium ($P = 0.009$) and phosphorus ($P < 0.001$) levels in 28-d-old broilers.

Bone properties and composition

The physical traits of the femur, tibia, and metatarsus of broilers at the three sampling days are summarized in Tables 5, 6, and 7, respectively, for analysis of the effects of

Table 2. Effect of stocking density and dietary supplementation with 25-hydroxycholecalciferol and additional vitamin E on the growth performance of broilers[1,2,3]

Item	ADG, g/d			ADFI, g/d			FCR, g/g			Mortality, %		
	Days 1 to 14	Days 15 to 28	Days 29 to 42	Days 1 to 14	Days 15 to 28	Days 29 to 42	Days 1 to 14	Days 15 to 28	Days 29 to 42	Days 1 to 14	Days 15 to 28	Days 29 to 42
Density												
High	28.55[b]	76.92	60.41	31.81	91.39	128.6	1.12[a]	1.19[a]	2.15	1.38	0.79	1.50
Low	32.18[a]	83.79	55.73	31.68	88.61	128.8	0.99[b]	1.12[b]	2.34	1.33	0.78	1.47
Diet												
Normal	29.87	82.72	55.31	31.58	90.82	129.3	1.07	1.16	2.36[a]	1.26	0.75	1.52
25-(OH)D3+VE	30.86	77.98	60.84	31.89	89.18	128.1	1.05	1.16	2.13[b]	1.45	0.83	1.45
Interaction												
HD*Normal	28.52	76.82	56.43	31.95	91.01	128.1	1.12	1.19	2.28	1.44	0.79	1.55
HD*25-(OH)D3+VE	28.58	77.01	64.39	31.65	91.78	129.2	1.11	1.19	2.02	1.32	0.81	1.45
LD*Normal diet	31.21	88.62	54.17	31.21	90.63	130.5	1.01	1.12	2.44	1.08	0.71	1.49
LD*25-(OH)D3+VE	33.14	78.95	57.28	32.14	86.58	126.9	0.98	1.11	2.23	1.57	0.85	1.45
SEM	1.26	1.48	3.06	0.96	2.28	4.23	0.03	0.029	0.09	0.35	0.19	0.28
P value												
Density	0.009	0.074	0.158	0.899	0.250	0.980	0.001	0.015	0.07	0.753	0.852	0.788
Diet	0.437	0.632	0.096	0.75	0.489	0.786	0.508	0.747	0.025	0.654	0.325	0.584
Interaction	0.468	0.550	0.455	0.531	0.318	0.596	0.774	0.606	0.749	0.455	0.699	0.435

[1]Means in a row with no common superscript are significantly different ($P < 0.05$).
[2]Means are based on 6 replicates per treatment for data on interaction effect and 12 replicates per treatment for data on main effect of stocking density and diet.
[3]25-(OH)D3+VE is half 25-(OH)D3 as a source of vitamin D3 and an additional 60 IU of vitamin E; ADFI, average daily feed intake; ADG, average of daily gain; HD, high stocking density; LD, low stocking density.

Table 3. Effect of stocking density and dietary supplementation with 25-hydroxycholecalciferol and additional vitamin E on serum antioxidant parameters in broilers[1,2,3]

Item	CAT, μ/mL			GSH-px, μ/mL			MDA, nmol/mL			SOD, U/mL		
	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42
Density												
High	4.07	4.82	2.51[b]	1,870	2,223	2,927	1.62	2.17	1.58	340.8	341.7	339.4
Low	4.65	3.69	3.22[a]	1,687	2,558	2,812	1.26	1.72	1.58	317.4	376.1	378.2
Diet												
Normal	3.99	3.52b	2.23	1,810	2,547	2,772	1.71[a]	2.05	1.76[a]	330.9	377.1	351.9
25-(OH)D3+VE	4.72	4.99a	3.44	1,746	2,233	2,968	1.17[b]	1.84	1.40[b]	327.2	340.7	365.7
Interaction												
HD*Normal	3.51[b]	4.12	1.19[c]	1,783[ab]	2,462	2,689[b]	1.98	2.38	1.71	345.9	365.9	336.8
HD*25-(OH)D3+VE	4.63[a]	5.51	3.84[a]	1,955[a]	1,983	3,165[a]	1.26	1.95	1.44	335.6	317.4	342.0
LD*Normal diet	4.48[a]	2.92	3.38[b]	1,836[ab]	2,632	2,854[ab]	1.44	1.71	1.81	315.9	388.2	367.0
LD*25-(OH)D3+VE	4.82[a]	4.47	3.05[b]	1,537[b]	2,483	2,770[ab]	1.08	1.73	1.35	318.8	363.9	389.4
SEM	0.61	0.42	0.35	91.03	161.0	110.0	0.24	0.24	0.15	27.33	19.58	26.58
P value												
Density	0.365	0.102	< 0.001	0.053	0.051	0.343	0.143	0.072	0.995	0.403	0.097	0.146
Diet	0.675	0.004	0.072	0.473	0.067	0.073	0.033	0.341	0.028	0.894	0.078	0.647
Interaction	0.042	0.854	< 0.001	0.016	0.32	0.015	0.455	0.38	0.56	0.811	0.555	0.793

[1]Means in a row with no common superscript are significantly different ($P < 0.05$).
[2]Means are based on 6 replicates per treatment for data on interaction effect and 12 replicates per treatment for data on main effect of stocking density and diet.
[3]25-(OH)D3+VE is half 25-(OH)D3 as a source of vitamin D3 and an additional 60 IU of vitamin E; CAT, catalase; GSH-px, glutathione peroxidase; HD, high stocking density; LD, low stocking density; MDA, malondialdehyde; SOD, superoxide dismutase.

Table 4. Effect of stocking density and dietary supplementation with 25-hydroxycholinesterase and additional vitamin E on serum calcium (Ca), phosphorus (P) and vitamin E levels.[1,2,3]

Item	Ca, µmol/dL			P, mmol/dL			VE, µg/mL		
	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42
Density									
High	28.71	16.89[a]	16.16	0.44	0.49[a]	0.45	293.6[a]	265.9	271.6
Low	25.83	11.83[b]	16.79	0.44	0.41[b]	0.47	324.0[b]	297.2	278.0
Diet									
Normal	26.34[b]	13.47	16.54	0.46	0.47	0.47	300.8	215.0[b]	225.9[b]
25-(OH)D3+VE	28.19[a]	15.25	16.43	0.41	0.44	0.44	316.8	348.1[a]	323.8[a]
Interaction									
HD*Normal	27.12	15.43	16.28	0.45	0.51	0.46	302.2[b]	176.0[b]	224.5
HD*25-(OH)D3+VE	30.28	18.36	16.04	0.42	0.48	0.43	285.0[b]	355.7[a]	318.8
LD*Normal diet	25.55	11.51	16.79	0.47	0.42	0.49	299.4[b]	253.9[ab]	227.3
LD*25-(OH)D3+VE	26.11	12.15	16.81	0.41	0.39	0.44	348.6[a]	340.6[a]	328.8
SEM	3.87	0.83	0.91	0.02	0.02	0.02	7.86	22.01	15.47
P value									
Density	0.191	0.009	0.595	0.764	<0.001	0.224	0.004	0.169	0.718
Diet	0.010	0.570	0.856	0.053	0.130	0.134	0.085	<0.001	<0.001
Interaction	0.655	0.579	0.561	0.493	0.910	0.643	0.002	0.047	0.836

[1]Means in a row with no common superscript are significantly different ($P < 0.05$).
[2]Means are based on 6 replicates per treatment for data on interaction effect and 12 replicates per treatment for data on main effect of stocking density and diet.
[3]25-(OH)D3+VE is half 25-(OH)D3 as a source of vitamin D3 and an additional 60 IU of vitamin E; Ca, calcium; HD, high stocking density; LD, low stocking density; P, phosphorus.

Table 5. Effect of stocking density and dietary supplementation with 25-hydroxycholecalciferol and additional vitamin E on structural parameters of femur in broilers.[1,2,3]

Item	Length, cm			Weight, g			Strength, MPa		
	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42
Density									
High	4.33	5.97b	9.86	3.61	5.88b	10.08	51.86	71.58	45.97
Low	4.42	6.43a	9.73	3.55	6.77a	10.28	62.45	72.71	54.38
Diet									
Normal	4.36	6.33	9.68	3.58	6.23	10.02	53.71	61.86	46.72
25-(OH)D3+VE	4.39	6.07	9.91	3.58	6.40	10.34	60.61	82.43	53.63
Interaction									
HD*Normal	4.40[ab]	6.22	9.85	3.56	5.68	9.9	53.82	53.55[b]	40.76
HD*25-(OH)D3+VE	4.26[b]	5.72	9.87	3.65	6.08	10.26	49.9	89.61[a]	51.18
LD*Normal diet	4.32[b]	6.43	9.51	3.59	6.77	10.13	53.59	70.17[ab]	52.68
LD*25-(OH)D3+VE	4.52[a]	6.42	9.95	3.51	6.72	10.42	71.31	75.25[ab]	56.07
SEM	0.09	0.16	0.14	0.16	0.29	0.51	9.01	6.57	4.11
P value									
Density	0.688	0.008	0.386	0.775	0.009	0.719	0.270	0.863	0.060
Diet	0.272	0.112	0.129	0.979	0.564	0.541	0.465	0.007	0.116
Interaction	0.041	0.136	0.168	0.630	0.460	0.951	0.260	0.031	0.411

[1]Means in a row with no common superscript are significantly different ($P < 0.05$).
[2]Means are based on 6 replicates per treatment for data on interaction effect and 12 replicates per treatment for data on main effect of stocking density and diet.
[3]25-(OH)D3+VE is half 25-(OH)D3 as a source of vitamin D3 and an additional 60 IU of vitamin E; HD, high stocking density; LD, low stocking density.

the experimental treatments on bone. At day 14, a significant interaction between dietary vitamins and stocking density was observed in the femur ($P = 0.041$) and metatarsal ($P = 0.004$) length, with the low-density stocking vitamin diet treatment group having higher bone length. At day 28, however, the data showed an interactive effect of the two factors on femur bone strength ($P = 0.031$) and metatarsal length ($P = 0.04$), with the high-density vitamin treatment group exhibiting

Table 6. Effect of stocking density and dietary supplementation with 25-hydroxycholecalciferol and additional vitamin E on tibial structural parameters in broilers.[1,2,3]

Item	Length, cm			Weight, g			Strength, MPa		
	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42
Density									
High	5.99	8.11	9.65[b]	5.41	7.94	13.42	41.28	47.91	27.63
Low	6.12	8.26	9.93[a]	5.44	8.66	14.37	37.32	51.62	31.66
Diet									
Normal	6.12	8.08	9.76	5.49	7.92	13.99	38.37	51.87	27.22
25-(OH)D3+VE	5.99	8.29	9.83	5.35	8.68	13.80	40.23	47.66	32.07
Interaction									
HD*Normal	6.18	7.98	9.72	5.42	7.42	13.35	37.24	50.47	25.01
HD*25-(OH)D3+VE	5.80	8.22	9.58	5.39	8.45	13.48	45.31	45.35	30.24
LD*Normal diet	6.06	8.18	9.78	5.56	8.42	14.62	39.49	53.27	29.42
LD*25-(OH)D3+VE	6.17	8.35	10.08	5.31	8.90	14.12	35.15	49.96	33.89
SEM	0.14	0.11	0.12	0.26	0.45	0.65	3.55	3.52	3.08
P value									
Density	0.331	0.133	0.029	0.911	0.103	0.167	0.278	0.323	0.190
Diet	0.301	0.07	0.503	0.604	0.093	0.787	0.605	0.263	0.119
Interaction	0.072	0.743	0.078	0.695	0.516	0.64	0.096	0.806	0.899

[1]Means in a row with no common superscript are significantly different ($P < 0.05$).
[2]Means are based on 6 replicates per treatment for data on interaction effect and 12 replicates per treatment for data on main effect of stocking density and diet.
[3]25-(OH)D3+VE is half 25-(OH)D3 as a source of vitamin D3 and an additional 60 IU of vitamin E; HD, high stocking density; LD, low stocking density.

Table 7. Effect of stocking density and dietary supplementation with 25-hydroxycholecalciferol and additional vitamin E on structural parameters of the metatarsus in broilers.[1, 2, 3]

Item	Length, cm			Weight, g			Strength, MPa		
	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42
Density									
High	4.11	6.17	7.05	3.63	6.04	6.87	60.78	28.12	20.38[b]
Low	4.24	6.17	7.17	3.47	6.29	7.56	54.31	27.94	27.81[a]
Diet									
Normal	4.17	6.07	7.01	3.58	6.10	6.87	61.43	28.24	24.49
25-(OH)D3+VE	4.18	6.26	7.22	3.52	6.23	7.56	53.65	27.82	23.70
Interaction									
HD*Normal	4.28[ab]	5.90[b]	7.00	3.68	5.90	6.82	67.18	28.94	17.73
HD*25-(OH)D3+VE	3.93[b]	6.44[a]	7.10	3.58	6.18	6.92	54.38	27.29	23.03
LD*Normal diet	4.05[ab]	6.28[ab]	7.02	3.47	6.29	6.92	55.68	27.53	31.25
LD*25-(OH)D3+VE	4.43[a]	6.07[ab]	7.33	3.46	6.28	8.20	52.92	28.35	24.37
SEM	0.11	0.17	0.16	0.19	0.12	0.54	7.44	2.08	3.09
P value									
Density	0.206	0.977	0.625	0.393	0.078	0.205	0.479	0.940	0.025
Diet	0.878	0.358	0.323	0.789	0.314	0.205	0.395	0.857	0.797
Interaction	0.004	0.040	0.717	0.832	0.286	0.275	0.579	0.599	0.061

[1]Means in a row with no common superscript are significantly different ($P < 0.05$).
[2]Means are based on 6 replicates per treatment for data on interaction effect and 12 replicates per treatment for data on main effect of stocking density and diet.
[3]25-(OH)D3+VE is half 25-(OH)D3 as a source of vitamin D3 and an additional 60 IU of vitamin E; HD, high stocking density; LD, low stocking density.

better metatarsal length development and a significant advantage in femur strength for the high-density stocking vitamin diets. Vitamin treatment increased the strength ($P = 0.007$) of the femur, and more significant changes occurred across stocking density treatments, with the low-density stocking group promoting femur length ($P = 0.008$) and weight ($P = 0.009$). There was no interaction observed between different diets and densities on the structural parameters of bone in 42-d-old broiler chickens. Similar to the performance at 28 d of age, there was a significant effect in the promotion of tibia

length (P = 0.029) and metatarsal strength (P = 0.025) in the lower-density stocking group. In conclusion, the dietary vitamin treatment provided improvement in bone development at higher densities, while the lower stocking density created a benign effect on skeletal development.

The composition of the femur, tibia, and metatarsus of broilers at the three sampling days are summarized in Tables 8, 9, and 10. Significant interaction effects between dietary vitamins and stocking density were observed in the composition of skeletons at 14 d of age, with significant interaction effects for the phosphorus content of femurs, ash, and phosphorus contents of metatarsals (P < 0.05), and undoubtedly a more significant advantage for the lower-density vitamin treatment group. The vitamin diet treatment was significantly associated with higher ash (P = 0.025) and phosphorus (P = 0.003) contents in the metatarsals being detected. In contrast, low stocking density significantly contributed to the ash content of the tibia and the phosphorus content of the metatarsus in all bones at 14 d of age (P < 0.05). At 28 d of age, a significant interaction between dietary vitamins and stocking density was detected for phosphorus content in the metatarsals (P = 0.001), with phosphorus being significantly higher in the high-density stocking vitamin group than in the low-density normal diet group. The vitamin diet treatment significantly increased the calcium and phosphorus levels in the metatarsals at this stage (P < 0.05), while the metatarsal phosphorus content was also significantly upregulated by the high-density stocking treatment (P = 0.001). Forty-two-day-old broilers had a significant interaction between femur phosphorus content (P = 0.048) and metatarsal ash (P = 0.001). At day 42, the vitamin treatment significantly increased the ash content of femur bones (P = 0.035), while the vitamin treatment group was significantly upregulated in the high-density stocking group. There was a significant decrease in ash, calcium, and phosphorus content of tibiae in response to increased stocking density (P < 0.05). Thus, the low-density vitamin treatment group maintained relatively high levels of inorganic matter throughout the test period, while the high-density vitamin treatment group upregulated bone inorganic matter levels at 28 d of age.

Carcass characteristics

The slaughter performance of broilers is shown in detail in Table 11. A significant interaction between dietary vitamins and stocking density was observed for carcass yield (P = 0.041). Dietary treatment did not have a significant effect on slaughter traits, while high stocking density significantly increased the broiler leg-muscle ratio (P = 0.017) and meat yield efficiency (P = 0.001). We can conclude that high-density stocking improved the slaughter performance of broilers by increasing the leg-muscle ratio and meat yield efficiency.

Discussion

With a significant correlation between stocking density and broiler health, it is paramount that poultry producers are able to maximize meat production per square meter of space and prevent production losses due to crowding stress (Abudabos et al., 2013). Previous studies have shown that crowded environments lead to a disruption in the balance between pro-oxidant and antioxidant systems, which in turn promotes oxidative damage in broiler tissues (Magnuson et al., 2020). The serum antioxidant enzymes SOD, CAT, and GSH-px are

Table 8. Effect of stocking density and dietary supplementation with 25-hydroxycholecalciferol and additional vitamin E on the content of ash, calcium (Ca) and phosphorus (P) in the femur of broilers.[1, 2, 3]

Item	Ca, %			P, %			Ash, %		
	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42
Density									
High	13.24	15.97	16.89	12.68	15.81	16.89	36.09[b]	45.62	41.51
Low	13.46	16.69	16.41	12.71	16.09	16.41	37.55[a]	45.55	40.82
Diet									
Normal	13.19	16.08	16.43	12.48	16.04	16.43	36.60	45.51	40.47[b]
25-(OH)D3+VE	13.52	16.57	16.87	12.91	15.86	16.87	37.03	45.66	41.86[a]
Interaction									
HD*Normal	13.21	15.91	17.36	12.81[ab]	15.99	17.36	36.44	45.86	40.95
HD*25-(OH)D3+VE	13.27	16.02	16.41	12.56[ab]	15.62	16.41	35.73	45.37	42.07
LD*Normal diet	13.16	16.25	15.49	12.16[b]	16.09	15.49	36.76	45.16	39.98
LD*25-(OH)D3+VE	13.76	17.12	17.33	13.25[a]	16.10	17.33	38.33	45.94	41.65
SEM	0.41	0.45	0.85	0.26	0.23	0.85	0.64	0.63	0.67
P value									
Density	0.586	0.124	0.527	0.936	0.211	0.527	0.032	0.919	0.265
Diet	0.417	0.284	0.553	0.130	0.407	0.553	0.505	0.822	0.035
Interaction	0.511	0.408	0.173	0.020	0.390	0.173	0.087	0.326	0.657

[1]Means in a row with no common superscript are significantly different (P < 0.05).
[2]Means are based on 6 replicates per treatment for data on interaction effect and 12 replicates per treatment for data on main effect of stocking density and diet.
[3]25-(OH)D3+VE = half 25-(OH)D3 as a source of vitamin D3 and an additional 60 IU of vitamin E; Ca = calcium; HD = high stocking density; LD = low stocking density; P = phosphorus.

Table 9. Effect of stocking density and dietary supplementation with 25-hydroxycholecalciferol and additional vitamin E on the content of ash, calcium (Ca) and phosphorus (P) in the tibia of broilers.[1, 2, 3]

Item	Ca, %			P, %			Ash, %		
	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42
Density									
High	13.48	15.67	15.79[b]	11.88	15.30	13.89[b]	35.79[b]	44.06	40.34[b]
Low	14.37	16.08	17.72[a]	12.77	14.98	14.80[a]	37.54[a]	44.51	42.40[a]
Diet									
Normal	13.68	15.91	16.96	12.45	14.93	14.53	37.04	44.13	41.38
25-(OH)D3+VE	14.17	15.88	16.55	12.21	15.35	14.16	36.19	44.44	41.36
Interaction									
HD*Normal	13.72	15.71	15.85	12.13	15.05	14.04	36.06	43.65	39.69
HD*25-(OH)D3+VE	13.24	15.63	15.72	11.63	15.55	13.73	35.31	44.47	40.99
LD*Normal diet	13.64	16.10	18.06	12.76	14.81	15.01	38.01	44.61	43.07
LD*25-(OH)D3+VE	15.09	16.06	17.38	12.78	15.14	14.59	37.06	44.41	41.72
SEM	0.69	0.75	0.71	0.49	0.33	0.38	0.68	0.67	0.79
P value									
Density	0.199	0.647	0.007	0.074	0.331	0.020	0.003	0.638	0.013
Diet	0.478	0.763	0.529	0.619	0.207	0.322	0.613	0.497	0.971
Interaction	0.157	0.541	0.666	0.589	0.790	0.878	0.565	0.437	0.091

[1]Means in a row with no common superscript are significantly different ($P < 0.05$).
[2]Means are based on 6 replicates per treatment for data on interaction effect and 12 replicates per treatment for data on main effect of stocking density and diet.
[3]25-(OH)D3+VE is half 25-(OH)D3 as a source of vitamin D3 and an additional 60 IU of vitamin E; Ca, calcium; HD, high stocking density; LD, low stocking density; P, phosphorus.

Table 10. Effect of stocking density and dietary supplementation with 25-hydroxycholecalciferol and additional vitamin E on the content of ash, calcium (Ca) and phosphorus (P) in the metatarsus of broilers.[1, 2, 3]

Item	Ca, %			P, %			Ash, %		
	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42
Density									
High	13.28	16.09	17.08	11.16[b]	15.63[a]	14.99	33.06[b]	45.02	43.24
Low	14.37	15.86	17.16	12.24[a]	14.62[b]	15.30	35.00[a]	43.32	43.54
Diet									
Normal	13.31	15.62[b]	16.68	11.17[b]	14.80[b]	14.83	33.26[b]	43.59	42.98
25-(OH)D3+VE	14.35	16.34[a]	17.55	12.23[a]	15.46[a]	15.47	34.79[a]	44.75	43.80
Interaction									
HD*Normal	12.97	15.85	17.06	11.18[b]	15.58[a]	15.02	33.24[b]	45.21	44.07[a]
HD*25-(OH)D3+VE	13.59	16.34	17.09	11.14[b]	15.68[a]	14.97	32.87[b]	44.83	42.41[b]
LD*Normal diet	13.64	15.38	16.30	11.17[b]	14.01[b]	14.63	33.29[b]	41.97	41.88[b]
LD*25-(OH)D3+VE	15.10	16.34	18.01	13.31[a]	15.23[a]	15.96	36.71[a]	44.67	45.19[a]
SEM	0.72	0.36	0.45	0.32	0.16	0.38	0.63	0.88	0.62
P value									
Density	0.086	0.513	0.851	0.002	0.001	0.426	0.006	0.063	0.631
Diet	0.099	0.05	0.062	0.003	0.001	0.099	0.025	0.194	0.184
Interaction	0.485	0.511	0.071	0.002	0.001	0.077	0.007	0.109	0.001

[1]Means in a row with no common superscript are significantly different ($P < 0.05$).
[2]Means are based on 6 replicates per treatment for data on interaction effect and 12 replicates per treatment for data on main effect of stocking density and diet.
[3]25-(OH)D3+VE = half 25-(OH)D3 as a source of vitamin D3 and an additional 60 IU of vitamin E; Ca = calcium; HD = high stocking density; LD = low stocking density; P = phosphorus.

critical in protecting animals from oxidative damage, while excessive oxidative stress can cause lipid oxidation in tissues and the production of the harmful substance MDA. In the present study, higher levels of GSH-px were detected in the serum of low-density-farmed broilers at days 14 and 28, and the high-density treatment also elevated MDA levels in

Table 11. Effect of stocking density and dietary supplementation with 25-hydroxycholinesterase and additional vitamin E on carcass characteristics in broilers.[1, 2, 3]

Item	Carcass yield, %	Abdominal fat, %	Pectoralis muscle, %	Leg muscle, %	Meat yield efficiency, kg/m^2
Density					
High	82.48	1.59	6.79	20.99[a]	48.75[a]
Low	83.86	1.65	6.72	18.82[b]	37.58[b]
Diet					
Normal diet	83.02	1.72	6.72	20.02	43.40
25-(OH)D3+VE diet	82.88	1.58	6.79	19.79	42.93
Interaction					
HD*Normal diet	82.03[b]	1.76	6.53	21.11	50.43
HD*25-(OH)D3+VE diet	82.95[ab]	1.42	7.05	20.88	47.06
LD*Normal diet	84.01[a]	1.66	6.91	18.93	36.37
LD*25-(OH)D3+VE diet	82.72[ab]	1.65	6.53	18.7	38.79
SEM	0.52	0.15	0.23	0.83	1.48
P value					
Density	0.096	0.684	0.750	0.017	0.001
Diet	0.864	0.344	0.778	0.791	0.763
Interaction	0.041	0.280	0.061	0.997	0.080

[1]Means in a row with no common superscript are significantly different ($P < 0.05$).
[2]Means are based on 6 replicates per treatment for data on interaction effect and 12 replicates per treatment for data on main effect of stocking density or diet.
[3]25-(OH)D3+VE is half 25-(OH)D3 as a source of vitamin D3 and an additional 60 IU of vitamin E; HD, high stocking density; LD, low stocking density.

broilers at day 28 and reduced CAT at day 42, suggesting that higher-density farming caused more severe oxidative stress. As previously reported, higher-density stocking broilers had higher levels of MDA and lower levels of SOD and GSH-px in serum than the low-density treatment (Nasr et al., 2021). Nonetheless, we did not observe a change in mortality, which can be explained by the fact that the intensity of oxidative stress did not reach excessively high levels due to the stable environmental control that removes excess heat in the large chicken houses of the commercial study. High-density stocking has been shown to reduce tibial ash content and bone length at 42 d of age, which in turn affects the walking ability and welfare of the birds, ultimately reducing body weight in broilers. This indicates that the effects of high-density farming on leg development in broilers due to a lack of sufficient activity can be very negative (Sun et al., 2018, Karaarslan and Nazlıgül, 2018). Similar bone development results were found in broilers at 28 and 42 d of age in this study. Under oxidative stress, more energy is redistributed to support the antioxidant defense system and immune system, meaning that production performance is compromised as a result (Wang et al., 2023). High-density broiler rearing reduced ADG in the first two weeks and increased FCR in broilers in the first four weeks, which showed the same trend as previous studies. It is worth noting that the results showed that broilers at 29 to 42 d of age showed a lower FCR under high-density conditions and that the FCR of broilers was related to the energy consumption of broilers in their daily activities, a phenomenon that can be explained by the fact that higher stocking densities limit excessive exercise, leading to lower energy consumption (Simitzis et al., 2012). In the present study, high stocking densities caused oxidative damage in broilers, affecting the length of bone development and causing stage-specific effects on production performance.

In general, vitamin E supplementation in the diet can alleviate the stress to which birds are subjected, both acutely and chronically (Hashem et al., 2017). This improvement can be understood in two ways. First, under stress, the demand for vitamin E increases to scour the body of free radicals, thereby promoting health (Bortoluzzi et al., 2020). Furthermore, when lipid peroxidation takes place, vitamin E protects the docosahexaenoic and arachidonic acids in cell membranes from peroxidation and thus protects the stability of cell membranes (Panda and Cherian, 2014). In our research, it was clear that the use of 25-(OH)D3 and the addition of vitamin E significantly increased serum CAT activity in broiler chickens raised at high density at 42 d of age, which further confirms that the 25-(OH)D3+VE dietary treatment alleviated the oxidative stress experienced by broilers due to high-density stocking. Previous research showed that an additional 67 IU/kg of vitamin E in the diet was shown to increase α-tocopherol concentrations in breast meat and improve lipid oxidation (Pitargue et al., 2019). A total intake of 100 IU/kg of vitamin E in the diet increased the unsaturated fatty acid content and improved the antioxidant capacity in broiler muscle after heat stress (Mazur-Kuśnirek et al., 2019). The additional inclusion of 100 IU/kg vitamin E has been found to alleviate oxidative stress challenges in broiler chickens under high-density farming conditions by reducing the levels of plasma corticosterone (Shehata et al., 2022). The mechanisms by which vitamin D functions as an antioxidant are not very well studied, but studies have shown that 25-(OH)D3, an activated form of vitamin D, can reduce serum cortisol levels and increase vitamin E levels (Rey et al., 2020). Recent research conducted on laying hens has shown that the inclusion of an additional 69 μg/kg of 25-(OH)D3 in the diet can improve intestinal barrier function and antioxidant capacity in high-density stocking conditions (Wang et al., 2021a). Vitamin D has also been shown to alleviate oxidative stress in mice with acetaminophen-induced

acute liver injury (Wang et al., 2021b). Furthermore, human studies have reported that serum GSH-px and MDA levels in patients with depression were also improved with vitamin D supplementation, suggesting that oxidative stress was reduced (Ostadmohammadi et al., 2019). In this study, the dietary treatments upregulated serum vitamin E levels at all ages, suggesting that the additional supplementation of vitamin E gave the birds a relatively adequate reserve of antioxidant capacity throughout the broiler stocking cycle.

Bone strength and development in poultry are inextricably related to calcium and phosphorus absorption and metabolism (Chou et al., 2009). Vitamin D3 and its metabolites could upregulate the gene expression of intestinal calcium-binding proteins and inorganic phosphate transporters to improve calcium and phosphorus availability in broilers (Han et al., 2022). As an activated form of vitamin D3, 25-(OH)D3 has greater bioavailability (Hsu et al., 2021). The results of this study showed that serum calcium was upregulated at 14 d of age as a result of the addition of 25-(OH)D3. A significant increase was also observed in serum calcium and phosphorus levels as a consequence of higher stocking density as age increased. According to previous studies, it is possible that stress due to crowding increases the calcium and phosphorus requirements of birds, and higher serum calcium and phosphorus levels have been reported in studies of broilers and laying hens under high-density farming conditions (Lallo et al., 2012; Incharoen et al., 2021). Bone fracture strength is positively correlated with bone density, and bone mass can be predicted by bone fracture strength (Hester et al., 2004). Stress inhibits the longitudinal growth of leg bones by suppressing the number of proliferating chondrocyte populations in the bone growth plates of broiler chickens, which in turn determines the rate of bone growth (Luo et al., 2013). Caged poultry suffer from reduced physical activity leading to compromised bone health and reduced strength of the metatarsals and femurs (Onbaşılar et al. 2020). Higher-density poultry results in reduced frequency of mobility, which may result in longer sitting times for the birds, causing more pressure and challenge to the leg bones (Ma et al., 2020). In the current study, the 25-(OH)D3+VE dietary treatment significantly improved bone strength in the femur and the length of metatarsus at 28 d of age in high stocking density, similar to the results of previous studies (Fritts and Waldroup, 2003). Broiler bone strength is influenced by the ash content of the bone, and the amount of calcium and phosphorus retained in the bone is suggested to be positively correlated with the ash content, as reflected in our data (Onyango et al., 2003; Shao et al., 2019). Our study confirmed that stocking density influenced serum calcium and phosphorus content, bone growth length, and bone strength and that the use of a 25-(OH)D3+VE diet alleviated the oxidative stress caused by high-density stocking in broilers, which improved bone composition and development.

According to our results, despite the oxidative stress caused by high-density rearing, the final production performance of broilers was not negatively affected. Higher densities have also been shown in past studies to potentially improve nutrient digestibility, leading to better FCR results (Feddes et al., 2002). Due to better biological availability, higher circulating levels of 1,25-(OH)2 vitamin D3 were able to modulate the immune response to counteract stress-mediated inhibition of immunity and growth performance, while additional vitamin E was able to scavenge free radicals generated by normal metabolism and oxidative stress lipid peroxidation in previous studies (Wideman Jr et al., 2015; Zdanowska-Sąsiadek et al., 2016). We demonstrated that 25-(OH)D3+VE dietary treatment alleviated oxidative stress in broilers, improved leg bone development, and ultimately improved FCR in broilers. The carcass characteristics of broilers are important in poultry production, as they serve as an important economic indicator in the chain from farm to fork. In this study, broilers raised at high density without a 25-(OH)D3+VE diet showed poor carcass ratios. High-density rearing impairs muscle and bone growth and affects the process by which insulin-like growth factor-1 induces skeletal muscle growth, which subsequently increases the efficiency of protein synthesis (Shehata et al., 2022). the carcass ratio is the criterion for assessing the slaughter value of broilers, while meat production per square meter reflects the real market value of broilers (Al-Baadani, et al. 2023). The reduction in carcass yield was caused by high-density stocking in broiler chickens, and we also observed that high-density rearing significantly increased meat production per square meter, and the yield of leg muscles was enhanced under high-density conditions. The vitamin diet treatment appears to be a promising solution, as vitamin supplementation improved antioxidant capacity and bone development in broilers raised at high density, offsetting the rise in feed costs associated with vitamin use and making broiler production more economically viable.

Conclusions

Overall, the use of the 25-(OH)D3+VE diet effectively suppressed oxidative stress caused by high stocking density and alleviated the negative impacts of high stocking density on bone development in commercial broiler farms. Furthermore, high-density stocking increased the proportion of leg muscles and improved meat production per square meter. Both factors contribute positively to the economic efficiency of broiler production.

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Authors' contributions

CX, ZS, BS, and LC conceived and designed the experiments and wrote the paper. XP and QZ performed the experiments and analyzed the data. NE and MS provided advice and revised the article. All authors have read and approved the final manuscript.

Conflict of interest statement.

The authors declare no real or perceived conflicts of interest.

Ethics approval and consent to participate

All experimental procedures were approved by the Ethics Committee of Shandong Agricultural University and performed in accordance with the Guidelines for Experimental

Animals of the Ministry of Science and Technology (Beijing, People's Republic of China).

Consent for publication

Not applicable.

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