p-ISSN 2615-787X e-ISSN 2615-790X
Accredited by Directorate General of Higher Education, Research, and Technology, Republic of Indonesia, No. 225/E/KPT/2022

Tropical Animal Science Journal, December 2023, 46(4):461-470
DOI: https://doi.org/10.5398/tasj.2023.46.4.461
Available online at https://journal.ipb.ac.id/index.php/tasj

Performance, Egg Quality, Bone Health, and Immunity Assessments of Lohmann Laying Hens Supplemented with Vitamin D3 in the Diet

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(Received 01-07-2023; Revised 25-09-2023; Accepted 26-09-2023)

ABSTRACT

Bone health in laying hens is associated with production performance and physical egg quality. This study aimed to evaluate the effects of a diet supplemented with Nutricell Eggstra (vitamin D_3 enriched with canthaxanthin) on the production performance, egg quality, bone mineral, and immunity of laying hens. Herein, 150 Lohmann laying hens were divided into three treatments with five replicates. The treatment diets were: T1= diet without Nutricell Eggstra supplementation (control), T2= diet supplemented with 1.00 kg/ton Nutricell Eggstra, and T3= diet supplemented with 1.50 kg/ton Nutricell Eggstra. Compared to the control diet, supplementation of Nutricell Eggstra at doses of 1.00 kg/ton and 1.50 kg/ton significantly increased ($p<0.05$) hen day production, egg mass, egg index, yolk index, albumen weight, yolk color, eggshell thickness, eggshell strength, vitamin D_3 content in egg yolk, heterophil, and heterophil/lymphocyte ratio. Supplementation of Nutricell Eggstra at the studied doses significantly decreased ($p<0.05$) the feed conversion ratio, yolk weight, eggshell porosity, and lymphocyte content. Nutricell Eggstra supplementation did not affect feed consumption, egg weight, albumen index, albumen weight, eggshell weight, Haugh unit, and tibia bone minerals. In conclusion, supplementation of 1.00 kg/ton Nutricell Eggstra yielded the best performance for Lohmann laying hens aged 36-39 weeks, while supplementation of 1.50 kg/ton Nutricell Eggstra produced the best quality eggs.

Keywords: bone; egg quality; immunity; Lohmann hens; vitamin D_3

INTRODUCTION

Owing to the exponential increase in the human population, there has been an exponential increase in the demand for table eggs worldwide (El-Sabrout *et al.*, 2022). Globally, eggs have become one of the main animal protein sources for society, based on data from the Food and Agriculture Organization of the United Nations (2022), accounting for 6% of the total hen egg production in the world, Indonesia ranks fourth in hen egg production after China, India, and the United States of America. According to the Central Bureau of Statistics, the demand for hen eggs in Indonesia increases annually. The demands for hen eggs in 2020, 2021, and 2022 were 4,895,998, 5,066,543, and 5,310,278 tons, respectively. High egg-producing laying hens suffer from osteoporosis during the laying period because the medullary and structural bones are progressively absorbed to supply calcium (Ca) for eggshell formation. This has become a severe economic and welfare problem for the poultry industry (Chen *et al.*, 2020). Attia *et al.* (2020) reported that the production performance and eggshell quality of laying hens were significantly influenced by their bone qualities, and farmers may experience economic losses owing to the reduced quality of their bones and eggshells.

Ensuring a balance between the medullary bone and structural formation is crucial for bone quality and egg production (Adhikari *et al.*, 2020). During eggshell synthesis, blood Ca is rapidly mobilized, and its level decreases stimulating the secretion of parathyroid hormones (PTH), which promotes bone resorption to maintain Ca homeostasis (Sinclair-Black *et al.*, 2023). When bone resorption exceeds formation, the bone matrix becomes fragile and prone to fracture (Duran *et al.*, 2018). Improving feed nutrition is a possible solution. The feed nutrition strategy focuses on maintaining bone health and the physical quality of eggs and improves the production performance and nutritional content of eggs for human consumption (El-Sabrout *et al.*, 2022).

Vitamin D is an essential fat-soluble vitamin for Ca and phosphorus (P) metabolism and maintaining bone integrity in chickens (Adhikari *et al.*, 2020). Vitamin D also plays a role in Ca and P absorption and homeostasis, PTH regulation, bone mineralization and mobilization, and the control of bone abnormalities (Garcia *et al.*, 2013). Two classes of vitamin D physiologically influence hormones: ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). Vitamin D_2 and vitamin D_3 are hydroxylated in the liver by 25-hydroxylase to become $25OHD_2$ / $25OHD_3$, which is then hydroxylated in the kidney by 1α-hydroxylase into the active form $1,25(OH)_2D_3$ (Holick & Chen, 2008). The active form synergizes with PTH, which involves intestinal Ca absorption, skeletal Ca deposition, and Ca resorption from bone tissue when plasma Ca levels are low (Saunders-Blades & Korver, 2014).

Supplementation of vitamin D_3 in the laying hen diet has been reported to increase egg production and physical quality (Geng *et al.*, 2018), as well as improve the tibia bone quality of laying hens during the egg-laying period (Li et *al.*, 2021). The vitamin D_3 in the feed is absorbed into the bloodstream and transferred to the egg yolk through the ovaries. The increase in the vitamin D_3 content of eggs is in line with the increase in vitamin D_3 in the diet (Yao *et al.*, 2013). Visual appearance, especially color, is vital in food selection, including eggs. Using carotenoids to pigment animal products to enhance egg yolk color is widely accepted in the poultry industry. Canthaxanthin is a carotenoid commonly used in diets, contributing to the orange-red color of egg yolk. This pigment also has the potential as an antioxidant for birds and their derivative products (Faruk *et al.*, 2018).

The efficiency of vitamin D_3 supplementation depends on several factors, such as dose, age, production phase, and duration of the study. Information regarding vitamin D_3 enriched with canthaxanthin has not been widely studied. Hence, we hypothesized that laying hens require an appropriate vitamin D_3 content during the production stage, and supplementation with Nutricell Eggstra as a vitamin D_3 source enriched with canthaxanthin in the diet may increase the production, egg quality, bone minerals, and profits for laying hen farmers. This study evaluated the effects of Nutricell Eggstra supplementation in the diet on performance, egg quality, bone mineral content, and immunity of Lohmann laying hens aged 36-39 weeks.

MATERIALS AND METHODS

Ethical Approval

All experimental procedures were approved by the Animal Ethics Committee of IPB University according to the Guidelines for the Care and Use of Animals (Number: 041-2023 IPB).

Animal and Diets

This study was conducted at the Department of Nutrition and Feed Technology, Faculty of Animal Science, IPB University, between October and December 2022. This research used 150 Lohmann laying hens aged 36–39 weeks, which were distributed according to a completely randomized design into three experimental treatments, with five replicates of ± 10 birds each. The supplement product used was Nutricell Eggstra, which is vitamin D_3-enriched canthaxanthin from PT. Nutricell Pacific (Table 1). The treatments were as follows: T1= diet without Nutricell Eggstra supplementation (control), T2= diet supplemented with 1.00 kg/ton Nutricell Eggstra, and T3= diet supplemented with 1.50 kg/ton Nutricell Eggstra. The birds were kept in individual cages equipped with a feeding trough and nipple drinker. The temperature generated during the research was 25.20-31.65 °C with humidity 72.65%-96.64%. Birds were offered feed twice a day (07.00 and 16.00 h) with a mash diet based on the nutritional requirements of Lohmann laying hens (Lohmann Management Guide, 2019) (Table 2) 110 g/bird/day. The laying hens were reared in an open house system with a concrete floor and rice husk litter.

Performance of Laying Hens

The performance of laying hens was measured at 36-39 weeks of age. According to the Lohmann Management Guide (2019), this age range is classified as the egg-laying period Phase 1. The diet for Phase 1 was designed to meet the maximum egg mass production requirement. Supplementation with vitamin D_3 during the rearing and laying stages could promote bone development and protect against bone loss during egg production, which may benefit the birds during the later laying period. Feed consumption was recorded daily and calculated as the difference between the amount of feed given and the remaining feed. The feed conversion ratio (FCR) was obtained by calculating the ratio between the amount of feed consumed and the mass of eggs produced. Hen day production (HDP) was calculated as the ratio of the number of eggs produced to the number of laying hens. Egg weight was calculated based on the results of daily egg weighing during rearing, and egg mass was calculated by multiplying egg production by egg weight.

Egg Quality

In total, 90 eggs (six eggs per replicate) were used for physical egg quality measurements. The measured physical quality variables included the egg index, percentage of albumen weight, yolk weight, eggshell weight, Haugh unit, yolk color, eggshell color, eggshell thickness, eggshell strength, and eggshell porosity. An electronic digital balance was used to measure the egg, yolk, eggshell, and albumen weights. Albumen and eggshell thicknesses were measured using a digital micrometer. The Haugh unit (HU) was calculated using the Haugh formula (Haugh, 1937), $HU = 100 \log [H + 7.57 - 1.7W^{0.37}]$, where H was the albumen thickness (mm) and W was the weight of the entire egg (g). The shape index was calculated as the ratio of the egg width to the egg length. Eggshell strength was measured using an RH-DW200 Eggshell Strength Tester (Guangzhou RunHu Instruments Co., Ltd.). The eggshell color and eggshell porosity were as-

Table 1. Product specification of Nutricell Eggstra (kg)

Material	Amount	Unit
Vitamin D_3	3,500,000	IU
Canthaxanthin	5.5	g
Antioxidant*	1.0	g

Note: *BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), ethoxyquin.

Table 2. The composition and nutritional value of the experimental diets

Items	Treatments		
	T1	T2	T3
Ingredients			
Corn (%)	55.50	55.50	55.50
Wheat bran (%)	2.50	2.50	2.50
Soybean meal (%)	18.00	18.00	18.00
Fish meal (%)	7.00	7.00	7.00
Corn gluten meal (%)	4.60	4.60	4.60
Crude palm oil (%)	2.00	2.00	2.00
$CaCO_3$ (%)	8.50	8.50	8.50
Dicalcium phosphate (%)	1.00	1.00	1.00
NaCl (%)	0.30	0.30	0.30
Premix (%)[3]	0.50	0.50	0.50
DL-methionine (%)	0.10	0.10	0.10
Total	100	100	100
Supplementation of Nutricell Eggstra (kg/ton)	0.00	1.00	1.50
Nutrients content			
Dry matter (%)[1]	89.58	89.58	89.58
Metabolizable energy (kcal/kg)[2]	2,855	2,855	2,855
Gross energy (kcal/kg)[1]	3,608	3,608	3,608
Crude protein (%)[2]	18.90	18.90	18.90
Crude fat (%)[1]	3.01	3.01	3.01
Crude fiber (%)[1]	4.96	4.96	4.96
Calcium (%)[1]	3.67	3.67	3.67
Total phosphorus (%)[1]	1.49	1.49	1.49
Available phosphorus (%)[2]	0.60	0.60	0.60
Lysine (%)[2]	1.10	1.10	1.10
Digestible lysine (%)[2]	0.99	0.99	0.99
Methionine (%)[2]	0.50	0.50	0.50
Digestible methionine (%)[2]	0.50	0.50	0.50
Methionine + cysteine (%)[2]	0.90	0.90	0.90
Digestible methionine + cysteine (%)[2]	0.70	0.70	0.70

Note: [1]Analysis results from PAU Laboratory IPB University 2023.
[2]Based on formulation calculations using least cost formulation.
[3] 5 kg premix contains: vitamin A 12.500 IU; vitamin D3 2.500 IU; vitamin E 10.000 mg; vitamin K3 2.000; vitamin B1 2.000 mg; vitamin B2 4.000 mg; vitamin B6 1.000 mg; vitamin B12 12,000 mcg; vitamin C 40.000 mg; niacin 40.000 mg; biotin 200 mg; l-arginine 10.000 mg; l-threonine 15,000 mg; Dl-methionine 5.000 mg; l-lysine 125.000 mg; folic acid 500 mg; ferrous 30.000 mg; Mn 60.000 mg; Cu 5.000 mg; iodide 200 mg; Se 200 mg; and Co 200 mg.
T1= diet without Nutricell Eggstra supplementation; T2= diet supplemented with 1.00 kg/ton Nutricell Eggstra; T3= diet supplemented with 1.50 kg/ton Nutricell Eggstra.

sessed using the grading and scoring systems provided by Nutricell Eggs-Pert, a tool from PT Nutricell Pacific. The yolk color index was determined using the yolk color fan scale. The measured variables of egg chemical quality included vitamin D_3 content in the egg yolk. The vitamin D_3 content was analyzed using liquid chromatography mass spectrometry (Shimadzu LCMS 8045 2022).

Blood Profile Analysis

Blood hematology profile analysis included the number of erythrocytes and leukocytes, as well as the hematocrit, hemoglobin, and differential leukocyte counts. The numbers of erythrocytes and leukocytes were calculated using the Neubauer counting chamber method and observed under a microscope. The number of red and white blood cells were calculated using Hayem's and Reez-Ecker's diluents. Hemoglobin was analyzed using the Sahli method with HCl (0.1 N). Differential leukocyte counts were performed by preparing smears with May Grunwald-Giemsa staining (Schalm, 1961) and observed using a microscope. Monocytes lymphocytes, heterophils, basophils, and eosinophils were differentiated. The hematocrit value was measured using a microcapillary tube filled with blood until it reached 4/5. The end of the tube was plugged with a crest seal, the tube was centrifuged for 3 min, and the hematocrit value was measured with a microhematocrit tube reader.

Tibia Bone Minerals Analysis

Laying hens from each treatment were slaughtered according to the Islamic method at the end of the study to collect the tibia. The meat and tibia were soaked in hot water, cleaned, and analyzed for mineral content, namely Ca, P, and zinc (Zn) using the AOAC method (2005). Dry and mashed samples were weighed in a porcelain dish ±2–10 g, then put into a furnace for 4 h at 550 °C. Thereafter, 10 mL of 3 M HCl was added to the samples and heated on a hotplate for 10 min. The heated solution was then added to 100 mL of distilled water in a measuring flask and filtered. The responses of Ca and Zn were measured using an atomic absorption spectrophotometer, where P was measured spectrophotometrically.

Immune Organs

Immune organs, such as the spleen, thymus, and bursa of Fabricius were separated and weighed to calculate the weight percentages. The relative weight of the immune organs (thymus, spleen, and bursa of Fabricius) were computed using the body weight at slaughter (Lan *et al.,* 2017).

Statistical Analysis

Data were statistically analyzed using various tests. When a treatment yielded a significant result ($p<0.05$), the Duncan's multiple range test was used to determine significant differences among the mean values. Analysis of variance was performed using the SPSS Statistics software version 25 (2017).

RESULTS

Performance of Laying Hens

Nutricell Eggstra supplementation in the diet of Lohmann laying hens aged 36–39 weeks showed a significant effect ($p<0.05$) on HDP, egg mass production, and FCR, but it had no significant effect on feed consumption and egg weight (Table 3). Our results displayed an increase in HDP by 12.44% (T2) and 9.01% (T3) compared to the control (T1). Laying hens whose diets were supplemented with Nutricell Eggstra had a weekly increasing HDP value, whereas those that were only fed a control diet had a fluctuating HDP in line with egg mass production. While the FCR fluctuated throughout the experimental period, treatment with Nutricell Eggstra supplementation at a 1.00 kg/ton dose had the lowest FCR compared to other treatments. At 36 and 38 weeks, the FCR peaked for all the treatments. At 37 and 39 weeks, the FCR gradually decreased, but the control diet treated birds maintained a high FCR throughout. Nutricell Eggstra supplementation at 1.00 and 1.50 kg/ton did not have different FCRs, but both were significantly lower than the control diet. These results indicate that a Nutricell Eggstra supplemented diet had the highest feed efficiency, with a decrease in FCR of 11.61 (T2) and 9.37% (T3) compared to the control diet. The best income over feed cost (IOFC) value was achieved in laying hens supplemented with Nutricell Eggstra at as much as 1.00 kg/ton with a profit of IDR 26,257.69/bird.

Egg Quality

Laying hens supplemented with Nutricell Eggstra in their diets showed significantly increased ($p<0.05$) egg shape index, yolk index, percentage of albumen and egg yolk, yolk color, eggshell thickness, eggshell strength, eggshell porosity, and vitamin D_3 content in the egg yolk. However, it did not show a significant effect ($p>0.05$) on the albumen index, percentage of eggshell, HU, and eggshell color (Table 4). Eggshell color, thickness, and strength were correlated with eggshell exterior quality in this experiment. Nutricell Eggstra supplementation in the Lohmann laying hens diet resulted in an improved eggshell quality. According to the eggshell porosity score (%), its eggshell quality was classified as good (score 2) compared to the control diet, which scored an average (score 3). The level of Nutricell Eggstra supplementation was correlated with the vitamin D_3 content of the egg yolk, as an increase in the supplement's level significantly increased the Vitamin D_3 content.

Blood Profile

The effects of Nutricell Eggstra supplementation on blood profiles in the Lohmann laying hens diet at 36–39 weeks of age are shown in Table 5. The addition of the Nutricell Eggstra supplement significantly ($p<0.05$) decreased the percentage of lymphocytes and increased the rate of heterophiles and the ratio of heterophiles/lymphocytes (H/L). However, no significant differences

Table 3. Performance of Lohmann laying hens aged 36–39 weeks supplemented with Nutricell Eggstra (vitamin D3 enriched with canthaxanthin)

Variables	Treatments		
	T1	T2	T3
Feed consumption (g/bird/day)	105.46 ± 1.26	105.77 ± 1.22	105.15 ± 1.03
Hen day production (%)	83.86 ± 1.80^{c}	94.29 ± 0.56^{a}	91.42 ± 0.71^{b}
Egg weight (g)	56.29 ± 1.35	56.53 ± 0.22	56.71 ± 1.17
Egg mass (g/bird)	47.16 ± 1.85^{b}	53.30 ± 0.27^{a}	51.82 ± 1.27^{a}
Feed conversion ratio	2.24 ± 0.08^{a}	1.98 ± 0.30^{b}	2.03 ± 0.30^{b}
Income over feed cost (IDR/bird)*	6,845.09	26,257.69	24,476.04

Note: T1= diet without Nutricell Eggstra supplementation, T2= diet supplemented with 1.00 kg/ton Nutricell Eggstra; T3= diet supplemented with 1.50 kg/ton Nutricell Eggstra. Means in the same row with different superscripts are significantly different ($p<0.05$).
*Normal egg price on Farm IDR 22.500.00 per kg, vitamin D enriched egg price on Farm IDR 33,750.00 per kg and feed price IDR 7.740.00.

Table 4. Egg quality of Lohmann laying hens aged 36–39 weeks supplemented with Nutricell Eggstra (vitamin D3 enriched with canthaxanthin)

Variables	Treatments		
	T1	T2	T3
Shape index	0.78 ± 0.00^{ab}	0.77 ± 0.00^{b}	0.79 ± 0.00^{a}
Albumen index	0.13 ± 0.00	0.14 ± 0.01	0.13 ± 0.00
Yolk index	0.45 ± 0.00^{b}	0.47 ± 0.00^{a}	0.48 ± 0.00^{a}
Albumen (%)	59.48 ± 1.39^{b}	61.66 ± 0.54^{a}	62.17 ± 0.71^{a}
Yolk (%)	29.74 ± 1.64^{a}	26.99 ± 0.64^{b}	26.26 ± 0.45^{b}
Shell (%)	10.79 ± 0.67	11.37 ± 0.27	11.54 ± 0.45
Haugh unit	94.25 ± 0.67	95.80 ± 1.67	95.14 ± 1.33
Yolk color	6.40 ± 0.54^{c}	13.40 ± 0.54^{b}	14.80 ± 0.44^{a}
Eggshell color	13.20 ± 0.83	13.20 ± 0.83	13.60 ± 0.54
Eggshell thickness (mm)	0.35 ± 0.01^{b}	0.38 ± 0.01^{a}	0.38 ± 0.01^{a}
Eggshell strength (kgf)	2.60 ± 0.30^{b}	2.90 ± 0.07^{a}	3.01 ± 0.19^{a}
Eggshell porosity	3.00 ± 0.00^{a}	2.40 ± 0.54^{b}	2.00 ± 0.00^{b}
Vitamin D_3 content in egg yolk (mcg/100 g)	0.00 ± 0.00^{c}	4.37 ± 0.72^{b}	7.11 ± 1.23^{a}

Note: T1= diet without Nutricell Eggstra supplementation, T2= diet supplemented with 1.00 kg/ton Nutricell Eggstra; T3= diet supplemented with 1.50 kg/ton Nutricell Eggstra. Means in the same row with different superscripts differ significantly ($p<0.05$).

(p>0.05) were observed in hemoglobin, hematocrit, erythrocytes, leukocytes, basophils, eosinophils, and monocytes.

Tibia Bone Minerals

Supplementation of Nutricell Eggstra in the diet of Lohmann laying hens aged 36–39 weeks showed no significant effect (p>0.05) on the content of tibia minerals such as Ca, P, and Zn (Table 6).

Immune Organs

Supplementation with Nutricell Eggstra in the diet of Lohmann laying hens aged 36-39 weeks had no significant effect on the average weight and percentage of immune organs (Table 7).

DISCUSSION

Performance of Laying Hens

Egg production can be influenced by several factors such as strain, diet nutrients, health and rearing management, age at first laying, and peak egg production. The HDP was in line with Geng *et al.* (2018), vitamin D_3 supplementation of 500–3000 IU/kg can produce an HDP of 90.30%–94.03%, but this was still lower than the standard HDP of Lohmann laying hens aged 36–39 weeks, which was 95.30%–95.00% (Lohmann Management Guide, 2019). Setiyaningsih *et al.* (2023) reported that the combination of 25(OH)D_3 and vitamin C contained in Nutricell HyC® can increase HDP in broiler breeders at the age of 34-46 weeks. According to Geng *et al.* (2018), vitamin D_3 supplementation in laying hens can increase the levels of follicle-stimulating and luteinizing hormone or induce a feedback effect on hypothalamic and pituitary hormone secretion, which positively affects follicle development. This supports the observed positive effect of vitamin D_3 on the performance of laying hens in association with estrogen hormones. However, various studies have shown that the use of vitamin D_3 in laying hen diets does not affect egg production (Nascimento *et al.*, 2014; Adhikari *et al.*, 2020).

The average egg weight in this study was still lower than the standard egg weight of Lohmann laying hens aged 36-39 weeks, with a weight of 61.60-62.30 g (Lohmann Management Guide, 2019). According to Chen *et al.* (2020), there was no significant difference in egg weight, which could be related to the age of the birds and the duration of vitamin D_3 supplementation. Other factors affecting egg weight include genetics, body size, climate, egg production, proteins, and amino acids (especially methionine), which are essential in controlling egg size (Leeson & Summers, 2005). This is also supported by Pakpahan *et al.* (2023), who found that one of the factors affecting egg weight is the nutritional

Table 5. Blood profile of Lohmann laying hens aged 39 weeks supplemented with Nutricell Eggstra (vitamin D3 enriched with canthaxanthin)

Variables	Treatments		
	T1	T2	T3
Hemoglobin (g%)	8.68 ± 1.46	9.04 ± 1.45	9.28 ± 0.59
Hematocrit (%)	22.00 ± 2.34	19.80 ± 1.78	21.00 ± 2.00
Erythrocytes (10^6/mm^3)	2.01 ± 0.62	2.53 ± 0.93	2.42 ± 0.74
Leukocytes (10^3/mm^3)	13.72 ± 6.11	12.44 ± 9.20	11.09 ± 5.54
Lymphocytes (%)	55.70 ± 2.16[a]	51.74 ± 2.96[b]	52.85 ± 1.39[ab]
Monocytes (%)	3.06 ± 1.03	2.97 ± 0.91	2.79 ± 1.78
Heterophils (%)	31.31 ± 2.67[b]	34.56 ± 2.15[a]	35.13 ± 1.12[a]
Eosinophils (%)	2.19 ± 0.46	1.98 ± 0.69	1.69 ± 0.53
Basophils (%)	7.73 ± 1.40	8.73 ± 2.16	7.50 ± 1.37
Ratio H/L	0.56 ± 0.07[b]	0.67 ± 0.07[a]	0.66 ± 0.03[a]

Note: T1= diet without Nutricell Eggstra supplementation, T2= diet supplemented with 1.00 kg/ton Nutricell Eggstra; T3= diet supplemented with 1.50 kg/ton Nutricell Eggstra. Means in the same row with different superscripts differ significantly (p<0.05).

Table 6. Tibia bone minerals content of Lohmann laying hens aged 39 weeks supplemented with Nutricell Eggstra (vitamin D3 enriched with canthaxanthin)

Variables	Treatments		
	T1	T2	T3
Ca (%)	39.98 ± 1.13	38.45 ± 1.89	39.72 ± 1.28
P (%)	5.77 ± 0.49	5.12 ± 0.64	5.42 ± 0.45
Zn (ppm)	128.02 ± 7.05	127.86 ± 7.08	131.39 ± 8.48

Note: T1= diet without Nutricell Eggstra supplementation, T2= diet supplemented with 1.00 kg/ton Nutricell Eggstra; T3= diet supplemented with 1.50 kg/ton Nutricell Eggstra.

Table 7. Average weight and percentage of immune organs in Lohmann laying hens aged 39 weeks supplemented with Nutricell Eggstra (vitamin D3 enriched with canthaxanthin)

Variables		Treatments		
		T1	T2	T3
Thymus	(g)	0.08 ± 0.23	0.78 ± 0.33	1.02 ± 0.58
	(%)	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.03
Spleen	(g)	2.88 ± 1.26	2.80 ± 1.36	2.02 ± 0.45
	(%)	0.18 ± 0.08	0.17 ± 0.07	0.12 ± 0.02

Note: T1= diet without Nutricell Eggstra supplementation, T2= diet supplemented with 1.00 kg/ton Nutricell Eggstra; T3= diet supplemented with 1.50 kg/ton Nutricell Eggstra.

content of the diet. Egg weight in this study did not differ between treatments because they were fed the same nutrient content, especially protein and fat.

Nutricell Eggstra supplementation in the diet resulted in higher egg mass production than in the control diet. The results were positively correlated with HDP because egg mass production is affected by egg production, egg weight, and heat stress (Yan *et al*., 2022). The laying hens receiving a Nutricell Eggstra supplemented in the diet had higher egg mass production than those reported by Wen *et al.* (2019) that laying hens received a vitamin D_3 supplementation of 1681–68348 IU/kg had egg mass production of 50.40–51.80 g/bird. However, a lower egg mass production was observed compared to Geng *et al.* (2018), which was as high as 54.55–56.83 g/bird after receiving 500–3000 IU/kg of supplemented vitamin D_3. Setiyaningsih *et al.* (2023) reported that combining 25(OH)D_3 and vitamin C increased egg mass in broiler breeders at 34-46 weeks of age. The improvement in the gut microbial composition induced by adding vitamin D_3 also plays a role in maintaining gut health and improving egg-laying performance (Guo et *al.,* 2022).

Nutricell Eggstra supplementation in the diet resulted in higher feed efficiency when compared to the control diet. This resulted in laying hens receiving Nutricell Eggstra supplementation have a higher egg mass with the same feed consumption. Geng *et al.* (2018) reported that laying hens receiving vitamin D_3 supplementation of 500-3000 IU/kg had FCR 2.07-2.12, which was higher than that observed in this study. The average FCR in this study follows the standard FCR for Lohmann layer periods of 2.0–2.1 (Lohmann Management Guide, 2019). Vitamin D_3 supplementation in the diet of laying hens can influence physiological processes that collectively contribute to the improved egg production, bone health, and nutrient utilization, leading to an improved feed conversion ratio. Attia *et al*. (2020) found that vitamin D_3 supplementation in the diet of laying hens could influence various physiological processes that collectively contribute to better egg production, bone health, and nutrient utilization, leading to improved FCR. An improved microbial balance positively impacts gastrointestinal health and increases nutrient utilization. Guo *et al.* (2022) reported that adequate vitamin D_3 supplementation positively affected the intestinal bacterial microbiota. Vitamin D_3 increases the composition of gut microbes such as *Lactobacillales, Lactobacillaceae, Lactobacillus,* and *Bacilli*, which play an essential role in maintaining gut health and optimal egg production. *Bacillus* spp. are considered beneficial microbiota and used as feed additives and antibiotic alternatives due to the advanced feed absorption, which can lead to better growth and production efficiency, and affects FCR positively (Zhen *et al*., 2018), secret digestive enzymes, produces antimicrobial substances and possesses immune-regulatory and anti-inflammatory effects (Muras *et al*., 2021).

IOFC is used to determine profit. According to Afandi *et al.* (2020), increasing feed consumption and decreasing egg and egg mass production can reduce IOFC. The value of feed consumption for all treatments was relatively the same, even though treatment T2 or T3 required a higher total feed cost due to the additional cost of Nutricell Eggstra supplementation. However, this can be offset by egg mass production, which is higher than the control treatment, such that the difference between egg sales and the total diet cost incurred during the rearing period increases.

Egg Quality

The egg shape index is related to the shape of the egg, with an ideal value of 0.80 or oval in shape. The albumen index in this study was included in quality I, with a value of 0.134–0.175 (BSN, 2008). According to Obianwuna *et al.* (2022), protein in the diet can affect albumen viscosity. The thicker the albumen, the higher the albumen index required to maintain quality. The insignificant difference in the albumen index values in this study was aligned with the constant diet protein content in each treatment. Nutricell Eggstra supplementation significantly increased the yolk index compared to the control diet, and this value was included in quality I with a value of 0.458–0.521 (BSN, 2008). According to Khan *et al.* (2016), the factors that can affect the yolk index include storage time, temperature, vitelline membrane quality, and nutrition from the diet. The transfer of cholecalciferol to the egg yolk is highly efficient and responsive to dietary enrichment (Yao *et al.,* 2013). According to Geng *et al.* (2018), vitamin D_3 is involved in the regulation of hormone synthesis and secretion, including that of reproductive hormones, such as estrogen. Adequate vitamin D_3 levels may promote proper hormonal signaling, leading to optimal yolk development and potentially larger yolk size, contributing to an increased yolk index. Estrogen influences the development of the reproductive tract and plays a crucial role in egg yolk formation by stimulating the liver to produce the yolk precursors vitellogenin and very-low-density lipoprotein, which are the primary sources of yolk protein and lipids, respectively (Schneider, 2016).

The percentages of albumen and eggshells obtained were higher than those observed in a study by Wen *et al.* (2019) (57.60%–57.90% and 9.50%–9.70%, respectively), which used 1681–68348 IU/kg of vitamin D_3 supplement. The percentage of yolk weight obtained was higher than those observed in a study by Plaimast *et al.* (2015) (23.75%-25.53%), which used 2000–6000 IU/kg of vitamin D_3 supplementation. Salles *et al.* (2013) reported that vitamin D_3 supplementation in the diet can increase protein synthesis, which can contribute to albumen formation, indicating that laying hens do not experience a lack of protein during albumen formation but correlates with the egg yolk weight, which tends to be lower. The relationship between vitamin D_3 supplementation in the diet of laying hens and a decrease in egg yolk weight is not simple and depends on various factors, including the dosage of vitamin D_3, duration of supplementation, and overall diet composition (Chen *et al.,* 2020).

The higher the albumen content, the higher the HU value, indicating improved egg quality. The average HU value in this study was within the normal range according to the United States Department of Agriculture Standards (2000), and the eggs were categorized as AA-quality eggs (HU>72). The absence of a significant difference in the HU values is consistent with the results of the other studies on the different levels of vitamin D_3 supplementation (Plaimast *et al.*, 2015; Geng *et al.*, 2018; Chen *et al.*, 2020). According to North & Bell (1990), the HU value is generally more indicative of the length of egg storage, and it is not affected by the diet, provided the protein and metabolic energy are balanced. The HU value did not differ among treatments because the laying hens received a diet with the same protein and energy content, and the eggs were stored at the same place and for the same period.

The diet supplemented with 1.50 kg/ton Nutricell Eggstra had the highest egg yolk color score compared to other treatments. The results were higher than those of Attia *et al.* (2020), which used a vitamin D_3 supplementation of 800–1200 IU/kg and observed egg yolk color scores of 7.78–7.87. The color of the yolk depends on the source and levels of carotenoids in the diet (Nabi *et al.*, 2020). Commercial carotenoid pigments, such as canthaxanthin, are widely accepted and commonly used in the poultry feed industry, and Nutricell Eggstra contains 5.50 g/kg of canthaxanthin. Canthaxanthin is an important carotenoid that is efficiently distributed or stored in the yolk (Zhang *et al.*, 2011) and is responsible for producing the yellow-orange color of the egg yolk. A EFSA FEEDAP Panel study (2014) showed that the egg yolk color was significantly different when the canthaxanthin supplementation varied between 0–60 mg/kg of diet, namely 6.38–14.90. These results align with the color of the egg yolks in this study, where an improvement in the color of the egg yolks corresponded with an increase in vitamin D_3 supplementation and can be associated with the canthaxanthin content in the product.

The main factors that affect eggshell color are genetics, stress, age, and disease (infectious bronchitis) (Aygun, 2014). In addition to the type of pigment, eggshell color can also be affected by the concentration of pigments and the structure of the eggshell (Samiullah *et al.*, 2015). Nutricell Eggstra supplementation has the same shell thickness and contrasts with the control group with a lower thickness. This aligns with the result reported by Chen *et al.* (2020), who used a vitamin D_3 supplementation of 2760–5520 IU/kg, and obtained a thickness of 0.37–0.39 mm. According to Leeson & Summers (2005), the primary nutrients that affect eggshell thickness are Ca, P, and vitamin D_3. Nascimento *et al.* (2014) reported that supplementation of vitamin D_3 increases the percentage and thickness of eggshells. When the concentration of vitamin D_3 in the diet decreases, laying hens respond by reducing the thickness of the eggshells produced (Kaur *et al.*, 2013).

Eggshell strength plays a vital role in determining egg quality. The results showed lower eggshell strength than those reported by Geng *et al.* (2018), which had an eggshell strength of 3.23–3.94 kgf using a vitamin D_3 supplementation of 500–3000 IU/kg. The eggshell strength and thickness are correlated with an increase of eggshell strength and thickness in laying hens supplemented with vitamin D_3. The role of vitamin D_3 in Ca and P metabolism is fundamental for bone development and eggshell formation (Attia *et al.*, 2020). Increasing the strength of shells and reducing egg loss are important economic goals in commercial terms.

Microbial penetration into eggs can be affected by various factors, such as membrane components, eggshell characteristics (porosity and quality), temperature, humidity, and storage. Pathogenic bacteria and harmful microorganisms can enter eggs with high porosity (Gole *et al.*, 2014). The results of this study are in line with those reported by Setiyaningsih *et al.* (2023), which also found that the combination of 25(OH)D_3 and vitamin C contained in Nutricell HyC® could significantly decrease the eggshell porosity compared to the control diet in broiler breeders. Vitamin D_3 contained in Nutricell Eggstra could potentially enhance the absorption of Ca and P in the small intestine and promote Ca deposition within the bones. Fritts and Waldroup (2003) stated that Ca deposition is particularly significant in creating calcium carbonate ($CaCO_3$) during eggshell formation within the uterus, which lasts for 20-21 h. Wang *et al.* (2020) noted that supplementation with vitamin D_3 in the diet of laying hens increased the levels of serum vitamin D_3 and the presence of carbonic anhydrase serum. This enzyme plays a critical role in facilitating the deposition of $CaCO_3$ during eggshell formation, thereby contributing to the ideal eggshell porosity.

The transfer of vitamin D_3 from the diet to egg yolks was highly efficient and responsive to the enrichment level in the diet. The results of this study correlate with those obtained by Yao *et al.* (2013), in which an increase in the vitamin D_3 content of eggs will be in line with the increase in vitamin D_3 in the diet. Additionally, vitamin D_3 was absorbed into the bloodstream and transferred to egg yolks through the ovaries. Plasma cholecalciferol is precipitated in egg yolk by forming a complex with the protein binding to vitamin D. The supplementation of Nutricell Eggstra in diets aims to optimize the productivity of laying hens and improve egg quality by providing added value to products rich in vitamin D_3. Bio-fortification of animal foods enriches food products, where the nutrients of interest are added through animal feed. Eggs have garnered significant attention as a vehicle for vitamin D bio-fortification and enrichment. The economic impact of promoting enriched eggs as an effective food source increases vitamin D intake, productivity, and profits for farmers.

Blood Profile

The average hemoglobin level in this study ranged from 8.68–9.28 g%, and these results were within the normal range. Laying hens are resistant to internal physiological changes in an effort to maintain a balance in the internal body environment. Thus, the hematocrit value tends to decrease during egg laying due to the process of hemodilution, namely an increase in blood plasma volume and erythrocyte mass. Plasma levels return to normal when the last follicle ovulates (Hu *et al.*, 2021).

Pantaya & Utami (2020) stated that the number of erythrocytes is closely related to hemoglobin and hematocrit levels. Nutricell Eggstra supplementation in the diet did not interfere with the average erythrocyte counts and the birds could metabolize their nutrients properly. Laying hens reared at high ambient temperatures are highly susceptible to infectious diseases and vitamin D deficiency decreases the cellular immune response in hens. Vitamin D_3 supplementation has been shown to have anti-inflammatory activity in lipopolysaccharide induced avian immune cells (Attia *et al.*, 2020), enhancing the innate humoral immunity of birds and counteracting inflammatory responses when exposed to inappropriate immune reactions (Shojadoost *et al.*, 2015). Leukocytes play an active role in the body's defense system. Vitamin D plays an important role in enhancing pathogen-specific immune responses, and $1,25(OH)_2D_3$ has been shown to increase phagocytosis, differentiation, and production of antimicrobial peptides in macrophages (Martens *et al.*, 2020). The study by Saunders-Blades & Korver (2014) showed that substituting cholecalciferol with $25OHD_3$ in the diet increased the immune system by increasing leukocyte bactericidal activity and phagocytic responses.

Leukocyte differentials were classified according to the type of white blood cells. Lower lymphocyte levels in laying hens supplemented with vitamin D_3 indicate that the birds were not exposed to infections or disease agents, which is in line with the findings of Hofmann *et al*. (2020), which stated that a high lymphocyte level indicates that the birds were exposed to infection. Vitamin D can modulate the function of chicken lymphocytes by reducing cell proliferation, cytokine production, and phosphorylation of non-stimulated lymphocytes (Boodhoo *et al.*, 2016). However, vitamin D supplementation did not alter the ability of lymphocytes to undergo degranulation (Shojadoost *et al.*, 2015). This may be due to the differential effects of vitamin D on the functional abilities of T lymphocytes, which can inhibit immunopathology leading to T lymphocyte exhaustion without inducing general immunosuppression (Boodhoo *et al*., 2016). In the body's first line of defense, higher levels of heterophils in laying hens supplemented with vitamin D_3 are associated with the increased levels of vitamin D, which increases specificity.

According to Gross & Siegel (1983), stress levels in poultry can be categorized by the H/L ratio, birds with H/L ratios of 0.20 (low stress), 0.50 (moderate stress), and 0.80 (high stress). The H/L ratios obtained in this study indicated that the birds in each treatment experienced moderate stress levels. This suggests that birds experience heat stress owing to environmental temperature (Gross & Siegel., 1983). The increased H/L ratio observed in birds supplemented with vitamin D_3 could be due to the high egg production during the study.

Tibia Bone Minerals

The bone quality of laying hens is closely related to egg production and eggshell quality. According to Kerschnitzki *et al.* (2014), the medullary bone of birds changes rapidly during the oviposition cycle, and its mineral content decreases significantly during the shell formation process. The average tibia Ca and P mineral contents in this study ranged between 38.45%–39.98% and 5.12%-5.77%, respectively, which was higher than that observed by Attia *et al.* (2020), who supplemented feed with vitamin doses between 800–1200 IU/kg, namely 31.30%-32.60%, but with a lower P mineral content of 13.50%–14.30%.

According to Kakhki *et al.* (2019), maximizing bone mineralization is important because it increases bone strength and reduces problems related to leg weakness, morbidity, and mortality in birds. Vitamin D plays an important role in Ca and P absorption, bone mineralization, and the regulation of PTH (Garcia *et al.*, 2013). Vitamin D can regulate collagen maturation because of its role as a transcriptional regulator in bones to reduce collagen synthesis. Collagen is a prominent protein in bone, and maintaining proper collagen content is important for building bone health (Duran *et al*., 2018).

The average Zn content in the tibia in this study ranged between 127.86-131.39 ppm. Groff & Gropper (2000) stated that Zn minerals are required for alkaline phosphatase activity, which plays a role in Ca resorption in bone and is responsible for providing inorganic P where required. Schisler & Kienholz (1967) reported that vitamin D deficiency reduces Zn mineral levels in the bones of laying hens. There were no significant differences in the levels of Ca, P, and Zn in this study, which could be related to the age of the hens and the duration of vitamin D_3 supplementation.

Immune Organs

Immune organs are indicators of poultry health. The primary lymphoid organs, namely the bursa of Fabricius and thymus, produce lymphoid cells that function as specific immune systems, including fighting pathogenic bacteria (B lymphoid cells), fighting antigens in cells and destroying cells affected by viruses (T lymphoid cells) (Owen *et al.*, 2013). At the time of surgery, no bursa of Fabricius was observed in any treatment group. According to Ko *et al.* (2018), this occurs because the bursa of Fabricius is formed from the embryo and can disappear after the birds mature. The bursa of Fabricius is then replaced by the other lymphoid organs, such as the spleen, thymus, and ceca tonsils. Secondary lymphoid organs, such as the spleen, play an important role in the repair, maturation, and selection of lymphoid cells to deal with specific pathogens.

CONCLUSION

Supplementing the diet of laying hens with Nutricell Eggstra can potentially improve egg production and quality. The best production performance was observed with 1.00 kg/ton supplementation, while 1.50 kg/ton resulted in better yolk color, egg yolk vitamin D_3 content, and eggshell quality. However, supplementation did not affect the tibia bone mineral content or immunity. Further research is needed to explore the long-term effects of Nutricell Eggstra and Ca supplementation on the bone quality and immune response in laying hens.

CONFLICT OF INTEREST

W. W. Wardani, I. Akbar, and N. D. S. Putri are employees of PT. Nutricell Pacific, but no conflict of interest could be construed to influence the content of this study. R. Mutia serves as an editor of the Tropical Animal Science Journal but has no role in the decision to publish this article.

ACKNOWLEDGEMENT

The authors extend their gratitude to PT. Nutricell Pacific for providing the financial support for this research.

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