Animal Nutrition 19 (2024) 90–103

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Dietary supplementation with 25-hydroxyvitamin D_3 regulates productive performance, lipid metabolism and gut microbiota in aged laying ducks

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ARTICLE INFO

Article history:
Received 7 December 2023
Received in revised form
29 February 2024
Accepted 4 April 2024
Available online 22 August 2024

Keywords:
Laying duck
25-Hydroxyvitamin D_3
Lipid metabolism
Cecal microbiota

ABSTRACT

The aim of this study was to investigate the effect of dietary supplementation with 25-hydroxyvitamin D_3 (25(OH)D_3) on productive performance, lipid metabolism and gut microbiota in aged laying ducks. A total of 432 healthy Longyan ducks at 60-week of age were randomly allotted to 6 groups, each with 6 replicates of 12 ducks. Ducks were given a basal diet (without added 25(OH)D_3) or that diet supplemented with 800, 1600, 2400, 3200, or 4000 IU/kg 25(OH)D_3 for a total of 16 wk. Dietary supplementation with 25(OH)D_3 improved egg production, egg mass and average daily feed intake, and decreased the feed conversion ratio (FCR) of ducks during the whole trial period (linear, quadratic; $P < 0.05$). Supplementation with 25(OH)D_3 decreased very low-density lipoprotein (VLDL) content in yolk ($P = 0.008$), decreased high-density lipoprotein and low-density lipoprotein (LDL) content in plasma ($P = 0.002$). Hepatic index, VLDL, LDL, triglyceride and total cholesterol content in liver, nonalcoholic fatty liver activity score of liver and alanine aminotransferase activity in plasma were decreased with supplementation of 25(OH)D_3 (linear or quadratic; $P < 0.05$). The decreased hepatic apolipoprotein B 100 and lipoprotein lipase expression, and increased hepatic peroxisome proliferator-activated receptor-α and sterol regulatory element binding protein-1 expression resulted from 25(OH)D_3 supplementation (linear, quadratic; $P < 0.05$). Moreover, 25(OH)D_3 supplementation increased the villus/crypt ratio (linear, quadratic; $P < 0.05$) and expression of zonula occludens protein 1 and nuclear factor-κ-gene binding in duodenum ($P < 0.05$). The supplementation of 25(OH)D_3 reduced the abundance of Wittenberg polluted soil-2 bacteria, Synergistota, *Bacteroidales*, *Colidextribacter*, Eggerthellaceae, *Oscillospira*, *Oscillibacter*, *UCG-009*, Barnesiellaceae and *Lachnospiraceae_UCG-010* in cecal contents ($P < 0.05$). Dietary requirements for 25(OH)D_3 for ducks (60 to 76 wk), were estimated to be 3377 IU/kg for egg production, 3434 IU/kg for egg mass, and 3256 IU/kg for FCR. In summary, dietary 25(OH)D_3 supplementation improved productive performance and influenced liver and plasma lipid homeostasis in aged laying ducks, which may be associated with the reduction of bacteria involved in carbohydrate metabolism in the cecum. Supplementing the basal diet with 3250 to 3450 IU/kg 25(OH)D_3 is recommended for aged laying ducks (60 to 76 wk).

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Peer review under the responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2024.04.029

1. Introduction

Aged commercial laying poultry are known for their decreased productive performance after the long peak-laying period, leading to limited economic profit (England and Ruhnke, 2020; Van Den Brand et al., 2004). This is partly because of the disturbance in the lipid metabolism, which can result in the lipid droplet deposition in liver (Trott et al., 2014), affecting hepatic function. In aged laying hens, fatty liver hemorrhagic syndrome impairs the ability of the liver to synthesize yolk precursors, which reduces egg production and egg quality (Tůmová et al., 2017). In addition, impaired intestinal function with aging also contributes to decreased performance of aged laying hens (Rattanawut et al., 2018; Wistedt et al., 2019). Transcriptome analysis found that the homeostasis of lipid metabolism and antioxidant status in the intestine in aged laying hens was impaired compared with the young hens (Wang et al., 2019). Compared with hens at the late laying period, the population of *Bacteroides* in the cecal microbiome was enriched, and the pathway of carbohydrate biosynthesis and metabolism was enhanced in cecal contents in laying hens at the peak laying period; these might provide more energy to the hens and lead to higher levels of egg production (Yang et al., 2022). In this respect, several studies have focused on regulating lipid metabolism and gut health in aged birds (Han et al., 2023; W.W. Wang et al., 2020).

Vitamin D_3 is an essential nutrient influencing bone health and the dynamic balance of calcium and phosphorus, and improving eggshell quality in laying hens (Chen et al., 2020; Sinclair-Black et al., 2023). Dos Santos et al. (2021) found that dietary supplementation 25(OH)D_3 increased eggshell thickness in aged layers. Recent studies have discovered that vitamin D_3 played a pivotal role in lipid metabolism and intestinal homeostasis in vivo or in vitro (Lee et al., 2020; Fakhoury et al., 2020). The primary form of vitamin D_3 that circulates in the blood is 25-hydroxyvitamin D_3 (25(OH)D_3), which is of hepatic origin (Soares et al., 1995). It is one of the best markers of the vitamin D_3 status in animals (Bar et al., 2003). The commercial product, 25 (OH)D_3, has gained approval as a feed additive for animals, and shows better biological activity than vitamin D_3 in laying hens (J. Wang et al., 2020, 2021). It has been demonstrated that 25(OH)D_3 was more easily utilized than vitamin D_3 in birds (Bar et al., 1980). Dietary 25(OH)D_3 supplementation improved gut morphology in growing chickens (Chou et al., 2009) and intestinal barrier function in hens (Wang et al., 2023). In addition, 25(OH)D_3 played an anti-injury role in intestinal barrier function and affected colonization and composition of intestinal microbes (Zhang and Piao, 2021). Vitamin D influenced the gut microbiome, and its deficiency resulted in dysbiosis by increasing the abundance of Bacteroidetes and Proteobacteria phyla, leading to greater susceptibility to injury in the guts of mice (Ooi et al., 2013). Several studies have observed a correlation between vitamin D_3 and lipid metabolism. Vitamin D plays a role in reducing lipid accumulation and inhibiting fat formation in 3T3-L1 preadipocytes (Rayalam et al., 2008). It has been observed that the absence of adipose tissue-specific vitamin D receptor in female mice can lead to an increase in visceral fat mass (Matthews et al., 2016). The expression of lipogenic genes, such as fatty acid synthase (*FAS*), acetyl-CoA carboxylase 1 (*ACC1*), and sterol regulatory element-binding protein-1c (*SREBP-1c*), was suppressed by dietary 1,25-dihydroxyvitamin D_3 supplementation in rats (Kang et al., 2015). 1,25-Dihydroxyvitamin D_3 suppressed biosynthesis of fat in preadipocytes and mesenchymal stem cells from mice, swine and humans through multiple targets including peroxisome proliferator-activated receptor-γ (*PPAR-γ*) (Mutt et al., 2014; Li et al., 2018).

A number of studies have indicated that laying performance, egg and bone quality, were optimized by inclusion of 1500 to 3300 IU/kg 25(OH)D_3 in diets for laying hens (Chen et al., 2020; Terry et al., 1999; Koreleski and Świątkiewicz, 2005). Given the lack of research on 25(OH)D_3 in laying ducks, the equivalent recommendation for such birds is lacking. Overall, the current research was aimed at studying the effect of dietary 25(OH)D_3 supplementation on productive performance, egg quality, lipid metabolism, gut morphology and microflora composition in aged laying ducks, to recommend a suitable supplemental level of 25(OH)D_3 for use in the laying duck industry.

2. Materials and methods

2.1. Animal ethics statement

The animal experimental protocol used in this study was based on the Chinese guidelines for animal welfare and was approved by the Animal Care and Use Committee of Guangdong Academy of Agricultural Sciences (2023006).

2.2. Diets and study design

A total of 432 healthy Longyan ducks at 60-wks were randomly allotted to 6 groups, each with 6 replicates of 12 ducks. The trial lasted for 16 wk. Ducks were fed a basal diet without 25(OH)D_3 supplementation or supplementation with 25(OH)D_3 (250 mg/kg; DSM, Heerlen, Netherlands) at 800, 1600, 2400, 3200, or 4000 IU/kg (1 IU = 0.025 μg). Each duck was raised in a cage equipped with a feeder and a nipple water dispenser. Diets and water were supplied ad libitum. The composition and nutrient levels of the basal diet are listed in Table 1. It was formulated according to the China National Standard (GB/T 41189-2021). The content of crude protein in the diet was determined by using the Kjeldahl method (GB/T 6432-2018). The contents of calcium and total phosphorus in the diet were determined by ethylenediaminetetraacetic acid complexometric titration (GB/T 6436-2018) and ammonium metavanadate colorimetric (GB/T 6437-2018) method respectively.

2.3. Sample collection

At the end of wk 4, 8, 12, and 16 of the trial periods, 3 eggs approximating the average egg weight for each replicate were

Table 1
Ingredients and nutrient levels of basal diets (air-dry basis, %).

Ingredients	Content	Nutrient level[2]	Content
Corn (CP 7.8%)	53.90	AME, MJ/kg	10.46
Soybean meal (CP 43%)	25.30	Crude protein	17.06
Wheat bran (CP 15.6%)	8.93	Calcium	3.59
Limestone	8.90	Total phosphorus	0.60
DL-Methionine	0.16	Available phosphorus	0.37
L-Lysine sulfate	0.03	Lysine	0.42
Calcium hydrogen phosphate	1.43	Methionine	0.88
Salt	0.35		
Premix[1]	1.00		
Total	100		

AME = apparent metabolizable energy; CP = crude protein.
[1] Premix provided the following per kilogram of diet: vitamin A 7500 IU, vitamin E 20.00 IU, vitamin K_3 2.50 mg, vitamin B_1 3.45 mg, vitamin B_2 6.00 mg, vitamin B_6 2.50 mg, vitamin B_{12} 0.02 mg, Choline chloride 600 mg, pantothenic acid 20.58 mg, folic acid 1.00 mg, biotin 0.20 mg, nicotinic acid 27.13 mg, Fe ($FeSO_4 \cdot H_2O$) 75.00 mg, Cu ($CuSO_4 \cdot 5H_2O$) 10 mg, Mn ($MnSO_4 \cdot H_2O$) 50.00 mg, Zn ($ZnSO_4 \cdot H_2O$) 43.75 mg, Se (Na_2SeO_3) 0.30 mg, I (KI) 0.44 mg.
[2] AME, available phosphorus, lysine and methionine were calculated according to China National Standard (GB/T 41189-2021), and the rest of the nutrient levels were measured.

randomly selected to measure egg quality. The measurements were completed on the day of collection.

At the end of trial, 2 ducks from each replicate were selected according to average body weight. After fasting for 12 h, blood (5 mL each tube, 2 tubes each duck) was obtained from the wing vein into evacuated tubes, containing heparin sodium, and centrifuged at 3000 × *g* for 10 min, and the plasma was collected and kept at −80 °C until analysis.

These ducks were then killed by cervical dislocation and exsanguination. The liver was immediately excised and weighed. The liver tissue sample (1 cm × 1 cm × 1 cm) was taken from the mid-right section and fixed in formaldehyde. A sample of middle liver was put into a 1.5-mL sterile tube, and the remaining liver tissue was wrapped in aluminum foil, and then immediately frozen in liquid nitrogen and stored at −80 °C until analysis. A segment (1 cm) was excised from the mid-duodenum and fixed in formaldehyde. The lumen would be rinsed with saline and a segment would be opened lengthwise for the mucosa to be removed by gentle scraping, before being transferred into nuclease-free tubes. These tubes were immediately placed into liquefied nitrogen and kept in the refrigerator at −80 °C until analysis. Similarly, the contents of the cecum were also scraped into an enzyme-free tube and immediately placed in liquid nitrogen, and then stored at −80 °C refrigerator until analysis.

2.4. Performance and egg quality

The number and weight of eggs and broken eggs, and the feed consumption were recorded daily. Egg production, average egg weight, egg mass, average daily feed intake, feed conversion ratio (FCR), and ratio of broken eggs were calculated every 4 wk.

An electronic analytical balance was used to measure egg, yolk and albumen weight, then the albumen and yolk ratio was calculated. The albumen height, yolk color, and Haugh unit were measured with an Egg Analyzer (Israel Orka Food Technology Ltd., Herzliya, Israel).

2.5. Lipid metabolism indices in plasma, liver and yolk

The indices of lipid metabolism included triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), very low-density lipoprotein (VLDL), insulin (INS), lipase (LPS), total cholesterol (TC), aspartate transaminase (AST) and alanine aminotransferase (ALT) in plasma, liver and yolk, and were analyzed by biochemical kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Plasma concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E_2) were measured by ELISA kits for ducks (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China).

2.6. Liver fat content

Crude fat content in liver was determined using Soxhlet extraction with diethyl ether. The liver tissues stored at −80 °C were thawed at room temperature, oven-dried at 105 °C to constant mass; defatted filter paper was dried the same way. The dried liver was ground and a sample of approximately 2 g was weighed precisely (±100 g), wrapped in filter paper and re-dried, as above. After Soxhlet extraction (Soxtec-2055, FOSS, Hilleroed, Denmark) with anhydrous diethyl ether, the remaining tissue and filter was dried, as before, and weighed. Hepatic fat was expressed relative to dry tissue weight as percentages.

2.7. Liver and duodenum histology

Hepatic and duodenal tissues fixed in formalin were dehydrated, embedded in paraffin wax, sectioned at 4 μm thickness, dewaxed, rehydrated and then stained with sodium fluorescein (NaFl) dye, which consists of the degree of steatosis, ballooning, and lobular inflammation, scored using the published scoring system (Kleiner et al., 2005). 3DHistech software (3DHistech Ltd., Budapest, Hungary) was used for morphological examination of duodenal samples. Villus height was measured from the tip of the villus to the villus–crypt junction, and crypt depth was defined as the depth of the invagination between two villi (Saadatmand et al., 2019). Villus height to crypt depth ratio was then calculated.

2.8. Expression of key genes involved in lipid metabolism and the intestinal barrier

Total RNA was extracted from tissue samples using the Trizol procedure, giving product of OD260/280 (from 1.8 to 2.0) for reverse transcription (Aikerui Bioengineering Co., Ltd., Hunan, China) to generate cDNA. Relative expression was determined by real-time fluorescence quantitative PCR (Bio-Rad CFX96, Hercules, CA) with primers (Table 2) prepared by Shenggong Bioengineering Co., Ltd. (Shanghai, China). Relative quantification used the $2^{-\Delta\Delta Ct}$ approach (Livak and Schmittgen, 2001) with β-actin used as the reference transcript.

2.9. Cecal microflora determination by 16S rRNA

The composition of the duck cecal microbiome was determined by 16S rRNA sequencing, performed by Meiji Biotechnology Co., Ltd. (Shanghai, China). After DNA extraction was complete, the quality of the extracted DNA was checked using 1% agarose gel electrophoresis. PCR amplification was performed with the TransStart FastPfu DNA Polymerase (Transgen Biotech, Beijing, China) and

Table 2
Primer sequence information.

Genes	Primer sequence (5′→3′)	Accession number
β-Actin	Forward: GCTATGTCGCCCTGGATTT	NM-001310421.1
	Reverse: GGATGCCACAGGACTCCATAC	
OVR	Forward: ACTGTCCAGCTGGGTTTGAG	NM_001310401.1
	Reverse: ACAGCTTTGCACACTCCTGTA	
PPARα	Forward: AATGGCTGACCTCCGACAAC	NM_001310383.1
	Reverse: ACTTGTCCCTGTAGATTTCCTG	
LXR	Forward: CCTGCTGCTCCTTACTCTGC	NM_001310423.1
	Reverse: GTGAAAGCCCTTCCTCCTCC	
SREBP1	Forward: CGTTGGTGAGCGGGGG	XM_038187023.1
	Reverse: CGTTGATGGAGGAGCGGTAG	
ApoB100	Forward: AGCCAGGCTGATTTCGTCAA	XM_005012904.5
	Reverse: CTCAACCCTGAAGGTGGCAT	
ApoVLDL-II	Forward:GGCATTGGTGATAGCTCTGATCCTG	NM_001310364.1
	Reverse: AGGTCCTGGTCCCAACAACTACTG	
LPL	Forward: CTTGCTGGGGTACAGTCTGG	XM_027446391.2
	Reverse: CGTAAGGGCATCAGCGTACT	
ACC	Forward: TATGGGTGTCTTCGCAGCAG	XM_038165892.
	Reverse: AGTCTGGGCTCCATGTCTCT	
FAS	Forward: CAGCGGCAGTTGGTCAGTT	AY613443.1
	Reverse: GGCTCTCTCTCACATTGGCAG	
INSR	Forward: CACGCACTACCTGGTGTTCT	XM_038169078.1
	Reverse: CAGGAACAACACTCTCCGCT	
ZO-1	Forward: TTGATGATTGCATGCAGCGG	XM_038184899.1
	Reverse: CATCTGCAAGACGGTCAGGT	
NF-κB	Forward: CCTCACCAGGAGGACAACAC	XM_038177856.1
	Reverse: TCAGTTTGCGGAAGGAGGTC	
TNF-α	Forward: ATTCCCCGAGGCACGAATTT	XM_027471963.2
	Reverse: TCACTATGAGAGGCCTGGCT	

OVR = ovarian very low-density lipoprotein receptor; *PPARα* = peroxisome proliferator-activated receptor-α; *LXR* = liver X receptor; *SREBP1* = sterol regulatory element binding protein-1; *ApoB100* = apolipoprotein B 100; *ApoVLDL-II* = apolipoprotein very low-density lipoprotein-II; *LPL* = lipoprotein lipase; *ACC* = acetyl-CoA carboxylase; *FAS* = fatty acid synthase; *INSR* = insulin receptor; *ZO-1* = zonula occludens protein 1; *NF-κB* = nuclear factor-κ-gene binding; *TNF-α* = tumor necrosis factor-α.

each sample was repeated three times. The amplified products from the same sample were mixed and electrophoresis was performed with 2% agarose gel for detection. Based on the preliminary electrophoresis quantification results, the QuantiFluor™-ST blue fluorescence quantification system (Promega Corporation, Wisconsin, USA) was used to detect and quantify the PCR products, and the products were mixed at an appropriate ratio according to the sequencing requirements of each sample. The Illumina library was constructed and sequenced according to the sequencing quantity requirements of each sample. Paired-end reads from Illumina sequencing were first combined based on overlap, while sequence quality was controlled and filtered. After identifying the samples, operational taxonomic unit (OTU) cluster analysis and species classification analysis were both performed. Based on the OTU, multiple diversity analysis and sequencing depth detection were performed. Based on taxonomic information, a statistical analysis of community structure at various levels was estimated.

2.10. Statistical analysis

Each replicate was taken as the experimental unit. Homogeneity of variance of the data was confirmed using the Explore tool of SPSS 22.0 software (IBM Corporation, Armonk, NY, USA). To assess the impact of dietary 25(OH)D_3 supplementation, one-way ANOVA was employed, then means were compared using Tukey's multiple range tests. Regression analysis was employed to test the linear (L) and quadratic (Q) effects using SPSS 22.0 software.

The formula ($Y = aX^2 + bX + c$) is used to estimate the recommended supplemental level of 25 (OH)D_3, given by the lowest level resulting in 95% of the maximal response. The microbiome analysis was carried out using the Majorbio Cloud platform (Shanghai, China; https://cloud.majorbio.com). Data are expressed as means and pooled SEM. $P < 0.05$ was considered to be significant. The software Origin 2021 (OriginLab, Northampton, UK) was used for figures.

3. Results

3.1. Productive performance

Dietary 25(OH)D_3 supplementation improved egg production, egg mass, and average daily feed intake ($P < 0.05$, Table 3), and the responses were linear and quadratic with increasing levels during the trial period (5 to 8 wk, 9 to 12 wk, 13 to 16 wk and 1 to 16 wk, $P < 0.05$). The FCR was decreased with dietary 25(OH)D_3 supplementation during the whole duration ($P < 0.001$), and there were linear and quadratic effects between them ($P < 0.05$). There were no differences among treatments in the ratio of broken eggs ($P = 0.692$). Compared with the control, diets supplemented with 25(OH)D_3 at 2400 to 4000 IU/kg increased egg production, egg mass and feed intake, and decreased FCR during the whole trial period ($P < 0.05$).

Table 3
Dietary 25(OH)D_3 supplementation improved productive performance in aged laying ducks.[1]

Item	25(OH)D_3, IU/kg						SEM	*P*-value		
	0	800	1600	2400	3200	4000		ANOVA	Linear	Quadratic
Egg production, %										
1 to 4 wk	83.9	87.1	87.8	89.3	88.8	89.1	0.67	0.164	0.014	0.020
5 to 8 wk	74.5^{b}	78.4^{b}	82.7^{ab}	87.2^{a}	88.2^{a}	88.8^{a}	1.27	<0.001	<0.001	<0.001
9 to 12 wk	67.8^{b}	74.1^{ab}	75.5^{ab}	80.0^{a}	81.3^{a}	80.2^{a}	1.25	0.007	<0.001	<0.001
13 to 16 wk	60.3^{b}	75.7^{a}	74.0^{a}	82.0^{a}	79.8^{a}	80.6^{a}	1.72	<0.001	<0.001	<0.001
1 to 16 wk	71.6^{b}	78.8^{a}	80.0^{a}	84.6^{a}	84.5^{a}	84.7^{a}	1.06	<0.001	<0.001	<0.001
Average egg weight, g										
1 to 4 wk	69.0	69.8	70.9	70.1	69.1	75.5	0.75	0.100	0.051	0.073
5 to 8 wk	70.2	70.6	71.1	71.7	70.1	71.3	0.21	0.114	0.264	0.318
9 to 12 wk	69.3	70.8	70.6	71.0	70.0	70.8	0.24	0.294	0.237	0.241
13 to 16 wk	69.1	70.1	69.2	68.6	69.3	70.7	0.47	0.839	0.593	0.644
1 to 16 wk	69.4^{b}	70.3^{ab}	70.5^{ab}	70.4^{ab}	69.6^{b}	72.1^{a}	0.26	0.036	0.030	0.075
Egg mass, g										
1 to 4 wk	57.8	60.9	62.3	62.7	61.4	67.3	0.85	0.360	0.003	0.013
5 to 8 wk	52.1^{c}	55.4^{bc}	58.9^{ab}	62.5^{a}	61.8^{a}	63.4^{a}	0.95	<0.001	<0.001	<0.001
9 to 12 wk	46.9^{b}	51.9^{ab}	51.9^{ab}	58.0^{a}	56.9^{a}	55.5^{a}	0.97	0.003	0.001	<0.001
13 to 16 wk	41.5^{b}	53.1^{a}	52.4^{a}	56.5^{a}	54.3^{a}	55.0^{a}	1.25	0.002	0.002	<0.001
1 to 16 wk	49.6^{c}	55.3^{b}	56.4^{ab}	59.9^{a}	58.6^{ab}	60.3^{a}	0.81	<0.001	<0.001	<0.001
Average daily feed intake, g										
1 to 4 wk	166	168	169	169	169	169	0.4	0.094	0.014	0.012
5 to 8 wk	162^{b}	166^{ab}	167^{a}	171^{a}	171^{a}	171^{a}	0.8	0.001	<0.001	<0.001
9 to 12 wk	162	164	165	169	168	167	0.8	0.083	0.010	0.014
13 to 16 wk	153^{b}	161^{a}	162^{a}	165^{a}	164^{a}	163^{a}	1.1	0.011	0.003	0.001
1 to 16 wk	161^{b}	165^{a}	166^{a}	168^{a}	168^{a}	168^{a}	0.7	0.003	<0.001	<0.001
FCR, g:g										
1 to 4 wk	2.89^{a}	2.77^{ab}	2.72^{ab}	2.70^{ab}	2.76^{ab}	2.55^{b}	0.031	0.049	0.005	0.020
5 to 8 wk	3.13^{a}	3.02^{ab}	2.85^{bc}	2.74^{c}	2.77^{c}	2.70^{c}	0.036	<0.001	<0.001	<0.001
9 to 12 wk	3.48^{a}	3.18^{b}	3.19^{b}	2.92^{b}	2.96^{b}	3.02^{b}	0.047	0.002	<0.001	<0.001
13 to 16 wk	3.69^{a}	3.05^{b}	3.13^{b}	2.96^{b}	3.04^{b}	2.99^{b}	0.064	0.003	0.003	0.001
1 to 16 wk	3.30^{a}	3.00^{b}	2.97^{bc}	2.83^{bc}	2.88^{bc}	2.82^{c}	0.035	<0.001	<0.001	<0.001
Broken egg rate, %										
1 to 4 wk	4.11	3.38	3.90	3.02	3.69	2.61	0.268	0.624	0.181	0.406
5 to 8 wk	5.16	4.57	4.98	4.10	4.10	3.86	0.283	0.745	0.132	0.328
9 to 12 wk	5.45	4.92	5.01	4.70	4.24	5.21	0.315	0.929	0.589	0.662
13 to 16 wk	5.08	5.34	4.65	4.52	4.35	2.19	0.436	0.367	0.048	0.083
1 to 16 wk	4.95	4.55	4.63	4.09	4.10	3.47	0.266	0.692	0.088	0.231

25(OH)D_3 = 25-hydroxyvitamin D_3; FCR = feed conversion ratio.
Within a row, values with different superscript letters differ at $P < 0.05$.
[1] Mean of 6 replicates (12 ducks per replicate) per treatment.

3.2. Egg quality

Dietary supplementation of 25(OH)D_3 decreased weight and ratio of yolk, and increased the albumen ratio at wk 12, 16 and the mean value for the total trial period ($P < 0.05$, Table 4), and the responses were quadratic ($P < 0.05$). At wk 12, albumen weight was increased with supplementation of 25(OH)D_3 ($P = 0.027$), and there was a quadratic effect of increasing dose ($P = 0.004$). Egg weight, albumen height, yolk color, and Haugh unit were not affected by dietary 25(OH)D_3 supplementation ($P > 0.05$). Compared with the control, decreased yolk weight and ratio resulted from supplementation with 800 IU/kg 25(OH)D_3, while albumen ratio was increased by 1600 IU/kg 25(OH)D_3 ($P < 0.05$).

3.3. Lipid metabolism

Dietary supplementation with 25(OH)D_3 affected the contents of TC, HDL, LDL and FSH in plasma of laying ducks ($P < 0.05$, Table 5). There was a quadratic effect between level of 25(OH)D_3 supplementation and TC content ($P < 0.001$) with the lowest value obtained with 2400 IU/kg. The contents of HDL and LDL decreased linearly and quadratically with increasing dietary 25(OH)D_3 levels ($P < 0.05$). Plasma concentration of FSH increased linearly and quadratically with dietary 25(OH)D_3 supplementation ($P < 0.05$). There were no significant changes to TG, VLDL, E_2 and LH in plasma with supplementation of 25(OH)D_3 ($P > 0.05$). Compared with the control, diets supplemented with 2400 and 3200 IU/kg 25(OH)D_3

Table 4
Dietary 25(OH)D_3 supplementation affected egg quality in aged laying ducks.[1]

Item	25(OH)D_3, IU/kg						SEM	*P*-value		
	0	800	1600	2400	3200	4000		ANOVA	Linear	Quadratic
Egg weight, g										
4 wk	69.6	68.4	70.4	69.2	69.4	68.8	0.35	0.714	0.750	0.833
8 wk	71.8	69.8	71.9	70.9	69.5	70.6	0.36	0.276	0.284	0.561
12 wk	71.4	71.1	72.2	72.3	71.9	70.2	0.35	0.532	0.639	0.273
16 wk	69.3	69.0	69.1	69.6	68.6	69.4	0.48	0.996	0.966	0.996
Mean	70.5	69.6	70.9	70.5	69.9	69.7	0.23	0.517	0.458	0.590
Yolk weight, g										
4 wk	22.9	22.6	22.4	22.7	22.7	22.4	0.17	0.962	0.658	0.860
8 wk	23.6	21.9	23.1	22.6	23.3	22.9	0.20	0.217	0.966	0.694
12 wk	24.3^{a}	23.2^{b}	22.8^{b}	22.7^{b}	22.9^{b}	23.1^{b}	0.16	0.043	0.031	0.003
16 wk	24.2^{a}	22.3^{b}	22.8^{ab}	22.9^{ab}	22.2^{b}	23.4^{ab}	0.21	0.043	0.361	0.044
Mean	23.7^{a}	22.5^{b}	22.8^{b}	22.7^{b}	22.8^{b}	23.0^{b}	0.12	0.039	0.213	0.028
Albumen weight, g										
4 wk	40.2	39.5	41.5	40.0	40.2	39.7	0.38	0.721	0.818	0.744
8 wk	41.7	41.4	42.1	41.6	39.7	41.2	0.26	0.107	0.121	0.298
12 wk	41.2^{bc}	41.5^{abc}	42.8^{ab}	43.2^{a}	42.5^{ab}	40.5^{c}	0.28	0.027	0.970	0.004
16 wk	38.8	40.6	41.5	41.0	40.8	39.9	0.41	0.488	0.512	0.113
Mean	40.5	40.7	42.0	41.5	40.8	40.3	0.21	0.140	0.804	0.039
Yolk ratio, %										
4 wk	33.0	33.0	31.8	32.8	32.8	32.7	0.31	0.907	0.849	0.846
8 wk	32.9	31.3	32.2	31.9	33.6	32.4	0.23	0.077	0.377	0.453
12 wk	33.9^{a}	32.7^{b}	31.5^{b}	31.4^{b}	31.8^{b}	32.9^{b}	0.22	<0.001	0.070	<0.001
16 wk	35.1^{a}	32.3^{c}	32.6^{ab}	32.9^{ab}	32.4^{c}	33.7^{b}	0.23	<0.001	0.198	0.001
Mean	33.7^{a}	32.3^{b}	32.0^{b}	32.3^{b}	32.6^{ab}	32.9^{ab}	0.16	0.022	0.406	0.003
Albumen ratio, %										
4 wk	57.6	57.6	59.0	57.8	57.8	57.6	0.32	0.818	0.953	0.700
8 wk	58.1	59.4	58.6	58.7	57.2	58.3	0.23	0.135	0.253	0.416
12 wk	57.6^{b}	58.4^{ab}	59.3^{a}	59.5^{a}	59.1^{a}	57.8^{b}	0.20	0.005	0.429	<0.001
16 wk	56.2^{c}	58.8^{ab}	59.1^{ab}	58.9^{ab}	59.4^{a}	57.5^{bc}	0.29	0.003	0.156	<0.001
Mean	57.4^{c}	58.5^{ab}	59.0^{a}	58.7^{ab}	58.4^{abc}	57.8^{bc}	0.17	0.041	0.677	0.004
Albumen height, mm										
4 wk	8.03	7.67	7.83	7.98	8.11	8.04	0.086	0.723	0.390	0.553
8 wk	7.76	7.69	7.98	7.82	7.91	7.92	0.100	0.970	0.533	0.813
12 wk	7.67	7.45	7.56	7.55	7.47	7.46	0.051	0.836	0.354	0.631
16 wk	7.61	7.09	7.56	7.37	7.20	7.27	0.084	0.447	0.367	0.654
Mean	7.77	7.48	7.73	7.68	7.67	7.67	0.048	0.622	0.971	0.938
Yolk color										
4 wk	4.39	4.67	4.50	4.61	4.45	4.39	0.052	0.552	0.616	0.326
8 wk	4.72	5.00	4.89	4.61	4.78	4.89	0.064	0.589	0.937	0.980
12 wk	4.61	5.03	4.72	4.89	4.61	4.78	0.065	0.396	0.854	0.734
16 wk	5.06	5.17	4.95	5.17	5.17	4.94	0.046	0.487	0.726	0.719
Mean	4.69	4.97	4.76	4.82	4.75	4.75	0.034	0.244	0.651	0.505
Haugh unit										
4 wk	87.2	85.0	85.6	88.1	87.4	87.1	0.53	0.545	0.392	0.660
8 wk	85.0	84.9	86.2	84.4	86.1	86.6	0.54	0.845	0.376	0.639
12 wk	84.9	83.2	83.3	83.7	83.5	83.6	0.36	0.794	0.478	0.480
16 wk	84.1	80.6	82.8	82.2	81.3	81.7	0.55	0.583	0.375	0.576
Mean	85.3	83.4	84.5	84.6	84.6	84.8	0.27	0.576	0.865	0.635

25(OH)D_3 = 25-hydroxyvitamin D_3.
Within a row, values with different superscript letters differ at $P < 0.05$.
[1] Mean of 6 replicates (3 eggs per replicate) per treatment.

Table 5
Dietary 25(OH)D_3 supplementation affected lipid metabolism indices in plasma, liver and yolk in aged laying ducks.[1]

Item	25(OH)D_3, IU/kg						SEM	*P*-value		
	0	800	1600	2400	3200	4000		ANOVA	Linear	Quadratic
Plasma										
TG, mmol/L	8.13	8.47	8.94	8.93	8.66	9.42	0.165	0.317	0.037	0.114
TC, mmol/L	4.01^{b}	3.54^{b}	2.66^{b}	2.26^{b}	3.46^{b}	5.86^{a}	0.296	0.003	0.160	<0.001
HDL, mmol/L	12.8^{a}	10.6^{ab}	10.1^{b}	9.2^{bc}	7.6^{c}	10.1^{b}	0.39	0.002	0.001	0.003
LDL, mmol/L	1.81^{a}	1.41^{b}	1.47^{ab}	1.50^{ab}	1.01^{c}	1.26^{bc}	0.061	0.002	0.001	0.003
VLDL, μg/mL	5.79	6.43	5.15	5.24	5.67	5.10	0.167	0.162	0.102	0.261
INS, μIU/mL	8.30^{ab}	9.28^{a}	9.14^{a}	7.01^{c}	7.53^{c}	8.02^{bc}	0.192	0.001	0.024	0.080
FSH, μIU/mL	1.55^{b}	1.42^{b}	2.03^{ab}	3.07^{a}	3.19^{a}	3.19^{a}	0.194	0.002	<0.001	<0.001
E_2, ng/mL	94.9	89.6	78.9	89.0	96.4	94.1	4.79	0.972	0.791	0.705
LH, mU/mL	3.53	4.70	2.53	2.62	2.88	3.56	0.260	0.138	0.332	0.291
Liver										
Body weight, kg	1.32	1.43	1.37	1.34	1.35	1.38	0.018	0.791	0.830	0.951
Liver weight, g	43.7	39.1	38.8	38.7	44.4	39.7	1.21	0.596	0.876	0.705
Liver index, %	3.33	2.75	2.83	2.91	3.25	2.84	0.076	0.120	0.596	1.480
LPS, μmol/g prot	94.3^{cd}	107^{bc}	119^{ab}	120^{ab}	128^{a}	82.7^{d}	3.28	<0.001	0.247	0.006
Liver fat rate, %	33.1	30.6	25.6	29.8	31.0	30.6	0.94	0.333	0.319	0.245
TG, μmol/g prot	76.6^{a}	61.6^{b}	55.3^{b}	55.1^{b}	57.9^{b}	56.5^{b}	1.77	<0.001	0.001	<0.001
TC, μmol/g prot	5.89^{b}	7.32^{a}	4.85^{bcd}	3.64^{d}	4.43^{cd}	4.98^{bc}	0.249	<0.001	0.003	0.003
HDL, μmol/g prot	3.03^{b}	2.67^{b}	2.67^{b}	0.50^{c}	3.18^{b}	3.96^{a}	0.198	<0.001	0.332	<0.001
LDL, μmol/g prot	37.4^{a}	19.7^{b}	10.9^{c}	10.8^{c}	12.4^{c}	12.6^{c}	1.73	<0.001	<0.001	<0.001
VLDL, μg/mg	0.024^{ab}	0.030^{a}	0.022^{b}	0.019^{b}	0.020^{b}	0.020^{b}	0.0011	0.029	0.019	0.065
Yolk										
TG, mmol/g prot	9.29^{b}	9.54^{b}	8.32^{b}	8.61^{b}	9.79^{b}	14.01^{a}	0.374	<0.001	0.001	<0.001
TC, mmol/g prot	26.6	25.2	25.0	26.6	26.3	26.6	0.32	0.497	0.499	0.452
VLDL, μg/mg	0.014^{a}	0.011^{b}	0.012^{ab}	0.012^{ab}	0.009^{b}	0.009^{b}	0.0004	0.008	0.001	0.003

25(OH)D_3 = 25-hydroxyvitamin D_3; TG = triglyceride; TC = total cholesterol; HDL = high-density lipoprotein; LDL = low-density lipoprotein; VLDL = very low-density lipoprotein; INS = insulin; FSH = follicle stimulating hormone; E_2 = estradiol; LH = luteinizing hormone; LPS = lipase.
Within a row, values with different superscript letters differ at $P < 0.05$.
[1] Mean of 6 replicates (2 ducks per replicate) per treatment.

had decreased LDL, HDL and INS contents, and increased FSH content in plasma ($P < 0.05$).

Dietary supplementation of 25(OH)D_3 affected the liver index and its lipase activity, along with hepatic contents of TG, TC, HDL, LDL and VLDL in laying ducks ($P < 0.05$, Table 5). The liver index and its VLDL content were linearly decreased ($P = 0.029$) and the contents of TG, TC and LDL in liver were linearly and quadratically decreased with increasing 25(OH)D_3 supplemental levels ($P < 0.05$). The responses of lipase activity and HDL contents were quadratic with 25(OH)D_3 supplementation in diets ($P < 0.05$). Remarkably, the lowest values of TG, TC, HDL, LDL and VLDL contents in laying ducks were observed with supplementation of 25(OH)D_3 at 2400 IU/kg. Supplementation with 25(OH)D_3 did not affect body weight, liver weight and liver EE content in laying ducks ($P > 0.05$). Dietary 25(OH)D_3 at 2400 and 3200 IU/kg increased LPS content, and decreased TG, TC and LDL content in liver ($P < 0.05$). The HDL content was increased with 4000 IU/kg, but decreased with 2400 IU/kg supplementation ($P < 0.001$).

The VLDL content in egg yolk decreased with increasing supplementation of 25(OH)D_3 ($P = 0.008$, Table 5), with linear and quadratic effects ($P < 0.05$) and lower values were observed with 3200 and 4000 IU/kg supplementation ($P < 0.05$). Dietary 25(OH)D_3 supplementation affected the TG content in egg yolk ($P < 0.001$); responses were linear and quadratic ($P < 0.05$), and values with 4000 IU/kg exceeded all other treatments ($P < 0.05$). There was no difference of TC content in egg yolk with the dietary supplementation of 25(OH)D_3 ($P = 0.497$).

3.4. Liver morphology and key indices of function in plasma

As shown in Fig. 1, dietary 25(OH)D_3 supplementation decreased the nonalcoholic fatty liver disease activity score (NAS) of liver and ALT content in plasma ($P < 0.05$). Compared with ducks fed the basal diet, lower NAS and ALT activity in plasma was observed with 2400 and 3200 IU/kg supplementation, respectively ($P < 0.05$). The AST activity in plasma was not affected by 25(OH)D_3 supplementation ($P = 0.757$).

3.5. Gene expression in the ovarian follicle and liver

As shown in Fig. 2A, the expression of ovarian very low-density lipoprotein receptor (*OVR*) gene in ovarian follicle was decreased with increasing supplementation of 25(OH)D_3 ($P < 0.001$), and responses were linear and quadratic ($P < 0.05$).

Dietary 25(OH)D_3 supplementation affected the hepatic expression of apolipoprotein B 100 (*ApoB100*), lipoprotein lipase (*LPL*), peroxisome proliferator-activated receptor-α (*PPARα*), liver X receptor (*LXR*), sterol regulatory element binding protein-1 (*SREBP1*) and apolipoprotein very low-density lipoprotein-II (*apoVLDL-II*) genes (Fig. 2B–J, $P < 0.05$). The mRNA abundance of *ApoB100* (quadratic, $P < 0.001$) and *LPL* (linear, quadratic; $P < 0.05$) decreased; while those of *PPARα* (linear, quadratic; $P < 0.05$) and *SREBP1* (linear, quadratic; $P < 0.05$) increased with increasing 25(OH)D_3 supplementation levels. The response of *LXR* transcripts was linear and quadratic with 25(OH)D_3 supplementation with lower values in 2400 and 3200 IU/kg treatments ($P < 0.001$). There was a quadratic effect between level of supplemental 25(OH)D_3 and *apoVLDL-II* transcript levels with lowest values were observed with 1600 and 2400 IU/kg supplementation ($P < 0.001$). Dietary supplementation of 25(OH)D_3 did not affect hepatic gene expression of *ACC*, *FAS* and *INSR* ($P > 0.05$). Compared with the control, dietary 25(OH)D_3 supplemental levels at 1600 to 4000 IU/kg increased hepatic transcripts of *PPARα* and *SREBP1*, and decreased those of *ApoB100* and *LPL* ($P < 0.05$).

3.6. Duodenal morphology and gene expression

Dietary supplementation of 25(OH)D_3 decreased duodenal crypt depth ($P = 0.006$) and increased the villus to crypt ratio (Fig. 3, $P = 0.020$), and the responses were linear and quadratic ($P < 0.05$).

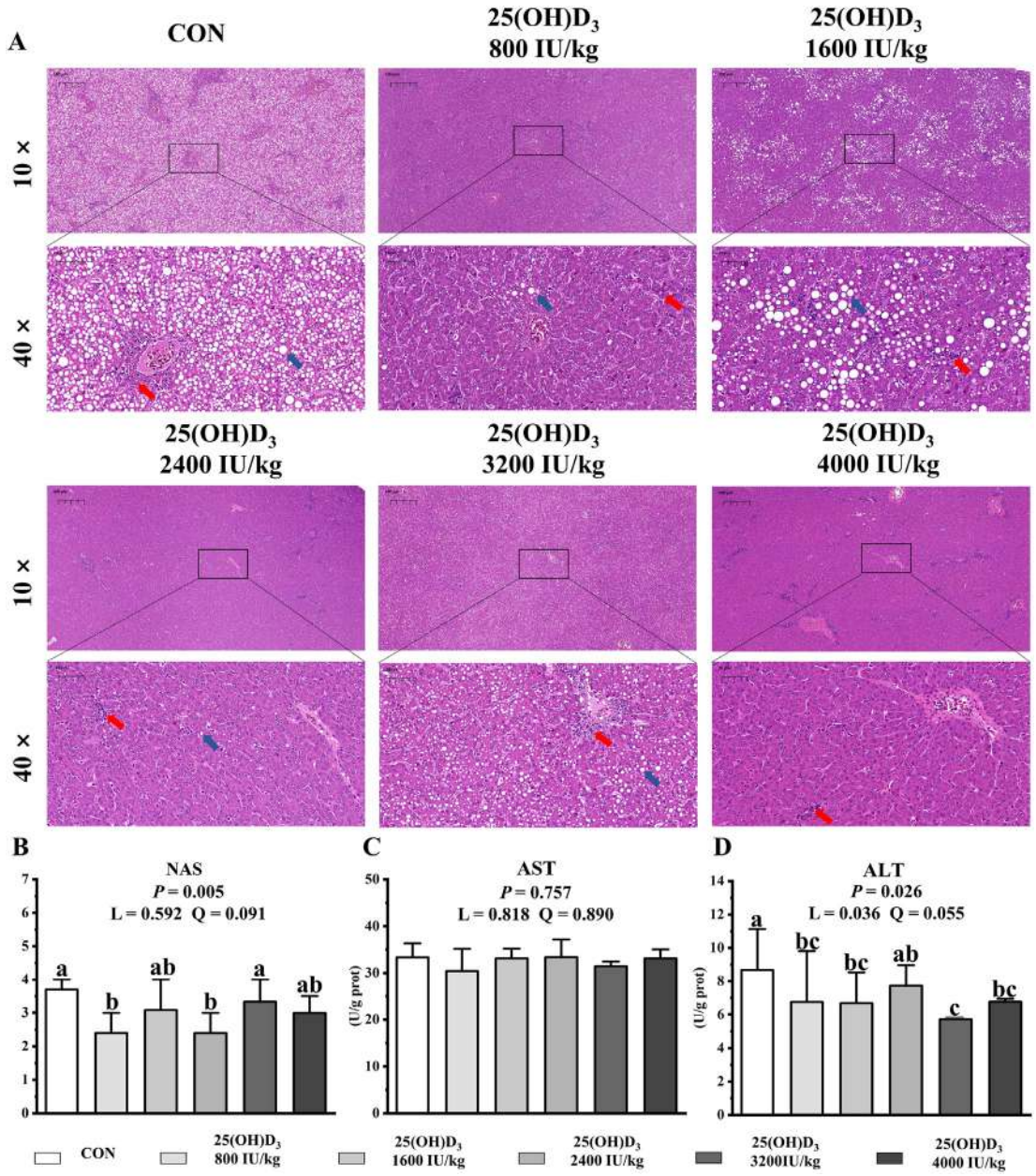

Fig. 1. Dietary 25(OH)D_3 supplementation reduced liver steatosis and improved its function in aged laying ducks. (A) The images of liver sections stained with hematoxylin and eosin. Steatosis is shown by the blue arrow. Inflammation or necrosis are shown by the red arrow. (B) The nonalcoholic fatty liver disease activity score (NAS) of the liver. (C–D) The activities of AST and ALT in duck plasma, respectively. AST = aspartate transaminase; ALT = alanine aminotransferase. [a–c]Bars with different superscript letters differ at $P < 0.05$. 25(OH)D_3 = 25-hydroxyvitamin D_3; L = linear effect; Q = quadratic effect; CON = control.

Lower values of crypt depth and higher values of villus to crypt ratio were observed in ducks fed 2400 and 3200 IU/kg 25(OH)D_3 compared with ducks fed the basal diet ($P < 0.05$). Transcripts of zonula occludens protein 1 (*ZO-1*) and nuclear factor-κ-gene binding (*NF-κB*) in duodenum of laying ducks were higher than in controls with 3200 and 800 IU/kg 25(OH)D_3 supplementation, respectively ($P < 0.05$). There was no impact of dietary 25(OH)D_3 supplementation on duodenal gene expression of tumor necrosis factor-α ($P = 0.359$).

3.7. Change of cecal microflora

Fig. 4A presents the results of OTU partition of cecal microorganisms in laying ducks. There was 951 common OTU. The controls acquired unique 11 OTU, whereas 21, 34, 11, 14 and 35 OTU were uniquely acquired from ducks supplemented with 800, 1600, 2400, 3200 and 4000 IU/kg of 25(OH)D_3, respectively. The number of OTUs decreased with the number of samples they show up (Fig. 4C), which indicated that the sample size in this study was sufficient. As shown in Fig. 4B, at the phylum level, there were mainly Firmicutes and Bacteroidetes in the cecal microorganisms in laying ducks, regardless of 25(OH)D_3 supplementation. The results of the beta diversity analysis are shown in Fig. 4D. The distance among the treatments was close, indicating that the addition of dietary 25(OH)D_3 had no effect on the beta diversity of cecal microflora in laying ducks ($P = 0.582$). Fig. 4E shows the number of OTUs and alpha diversity indexes including Shannon, abundance-based coverage estimator (ACE), Simpson and Chao1 indexes of cecal

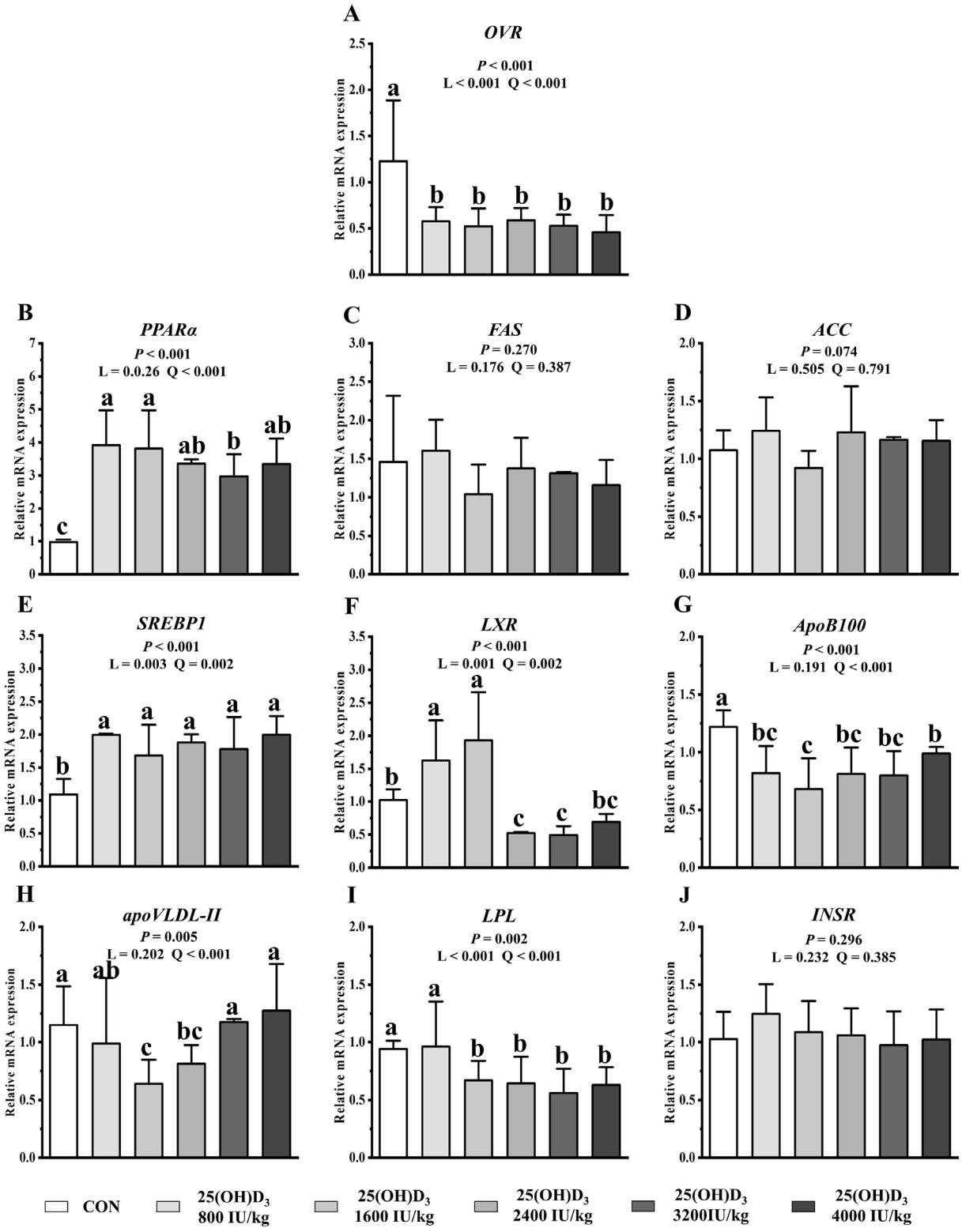

Fig. 2. Dietary 25(OH)D_3 supplementation affected the gene expression related lipid metabolism in ovarian follicle and liver in aged laying ducks. (A) The gene expression of *OVR* in ovarian follicle. (B–J) The genes expression of *PPARα*, *FAS*, *ACC*, *SREBP1*, *LXR*, *ApoB100*, *apoVLDL-II*, *LPL* and *INSR* in the liver, respectively. $^{a–c}$Bars with different superscript letters differ at $P < 0.05$. L = linear effect; Q = quadratic effect; CON = control; *OVR* = ovarian very low-density lipoprotein receptor; *PPARα* = peroxisome proliferator-activated receptor-α; *FAS* = fatty acid synthase; *ACC* = acetyl-CoA carboxylase; *SREBP1* = sterol regulatory element binding protein-1; *LXR* = liver X receptor; *ApoB100* = apolipoprotein B 100; *apoVLDL-II* = apolipoprotein very low-density lipoprotein-II; *LPL* = lipoprotein lipase; *INSR* = insulin receptor.

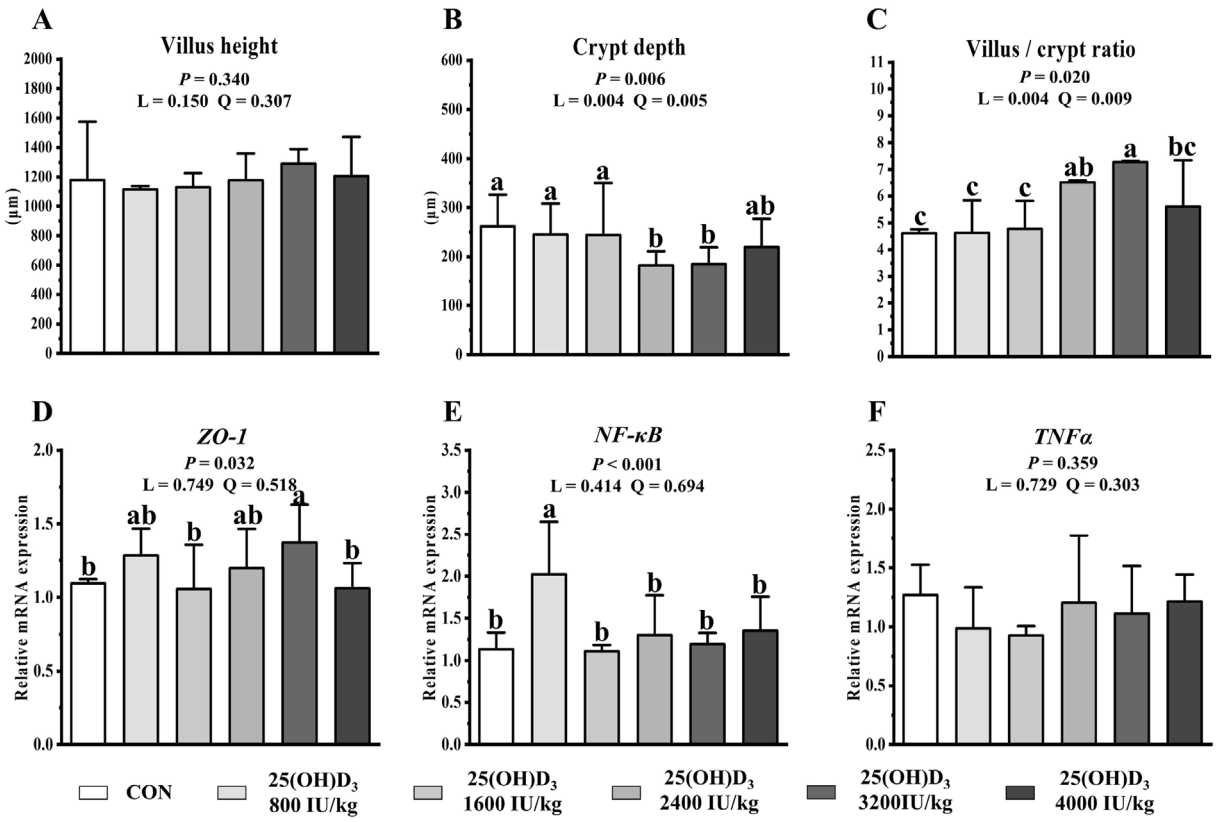

Fig. 3. Dietary 25(OH)D_3 supplementation improved duodenum morphology and mRNA values of tight junction proteins in aged laying ducks. (A–C) The indices of morphology in duodenum. (D–F) The indices of intestinal barrier in duodenum. $^{a-c}$Bars with different superscript letters differ at $P < 0.05$. L = linear effect; Q = quadratic effect; CON = control; *ZO-1* = zonula occludens protein 1; *NF-κB* = nuclear factor-κ-gene binding; *TNF-α* = tumor necrosis factor-α.

microorganisms in laying ducks. The results showed that dietary 25(OH)D_3 supplementation had no effect on the number of OTUs and alpha diversity of the cecal microflora ($P > 0.05$). Fig. 5A–E shows the differences in microbial composition between the controls and 25(OH)D_3 treatments in the phylum, class, order, family and genus levels, respectively. Dietary supplementation of 25(OH)D_3 decreased the cecal abundance of Wittenberg polluted soil-2 bacteria (WPS-2) and Synergistaceae at phylum, class and order levels ($P < 0.05$). In the family level, the abundances of Bacteroidota, WPS-2 and Synergistaceae were decreased ($P < 0.05$), and the abundance of Campylobacteraceae was increased with the supplementation of 25(OH)D_3 ($P = 0.022$). Dietary 25(OH)D_3 supplementation increased the abundance of *Faecalitalea*, *Ruminococcus*, XIII_AD3011 and *Campylobacter* ($P < 0.05$), but decreased those of *Colidextribacter*, Eggerthellaceae, *Bacteroidales*, WPS-2, *Oscillospira*, *Oscillibacter*, *UCG-009*, *Barnesiellace* and *UCG-010* at the genus level ($P < 0.05$). As shown in Fig. 5F, the functional prediction of cecal microorganisms with 25(OH)D_3 supplementation was similar to that of the controls, mainly focused on the function of amino acid transport and metabolism, energy production and conversion, carbohydrate transport and metabolism.

3.8. Estimation of the requirement of 25(OH)D_3 in diets

Dietary requirements for 25(OH)D_3 in aged laying ducks, as estimated from quadratic regression analyses of productive performance traits, are shown in Table 6. The requirements of 25(OH)D_3 for Longyan ducks from 60 to 76 wk of age were estimated to be 3377 IU/kg for optimizing egg production, 3434 IU/kg for egg mass, and 3256 IU/kg for FCR.

4. Discussion

Consistent with previous studies in laying hens (Akbari et al., 2019; Silva, 2017), the present study with laying ducks showed that dietary supplementation of 25(OH)D_3 improved productive performance, including increasing egg production, mass and feed intake, and decreasing FCR. This was possibly related to its positive effects on estrogen and intestinal status; 25(OH)D_3 supplementation increased plasma FSH content and altered intestinal mucosa in a manner consistent with improved function. The differentiation and development of gonads in ducks requires FSH (Ni et al., 2007), and a particular threshold concentration of FSH was necessary for continued follicular development (Liu et al., 2021). The current study has observed that 25(OH)D_3 supplementation increased feed intake of laying ducks, a finding that is consistent with those in pigs and broiler chicks (Driver et al., 2006; Flohr et al., 2014). Bello et al. (2014) found 25(OH)D_3 supplementation might increase nutrient absorption in broilers by enhancing small intestine morphology; comparable changes were noted here in laying ducks.

Supplementation with 25(OH)D_3 decreased yolk weight and ratio, increased albumen ratio decreased VLDL content in egg yolk, along with follicular expression of *OVR* in ovarian follicle. These results indicated that the lipid deposition in yolk was decreased with the supplementation of 25(OH)D_3 in diets. The lipids of yolk are imported into the follicle in the form of lipoproteins, mainly VLDL (Song et al., 2023), consisting one unit of ApoB and twenty-three units of ApoVLDL-II (Nii et al., 2020). In the process of egg yolk formation, only VLDL with particle diameter of 25 to 44 nm could accumulate in the developing follicles (Yang et al., 2013), and then the OVR was generally considered as a key factor regulating

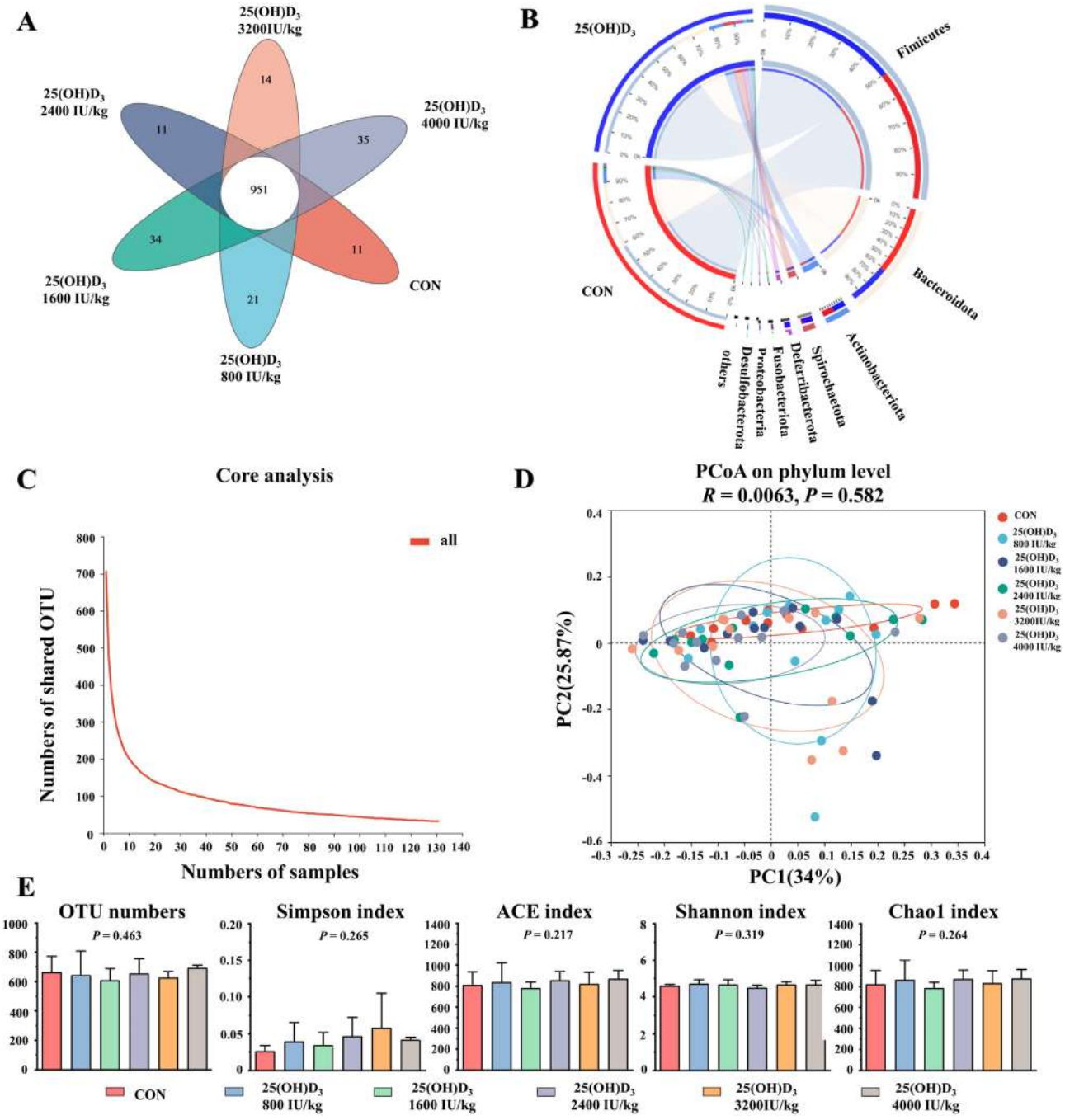

Fig. 4. Classification and diversity analysis of cecal microorganisms. (A) Venn diagram illustrates the numbers of unique and common operational taxonomic units (OUT) in each group. (B) The phylum-level species-sample association is displayed using the Circos plot. (C) The core analysis diagram is utilized to explain how the core OTU changed as sample size increased. (D) Beta diversity analysis was performed by principal co-ordinate analysis (PCoA), in which a sample is represented by each point. To distinguish the impacts among treatments, ellipses are used to identify the points that share the same color. (E) The number of OTUs in each group, and alpha diversity analysis. Alpha diversity indicated by Simpson index, abundance-based coverage estimator (ACE) index, Shannon index and Chao1 index.

the transport of VLDL and other yolk precursors to follicle (Elkin et al., 2012). These lipids are mainly synthesized by the liver, secreted into the blood, and then transported through the circulatory system to the ovary, where they are transferred into the follicles (Wang et al., 2022a). Therefore, supplemental 25(OH)D_3 decreased egg yolk here in part from downregulated follicular gene expression of *OVR*; changes in hepatic synthesis of lipids and lipoproteins would also have contributed. During yolk formation, apoVLDL-II interferes with the lipolytic action of LPL on VLDL, resulting in its triacylglycerol content remaining intact for the oocyte, with lipoprotein particles then being engulfed under the mediation of apoB100 (Walzem et al., 1999). The reduced follicular expression of *ApoB100* and *apoVLDL-II* genes from 25(OH)D_3 supplementation may have reduced the synthesis of egg yolk precursors, thereby reducing the weight of yolk.

The NAS reflects the degree of liver nonalcoholic fatty liver disease (Kleiner et al., 2005). The ALT, mainly present in the cytoplasm of liver cells, is involved in the decomposition and synthesis

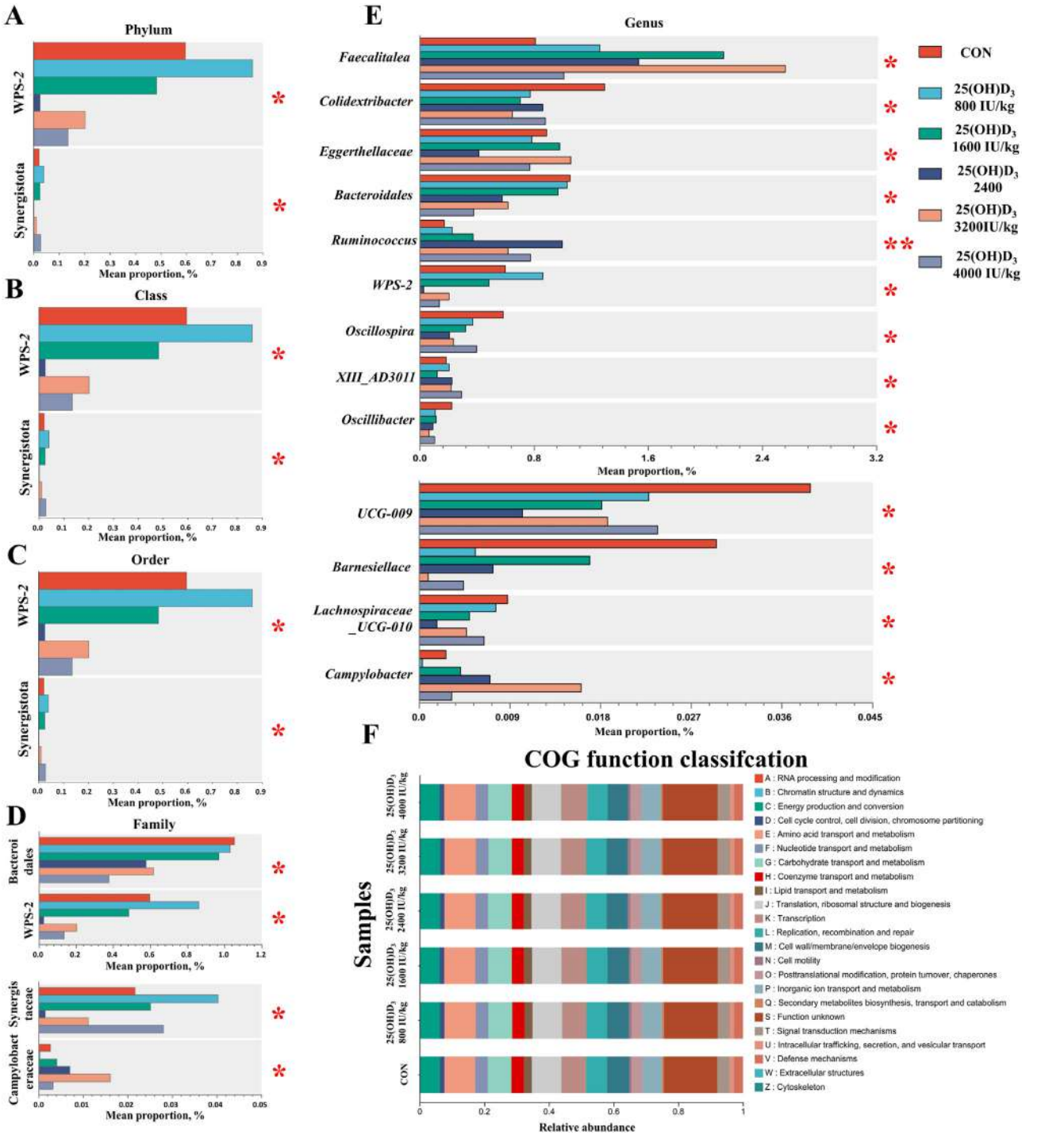

Fig. 5. Analysis of taxonomic composition and prediction of flora function. (A–E) These represent the differences in microbial composition among treatments. (F) COG function classification analysis. Different color blocks represented different functions, and their width indicated the abundance of the flora under this function. *P < 0.05, **P < 0.01. CON = control; COG = cluster of orthologous groups; WPS-2 = Wittenberg polluted soil-2 bacteria.

Table 6
Estimation of the dietary 25(OH)D_3 requirements based on quadratic regressions of egg production, egg mass and FCR on dietary 25(OH)D_3 supplemental levels.

Item	Time, wk	Regression equation[1]	R^2	P-value	Dietary 25(OH)D_3 requirement,[2] IU/kg
Egg production, %	1 to 16	$Y = -1.131X^2 + 7.64X + 72.061$	0.965	0.007	3377.54
Egg mass, g/d	1 to 16	$Y = -0.8334X^2 + 5.725X + 50.113$	0.937	0.016	3434.73
FCR, g:g	1 to 16	$Y = 0.041X^2 - 0.267X + 3.262$	0.926	0.020	3256.10

25(OH)D_3 = 25-hydroxyvitamin D_3; FCR = feed conversion ratio.
[1] Y is the dependent variable and X is the dietary 25(OH)D_3 supplemental level (IU/kg).
[2] The dietary 25(OH)D_3 supplemental level giving 95% of the maximal response.

of glutamic acid and pyruvic acid, and its increase in plasma indicates that liver cells are damaged (Yuan et al., 2007). The improvements in hepatic morphology and function with 25(OH)D_3 supplementation here was reflected in reduced NAS of liver and decreased ALT activity in plasma. Dietary with 25(OH)D_3 decreased the liver index, the contents of TC and LDL in plasma, hepatic contents of TG, TC, LDL and VLDL, and increased LPS content. These findings suggest that lipid metabolism and liver status of laying ducks were improved with 25(OH)D_3 supplementation. This outcome is consistent with Yin et al. (2012) who demonstrated that vitamin D_3 modulated lipid metabolism by attenuating hepatic steatosis in the livers of male adult rats. Hepatic lipid accumulation is closely related to *de novo* lipogenesis, lipid transport and lipid consumption (Cahill, 2006; McGarry and Foster, 1980). The protective effect of vitamin D_3 against hepatic steatosis was mediated by downregulating *SREBP-1c* and its target genes, *ACC* and *FAS* (Yin et al., 2012). Kang et al. (2015) suggested that 1,25$(OH)_2D_3$ downregulated lipogenesis-associated gene expression (such as *SREBP1* and *PPAR-γ*), which triggered a reduction in hepatic fat synthesis, and suppressed its deposition in adipose tissue. Similarly, the hepatic expression of genes related to the lipogenic proteins, such as *ApoB100*, *apoVLDL-II* and *LPL*, was downregulated here in aged laying ducks by 25(OH)D_3 supplementation. At the same time, hepatic expression of *PPARα* and *SREBP1* was increased. PPARα is predominantly expressed in liver (Yang et al., 2017) and plays an important role in maintaining lipid homeostasis by promoting fatty acid β-oxidation through regulating expression of the respective genes (Escher et al., 2001). The second transcription factor, SREBP1, regulates genes required for glucose metabolism, fatty acid and lipid synthesis (Ferré and Foufelle, 2010). Lipoprotein lipase is the enzyme responsible for the hydrolysis of TG-rich lipoproteins and plays a vital role in directing free fatty acids toward adipose and muscle tissues (Garenc et al., 2001). Therefore, the simultaneous upregulation of the gene expression of hepatic *SREBP1* and *PPARα*, and downregulation of hepatic *LPL* in the current study indicated that fat synthesis and β-oxidation in the liver were favored in laying ducks with supplementation of 25(OH)D_3. These changes in liver, to some extent, implied that overall metabolism in laying ducks was promoted, which might also contribute to the increased feed intake and egg production noted here; the accelerated metabolism could provide the energy required for production (Li et al., 2019; DePersio et al., 2015). The improved lipid metabolism in plasma and liver with 2400 IU/kg 25(OH)D_3 exceeded that with 4000 IU/kg, indicating no more benefit from higher levels of 25(OH)D_3 in diets of laying ducks. Wen et al. (2019) found that feeding Hy-LineW36 pullets at a higher level 68,348 IU of vitamin D_3 resulted in slower growth of young hens (to 17 wk) and ultimately decreased performance during laying (18 to 68 wk). Note that hepatic HDL content was significantly decreased here with 2400 IU/kg 25(OH)D_3 possibly due to the decreased content of TC; changes in the 2 variables with supplementation were similar. Taken together, these results suggested that appropriate supplementation of 25(OH)D_3 decreased the content of lipid and cholesterol in plasma and liver, reduced the NAS and supported the function and health of the liver, thus helping to maintain homeostasis of lipid metabolism in laying ducks at the late phase of production.

There are several recent reports of the positive role of vitamin D in maintaining intestinal homeostasis in laying hens (Guo et al., 2022). Most notably, vitamin D stabilized inter-epithelial junctions and promoted a balance between the gut microbiota and intestinal immunity (Fakhoury et al., 2020). In the current study, supplementation of 25(OH)D_3 decreased crypt depth, and increased the villus/crypt ratio and expression of *ZO-1* and *NF-κB* in the duodenum of laying ducks. Similarly, dietary 25(OH)D_3 supplementation increased the villus height and villus/crypt ratio in duodenum in breeder geese (Zhang et al., 2023). Expression of genes encoding E-cadherin and some tight junctional components (ZO-1, occludin, and claudins) in various cultured epithelial cells was enhanced by 1,25$(OH)_2D_3$ (Chen et al., 2015; Zhang et al., 2013). These findings indicate that 25(OH)D_3 improved the morphology and barrier function of intestine, which may be related to the vitamin changing the intestinal microbiota.

The beneficial association of microbiota, or their products (such as short-chain fatty acids), on intestinal homeostasis is established (Clark and Mach, 2016; Guo et al., 2022). There was no difference in the alpha and beta diversity of cecal microbiota among the present treatments, indicating no effect of 25(OH)D_3 supplementation on diversity of cecal microbiota in laying ducks, however, changes in the composition of cecal microbiota were observed. The abundance of WPS-2, Synergistota, *Bacteroidales*, *Colidextribacter*, Eggerthellaceae, *Oscillospira*, *Oscillibacter*, *UCG-009*, Barnesiellaceae and *Lachnospiraceae_UCG-010* were decreased with supplementation of 25(OH)D_3 in the present study. WPS-2 is an as-yet-uncultured bacterial clade, and it might be involved in metabolic processes and energy utilization in the intestine (Ji et al., 2021). Synergistota was found to be positively correlated with NH_3—N concentration which increases the absorption and utilization of nutrients (Wang et al., 2022b). *Bacteroidales* was mainly involved in the decomposition of carbohydrates in the intestine, producing butyric acid and acetic acid (Macfarlane and Macfarlane, 2003). From the functional roles of these changes, dietary 25(OH)D_3 supplementation mainly influenced the microbiota involved in the decomposition of carbohydrates. Similarly, the lack of sufficient vitamin D increased the abundance of bacteria in *Bacteriodetes* phyla (Assa et al., 2014; Ooi et al., 2013). Dietary 25(OH)D_3 supplementation increased the abundance of *Faecalitalea*, *Ruminococcus*, XIII_AD3011 and *Campylobacter* in the cecal microbiota in laying ducks here. The *Faecalitalea* can produce beneficial short-chain fatty acids (Heinken et al., 2014), which help to maintain intestinal mucosal health and enhance intestinal immune function (Feng et al., 2018). The *Ruminococcus* could alleviate obesity (Mueller et al., 2021) and showed anti-inflammatory responses (Wan et al., 2022). As a part of the intestinal flora, *Campylobacter* had no functional influence in mammals and birds (Harvey et al., 1999; Rath et al., 2021). In these respects, the supplementation of 25(OH)D_3 was beneficial in preserving intestinal integrity, health and immune function in laying ducks at the current study, which might be related to the increase of beneficial bacteria (*Faecalitalea* and *Ruminococcus*) in the cecal contents. Unfortunately, the functions of Eggerthellaceae, *Oscillibacte*, *Colidextribacter*, XIII_AD3011, *UCG-009*, Barnesiellaceae and *Lachnospiraceae_UCG-010* bacteria remain unclear. Overall, it can be inferred that 25(OH)D_3 supplementation can partly inhibit the carbohydrate decomposition and reduce lipid synthesis by decreasing the abundance of bacteria such as *Bacteroidales*. Additionally, it might contribute to maintaining intestinal barrier integrity and morphological normality by increasing the abundance of beneficial bacteria (*Faecalitalea* and *Ruminococcus*) in laying ducks.

For most variables examined here a diet containing 3250 to 3450 IU/kg of 25(OH)D_3 is recommended for laying ducks during the late laying period. This slightly exceeds the levels recommended by China National Standard (GB/T 41189-2021) for the peak phase of production in laying ducks (3000 vs. 2500 IU/kg), which possibly resulted from higher requirements for maintaining eggshell and bone quality of older ducks. Higher supplemental level of 25(OH)D_3 (5000 vs. 2500 IU/kg) could improve productive performance and eggshell quality in laying hens at the late phase of production (60 to 72 wk; Jing et al., 2022). As no feeding standard exists for 25(OH)D_3 in laying ducks at the late phase of production, the present data provide this needed information.

5. Conclusions

Dietary 25(OH)D_3 supplementation improved the productive performance, lipid metabolism and duodenal morphology, but reduced egg yolk weight in laying ducks. Its beneficial effect for lipid homeostasis stemmed from decreasing the abundance of bacteria involved in carbohydrate metabolism such as *Bacteroides* and increasing the abundance of beneficial bacteria (*Faecalitalea* and *Ruminococcus*) in the cecal contents. A diet that containing 3250 to 3450 IU/kg of 25(OH)D_3 is recommended for laying ducks during the late phase of production (60 to 75 wk).

Credit Author Statement

Yongyan Jin: Conceptualization, Writing - Original Draft, Writing - Review & Editing, Project administration, Formal analysis. **Chuntian Zheng:** Writing - Review & Editing, Supervision. **Huanting Xia:** Investigation, Resources, Project administration. **Xuebing Huang:** Review & Editing. **Kaichao Li:** Review & Editing. **Shuang Wang:** Investigation. **Weiguang Xia:** Investigation. **Shenglin Wang:** Validation. **Wei Chen:** Validation. **Chang Zhang:** Validation. **Yanan Zhang:** Writing - Review & Editing, Supervision.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgments

The authors sincerely thank Dr. W. Bruce Currie from Cornell University for his help in the presentation of this manuscript. This study was supported by the China Agricultural Research System (CARS-42-K13), Modern Agricultural Industry Technology System Innovation Team of Guangdong Province (2022KJ137), Special fund for scientific innovation strategy-construction of high-level Academy of Agriculture Science (202106TD, R2021PY-QY005).

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