

Dietary 25-hydroxyvitamin D improves intestinal health and microbiota of laying hens under high stocking density

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ABSTRACT The high stocking density is a major stress factor that adversely affects the health and performance of poultry. Therefore, the object of this study was conducted to explore whether dietary 25-hydroxyvitamin D (**25-OH-D₃**) could improve gut health of laying hens reared under high stocking density. A 2 × 2 factorial design was used in this 16-week study, in which 800 45-week-old Lohmann laying hens were allocated into two levels of dietary 25-OH-D₃ levels (0 and 69 μg/kg) and two rates of stocking densities [506 (low density, **LD**) and 338 (high density, **HD**) cm²/hen]. Compared with the layers with LD, the layers with HD had lower crypt depth in duodenum ($P_{(\text{Density})} < 0.05$), lower short chain fatty acid (propionic and butyric acid) contents in cecum ($P_{(\text{Density})} < 0.05$), and lower mRNA expression of intestinal barrier associated protein (claudin-1, mucin-1 and mucin-2). Exposed layer to HD also led to lower intestinal antioxidative capacity [superoxide dismutase (**SOD**), catalase (**CAT**), total antioxidant capacity (**T-AOC**), and higher malondialdehyde (**MDA**) content] in small intestine ($P_{(\text{Density})} < 0.05$),

lower bacterial abundance of *Bacteroidetes* (phylum), *Spirochaetes* (phylum) and *Bacteroides* (genus; $P_{(\text{Density})} < 0.05$), higher bacterial enrichment of *Lactobacillaceae* (genus) and *Firmicutes/Bacteroidetes* ratio ($P_{(\text{Density})} < 0.05$) in cecum. Dietary 25-OH-D₃ increased the villus height in duodenum and jejunum ($P_{(25\text{-OH-D}_3)} < 0.05$), decreased Chao 1 and ACE indexes in cecum ($P_{(25\text{-OH-D}_3)} < 0.05$), and it also up-regulated the mRNA expression of claudin-1, mucin-1 and mucin-2 ($P_{(25\text{-OH-D}_3)} < 0.05$). Layers treated with 25-OH-D₃ led to an enhanced antioxidative enzyme activity of CAT ($P_{(25\text{-OH-D}_3)} < 0.05$). Additionally, the effect of 25-OH-D₃ reversed the effect of HD on T-AOC and MDA content ($P_{(\text{Interaction})} < 0.05$). In HD layers, 25-OH-D₃ administration decreased the enrichment of *Bacteroidetes* (phylum), increased *Firmicutes* (phylum), and *Firmicutes/Bacteroidetes* ratio ($P_{(\text{Interaction})} < 0.05$). These results suggest that supplementing 25-OH-D₃ in diets may elevate gut health through the improvement of intestinal barrier function, antioxidative capacity and cecal microbiota composition in laying hens with high stocking density.

Key words: 25-hydroxycholecalciferol, stocking density, intestinal barrier, antioxidative capacity, microbiota

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INTRODUCTION

At present, the cage housing system (or conventional cage system) were the most commonly used rearing system for layers in China due to its better economic benefits. Stocking density referred to the space provided for

an individual hen in conventional cage system, which has been closely related to the welfare and stress of layers (Mench et al., 2011). Inadequate rearing density or high stocking density (**HD**) has been recognized as a source of chronic stress, which were shown to cause adverse effect on growth performance, egg production, egg weight, feed intake, feather pecking (Jalal et al., 2006; Saki et al., 2012; Kang et al., 2016; Li et al., 2019; Weimer et al., 2019; Wang et al., 2020). In addition, high stocking density is associated with chronic oxidative stress (Wu et al., 2018) and decreased the intestinal morphology and microbiota diversity in broilers and ducks (Sohail et al., 2010; Guardia et al., 2011; Wu et al., 2018).

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Vitamin D is generally involved in the calcium and phosphorus and maintains optimal Ca and P homeostasis for bone health and development (Lamberg-Allardt, 2006). Vitamin D is also found to maintain the intestine health by regulating the intestinal barrier integrity, controlling mucosal inflammation and microbiota homeostasis (Jin et al., 2015; Li et al., 2015; Cantorna et al., 2019; Zhang et al., 2020a,b; Fakhoury et al., 2020). The 25-hydroxyvitamin D (25-OH-D₃) is an active metabolite of vitamin D₃, which were shown to have better bioactivity than vitamin D. In our previous study, we found that dietary administration of 69 µg/kg 25-OH-D₃ improved the egg quality and tibia quality of layers under high stocking density (Wang et al., 2020); however, whether 25-OH-D₃ can alleviate the adverse effects of high stocking density on intestinal health is not clear. Therefore, the aim of the present study was to investigate the effects of 25-OH-D₃ on intestinal health and microbiota of laying hens with high stocking density.

MATERIALS AND METHODS

Birds, Diets, Management, and Sampling

The experimental protocol used in the study was approved by the Animal Care and Use Committee of the Sichuan Agricultural University (Chengdu, Sichuan, China). At 45 weeks of age, 800 Lohman pink-shell laying hens were randomly divided into 4 treatments with 10 replicates per treatment. Two 25-OH-D₃ levels (0 and 69 µg/kg; the vitamin D was supplemented at 5000 IU in basal diet) and two stocking densities [506 (low density, LD) and 338 (high density, HD) cm²/hen] were designed by 2 × 2 factor experiment. The total experimental period was 16 weeks. Laying hens were fed a complete feeding mixture in a mash form (Table 1), and the 69 µg/kg 25-OH-D₃ were premixed with 1 kg corn to make a premix prior to addition. Layers (LD: 4 hens per cage; HD: 6 layers per cage) in each replicate were raised in 8 adjacent cages (45 cm width × 50 cm length × 45 cm height). Room environment was controlled at 22°C by a daily lighting schedule of 16 h light and 8 h dark. Birds were allowed *ad libitum* access to water and feed.

At the end of 16 wk, 40 layers (10 replicates/treatment) were sacrificed by CO₂ suffocation, the intestine tissues (duodenum, jejunal) and cecum chyme were taken and then stored at -80°C till gene expression analysis.

Morphology of Intestinal Mucosa

Duodenum and jejunal mucosa (1 layer/replicate, 10 replicates/treatment) morphology were analyzed as described previously (Gong et al., 2021). Briefly, the intestinal segments were fixed in 4% paraformaldehyde, then embedded in paraffin, and stained with hematoxylin-eosin. Villus height and crypt depth were measured in 40 × magnification with a digital camera microscope

Table 1. Composition and nutrient level of basal diet (as-fed basis).

Item, %	Amount
Corn	59.06
Wheat bran	3.87
Soybean oil	1.50
Soybean meal (CP, 43%)	15.24
Corn gluten (CP, 60%)	5.00
Corn DDGS	5.00
Calcium carbonate (granular)	6.10
Calcium carbonate (powder)	2.50
Calcium hydrophosphate (powder)	0.94
NaCl	0.25
NaHCO ₃	0.10
L-Lysine HCl	0.16
DL-Methionine	0.01
Choline chloride, 60%	0.10
Vitamin premix ¹	0.02
Mineral premix ²	0.15
Total	100.00
Calculated nutrient content, %	
ME ³ , kcal/kg	2690
Analyzed nutrient levels, %	
Crude protein	15.82
Calcium	3.65
Total phosphate	0.64
Lysine	0.65
Methionine	0.33

¹Provided per kilogram of diets: VA 9950 IU, VB₁ 37.7 mg, VB₂ 12 mg, D-pantothenate 18.2 mg, VB₆ 7.55 mg, VB₁₂ 0.5 mg, VD₃ 5000 IU, VE 70 IU, VK₃ 4.47 mg, Biotin 4 mg, VC 195 mg, niacin acid 70.35 mg.

²Provided per kilogram of diets: Cu (as copper sulfate) 9.6 mg, Fe (as ferrous sulfate) 64 mg, Mn (as manganese sulfate) 121.5 mg, Zn (as zinc sulfate) 57 mg, I (as potassium iodide) 0.60 mg, Se (as sodium selenite) 0.36 mg.

³Calculated according to NRC (1994).

(BA400Digital, McAudi Industrial, Group Co., Ltd). A total of 10 intact villi and crypts were randomly selected in each sample. Then, the data included villus height, crypt depth and their ratio (V/C) was calculated.

Antioxidant Enzyme Activity of Jejunum

Superoxide dismutase (SOD) activity, total antioxidant capacity (T-AOC), catalase (CAT) and malondialdehyde (MDA) level in jejunum (1 layer/replicate, 10 replicates/treatment) were analyzed by using commercial kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions.

Intestinal Barrier Related Genes

Total RNA of jejunal mucosa (1 layer/replicate, 10 replicates/treatment) was extracted with TRIzol reagent (TaKaRa Biotechnology (Dalian Co., Ltd, Dalian, China) on basis of the manufacturer's instructions. The cDNA of samples was synthesized by using PrimeScript RT reagent kit and gDNA Eraser (TaKaRa Biotechnology (Dalian) Co., Ltd, Dalian, China). The primers of genes, listed in Table 2, were purchased from

Table 2. Primer sequences used to measure gene expression.

Genes	Orientation	Primer Sequences (5'-3')	Product size (bp)	Accession number
<i>β-actin</i>	Forward	GCTACAGCTTCACCACCACA	90	NM_205518.1
	Reverse	TCTCCTGCTCGAAATCCAGT		
<i>Occludin</i>	Forward	GCTGAGATGGACAGCATCAA	97	NM_205128.1
	Reverse	CCTCTGCCACATCCTGGTAT		
<i>ZO-1</i>	Forward	GAGCGCAAGTTTGAAAGTCC	135	XM_015278981.2
	Reverse			

AGGAGGCTGTGATGAGCTGTZO-2ForwardGAAAGCTCCAGCTGGTTGTC
 124NM_204918.1ReverseGGGAGAACGATCTGTTTGA *Mucin-1*ForwardGCCATCGTAGTTGTGTGTGG
 126XM_015279045.2ReverseTGCTTCAGGGTCTCTCCTGT *Mucin-2*ForwardTGCCAGCCTTTTATGCTCT
 80NM_001318434.1ReverseAGTGGCCATGGTTTCTTGTC *Claudin-1*ForwardGTCTTTGGTGGCGTGATCTT
 117NM_001013611.2ReverseTCTGGTGTAAACGGGTGTGA

Abbreviations: *ZO-1*, zonula occluden-1; *ZO-2*, zonula occluden-2.

TaKaRa Biotechnology (Dalian) Co., Ltd (Dalian, China). The real-time quantitative PCR with SYBR Premix Ex Taq reagents (TaKaRa Biotechnology, Ltd, Dalian, China) and a CFX-96 Real-Time PCR detection System (Bio-Rad Laboratories, Richmond, CA) were performed. The house keeping gene (β -actin) was chosen in order to correct for variance in the amount of RNA input in the reaction. The relative mRNA expression of occluding, *claudin-1*, **ZO-1** (*zonula occluden-1*), **ZO-2** (*zonula occluden-1*), *mucin-1* and *mucin-2* compared to the house keeping gene was obtained with previous methods (Wang et al., 2021). The specific primer sequences are shown in Table 2.

pH Value and Short Chain Fatty Acid Concentration of Cecal Digesta

1 g of cecal chyme (1 layer/replicate, 10 replicates/treatment) were taken to measure pH value with pH analyzer (pH-start, Germany). The short-chain fatty acids (SCFA; including acetic, propionic and butyric acid) were then analyzed by gas chromatographic system (Varian CP-3800, USA) as described by Yuan et al., (2016).

Microbiota Analysis in Cecal Digesta

Bacterial DNA was extracted from cecal digesta (6 replicates/treatment) using the QIAamp DNA Stool Mini Kit (QIAGEN, CA, Hamburg, Germany) according to the manufacturer's instructions. Total DNA was eluted in 50 μ L of elution buffer, confirmed by 1.2% agarose gel electrophoresis, and stored at -80°C until analysis in the PCR by LC-Bio Technology (Hangzhou, China). Before sequencing, the above 16S rDNA V3-V4 region of each sample was amplified with a set of primers targeting 16S rRNA gene region. Sequencing libraries were generated with NEB Next Ultra DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations, and index codes were added. The library quality was assessed, qualified and then sequenced using HiSeq2500 PE250. Sequencing and bioinformatics analysis were performed by Novogene Bioinformatics Technology Co. (Tianjin, China).

Statistical Analysis

Data were analyzed as a 2 \times 2 factorial arrangement of treatments by 2-way ANOVA analysis of variance

Table 3. Effect of stocking density and dietary 25-OH-D₃ supplementation on intestinal morphology of laying hens.

Item	25-OH-D ₃	Duodenum			Jejunum		
		Villus height, μ m	Crypt depth, μ m	V/C	Villus height, μ m	Crypt depth, μ m	V/C
Density							
Low	-	1205.31	224.45 ^{ab}	5.50	1033.11 ^b	191.94 ^b	5.49
Low	+	1345.47	233.23 ^a	6.32	1120.79 ^a	219.43 ^a	5.22
High	-	1202.31	196.08 ^b	6.17	1015.44 ^b	180.93 ^b	5.91
High	+	1290.57	202.23 ^b	6.59	1141.14 ^a	201.34 ^a	5.65
SEM		63.83	14.30	0.51	32.24	8.45	0.41
P-Value		0.24	<0.01	0.47	0.01	0.03	0.69
Main effect (P-Value)							
Density		0.52	<0.01	0.19	0.71	0.14	0.16
25-OH-D ₃		0.04	0.46	0.09	<0.01	0.34	0.37
Density*25-OH-D ₃		0.57	0.90	0.58	0.72	0.71	0.98

^{a,b}Means with different superscripts within a column differ significantly ($P \leq 0.05$).

¹Each mean represents 10 replicates per treatment, with 1 layer per replicate.

Abbreviations: 25-OH-D₃, 25-hydroxyvitamin D; V/C, ratio of villus height to crypt depth.

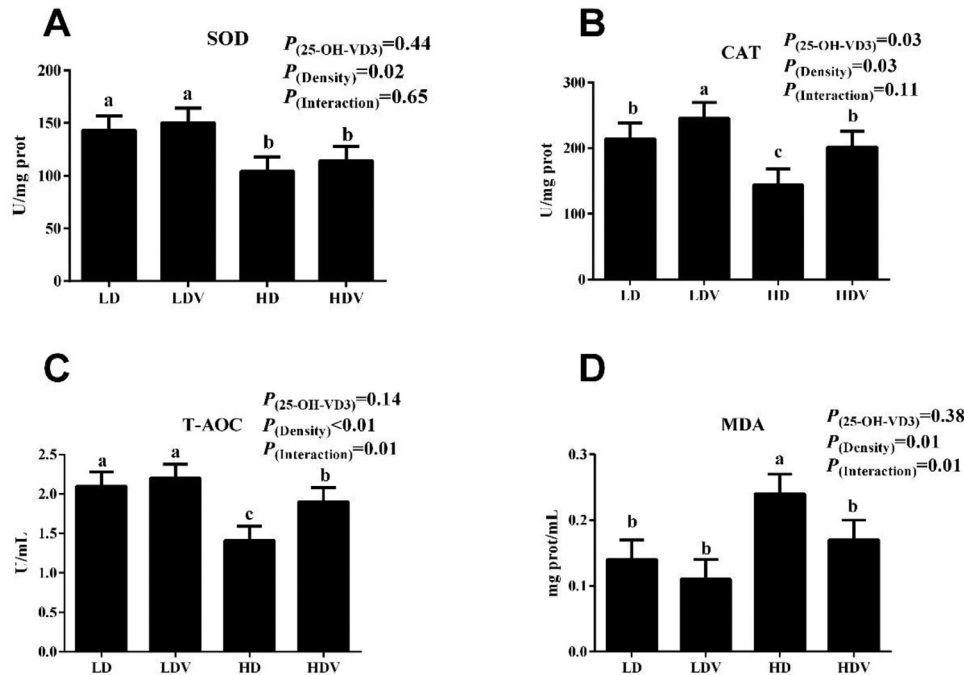


Figure 1. Effect of stocking density and dietary 25-OH-D₃ supplementation on antioxidant enzyme activity in jejunum of layers. Data are means \pm SEM represented by vertical bars or plot individual values. The activities of antioxidant enzymes, including (A) SOD, (B) CAT, (C) T-AOC, (D) SOD activity, and lipid peroxidation products (D) MDA levels. The difference N = 10. Statistical significance was evaluated by Tukey's range test, $P \leq 0.05$. Abbreviations: SOD, total superoxide dismutase; CAT, catalase; T-AOC, total antioxidant capacity; MDA, malondialdehyde.

using GLM procedure of SAS 9.2 software (SAS Institute Inc., Cary, NC). The model included the main effects of stocking density and 25-OH-D₃, as well as their interaction. Tukey's range test was used to determine significant differences among means. The significance was determined by $P \leq 0.05$.

RESULTS

Intestinal Morphology

As shown in Table 3, there was no significant different in duodenum and jejunal mucosa morphology (villus height, crypt depth and V/C) between LD and HD layers ($P > 0.05$), except the HD decreased the crypt depth in duodenum mucosa ($P_{(\text{Density})} < 0.05$). And dietary 25-OH-D₃ supplementation increased the villus height of duodenum and jejunum ($P_{(25\text{-OH-D}_3)} < 0.05$).

Antioxidative Capacity of Jejunum

Lower antioxidant enzymes activity, including SOD, CAT, T-AOC, and higher MDA content in small intestine were found in layers exposed to HD treatment (Figure 1; $P_{(\text{Density})} < 0.05$). Layers treated with 25-OH-D₃ led to an enhanced antioxidative enzyme activity of CAT ($P_{(25\text{-OH-D}_3)} < 0.05$), and also the effect of 25-OH-D₃ reversed the effect of HD on T-AOC and MDA content ($P_{(\text{Interaction})} < 0.05$).

The mRNA Expression Levels of Gut-Barrier Related Gene in Jejunal Mucosa

Compared with the layers with LD, the layers with HD had lower mRNA expression of intestinal barrier associated protein (claudin-1, mucin-1 and mucin-2) (Figure 2; $P_{(\text{Density})} < 0.05$), while the addition of 25-OH-D₃ up-regulated the mRNA expression of claudin-1, mucin-1 and mucin-2 ($P_{(25\text{-OH-D}_3)} < 0.05$).

Short Chain Fatty Acid Concentration and pH Value of Cecum Digesta

The content of propionic acid, butyric acid and total short-chain fatty acid content in cecum were lower in layers reared under HD situation (Table 4; $P_{(\text{Density})} < 0.05$). No effect of 25-OH-D₃ were observed to affect the short-chain fatty acid profiles and pH value of layers irrespective of space density ($P > 0.05$).

Alpha Diversity of Microbiota in the Cecal Digesta

As shown in Table 5, the community diversity (Shannon) and richness (Chao1 and ACE) indices of the microbiota in the cecal digesta of layers with HD were lower than those in the LD group ($P_{(\text{Density})} < 0.05$). Supplementing 25-OH-D₃ in the diets decreased the Chao 1 and ACE index of microbiota in the cecal digesta of layers ($P_{(25\text{-OH-D}_3)} < 0.05$).

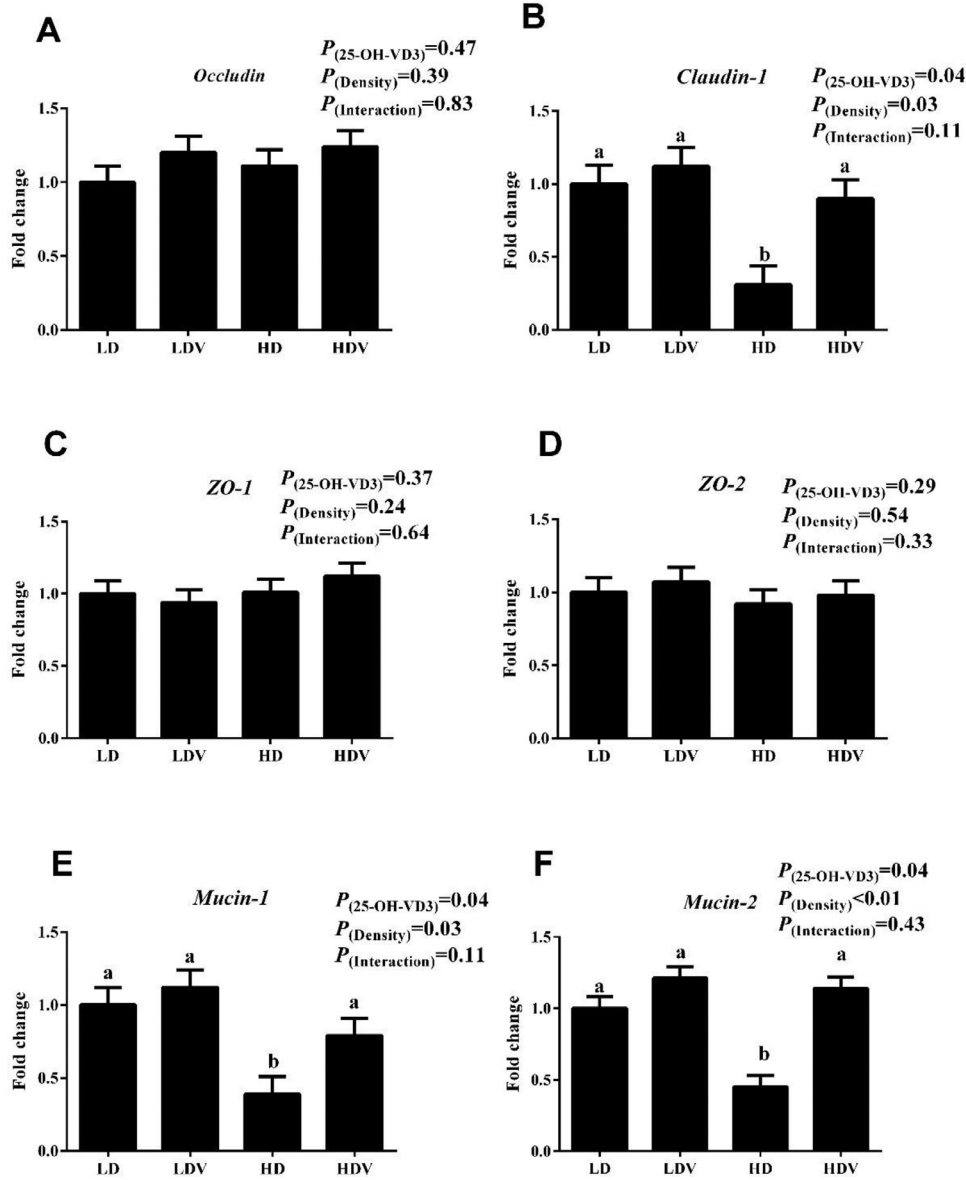


Figure 2. Effect of stocking density and dietary 25-OH-D₃ supplementation on jejunal barrier related gene expression. mRNA expression about tight junction protein (A) *Occludin*, (B) *Claudin-1*, (C) *ZO-1*, (D) *ZO-2*, and mucous barrier related protein (E) *Mucin-1* and (F) *Mucin-2*. The difference N = 10. Statistical significance was evaluated by Tukey's range test, $P \leq 0.05$. Abbreviations: *ZO-1*, zonula occluden-1; *ZO-2*, zonula occluden-2.

Table 4. Effect of stocking density and dietary 25-OH-D₃ supplementation on short chain fatty acid concentration and pH value of cecum ($\mu\text{mol/g}$).

Item		Acetic acid	Propionic acid	Butyric acid	Total	pH
Density	25-OH-D ₃					
Low	-	55.65	22.26	7.48	85.39	6.64
Low	+	53.69	23.44	8.85	85.98	6.90
High	-	43.11	17.91	5.78	67.80	6.86
High	+	50.31	20.17	7.73	78.21	6.80
SEM		6.44	3.36	1.28	4.78	0.14
P-Value		0.48	0.26	0.44	0.87	0.34
Main effect (P-Value)						
Density		0.25	0.03	0.02	0.01	0.53
25-OH-D ₃		0.70	0.48	0.21	0.59	0.30
Density*25-OH-D ₃		0.50	0.82	0.82	0.67	0.12

^{a,b}Means with different superscripts within a column differ significantly ($P \leq 0.05$).

¹Each mean represents 10 replicates per treatment, with 1 layer per replicate.

Abbreviations: 25-OH-D₃, 25-hydroxyvitamin D; V/C, ratio of villus height to crypt depth.

Table 5. Effect of stocking density and dietary 25-OH-D₃ supplementation on alpha diversity in cecum.

Item		Shannon	Simpson	Chao1	ACE
Density	25-OH-D ₃				
Low	-	7.12	0.98	740.34 ^a	739.81 ^a
Low	+	6.92	0.98	664.29 ^b	656.89 ^b
High	-	6.93	0.98	715.41 ^{ab}	702.18 ^{ab}
High	+	7.04	0.98	707.90 ^{ab}	697.34 ^{ab}
SEM		0.12	<0.01	27.80	22.00
P-value		0.42	0.61	<0.01	0.04
Main effect (<i>P</i> -Value)					
Density		0.72	0.27	0.64	0.93
25-OH-D ₃		0.61	0.53	0.05	0.04
Density*25-OH-D ₃		0.09	0.53	0.10	0.04

^{a,b}Means with different superscripts within a column differ significantly ($P \leq 0.05$).

¹Each mean represents 10 replicates per treatment, with 1 layer per replicate.

Abbreviations: 25-OH-D₃, 25-hydroxyvitamin D; V/C, ratio of villus height to crypt depth.

Beta Diversity and Composition of Microbiota in the Cecal Digesta

The shared and specific OTUs among 4 groups are shown in Figure 3A and the principal coordinate analysis plot were shown in Figure 3B. The microbiota of cecal sample was clearly differentiated between LD and LDV, as well as between HD and HDV groups, whereas the separation between HD and LDV could be hardly detected. As shown in Table 6, the relative abundance of the cecum at phylum level indicated that *Bacteroidetes* (LD 48.34%, LDV 60.28%, HD 50.63%, and HDV 40.50%) and *Firmicute* (LD 41.19%, LDV 32.26%, HD 41.06%, and HDV 51.21%) was the dominant phylum in all dietary treatments. The HD had lower *Bacteroidetes*, *Spirochaetes*, higher *Firmicute* abundance, and higher *Firmicute/Bacteroidetes* ratio ($P_{\text{Density}} < 0.05$). Administration of 25-(OH)-D₃ were shown to increase the *Bacteroidetes* and decreased *Firmicutes* enrichment as well their ratio in the cecum of LD layers, while it decreased the abundance of *Bacteroidetes* and increased *Firmicute* enrichment and their ratio in the cecum of HD layers ($P_{\text{Interaction}} < 0.05$). As shown in Figure 3D, E and F, HD increased *Copro-bacter* (genus, $P < 0.05$) and *Subdoligranulum* (genus, $P < 0.05$), decreased *unidentified_Lachnospiraceae* (genus, $P < 0.05$), *Clostridium_sp_Marseille-P3244* (Species) and *Parasullterlla_secunda* (species, $P < 0.05$). The HDV had higher *Alistipes* (genus, $P < 0.05$), *Bacteroidales* (genus, $P < 0.05$) and *Butyricoccus* (genus, $P < 0.05$) enrichment than that in HD layers ($P < 0.05$). These data showed that while density and 25-(OH)-D₃ caused microbial variations but did not change the dominant species at phylum level in the cecum, and also indicate that differences in microflora result from 25-(OH)-D₃ were different between HD and LD situation.

DISCUSSION

Deficiency in space allowance (HD) were reported to increase oxidative stress and had adverse effect on

intestinal health of broilers (Li et al., 2019; Magnuson et al., 2020). Reactive oxidative species (ROS) are byproducts of energy metabolism in tissues of animals and can be readily elevated by environmental stresses such as crowding (Surai et al., 2019). Oxidative stress resulted from stocking density or other stressors were proved to impair the health and production performance of layers (Kang et al., 2016; Abbas et al., 2020; Wang et al., 2020). Intestinal morphology changes with nutritional variations, stress, aging, and (or) disease and it determines the nutrient absorption capacity and are also closely related to the immune response (Celi et al., 2019). An increase in villus height indicated a larger absorption area, while deeper crypt health indicates that the villi in the small intestinal mucosa are atrophied and their absorptive capacity is decreased (Zhang et al., 2005). In the same study, we found that HD decreased the production performance and increased the corticosterone in layers (Wang et al., 2020), which indicated that the HD induced a stress in layers. At the same time, we didn't observe any difference in villus height caused by HD, however, as expected, HD decreased the crypt depth and antioxidative capacity of small intestine, as demonstrated by lower activities of antioxidant enzymes, SOD, CAT and T-AOC, and higher corresponding levels of MDA. Kritdayopas et al. (2019) also reported that exposed to HD reduced the duodenum, jejunal and ileal villus height in broilers. In previous study, high stocking density led to a reduction in activities of T-AOC, SOD and GSH-Px and higher MDA in serum of broilers and ducks (Wu et al., 2018; Li et al., 2019). We also found that 25-(OH)-D₃ supplementation improved CAT activity of small intestine of layers, and the protective effect of 25-(OH)-D₃ on T-AOC and MDA were more pronounced in layers suffered from HD. It has been demonstrated that both 25-(OH)-D₃ and its active hormonal form (1,25(OH)₂D₃) are essential for physiological functions, including damping down inflammation and oxidative stress (Nakai et al., 2014; Wimalawansa, 2019). Although, literatures about supplementation 25-(OH)-D₃ in layers exposed to HD is limited, previous studies also reported that 25-(OH)-D₃ and vitamin D₃ can alleviate the immune response and improve performance of layers against lipopolysaccharide or coccidia-induced immunological stress (Morris et al., 2014, 2015; Geng et al., 2018). This may indicate that 25-(OH)-D₃ exert protective effect against HD as indicated by intestinal morphology and antioxidative capacity.

The intestinal barrier function is one of the important components that maintain gut health and function, and it is generally determined by tight junction integrity of epithelial cells and mucus gel layer (Moret  and P rez-Bosque, 2009). The tight junctions including occluding, claudins and junction adhesion molecule (JAM) are the main component proteins of tight junctions and ZO-1 acts as a plaque protein connecting between those tight junction proteins and cytoskeletons (Ulluwishewa et al., 2011). Mucins are the main constituent of mucus gel layer on intestinal mucosa. The decreased expression of

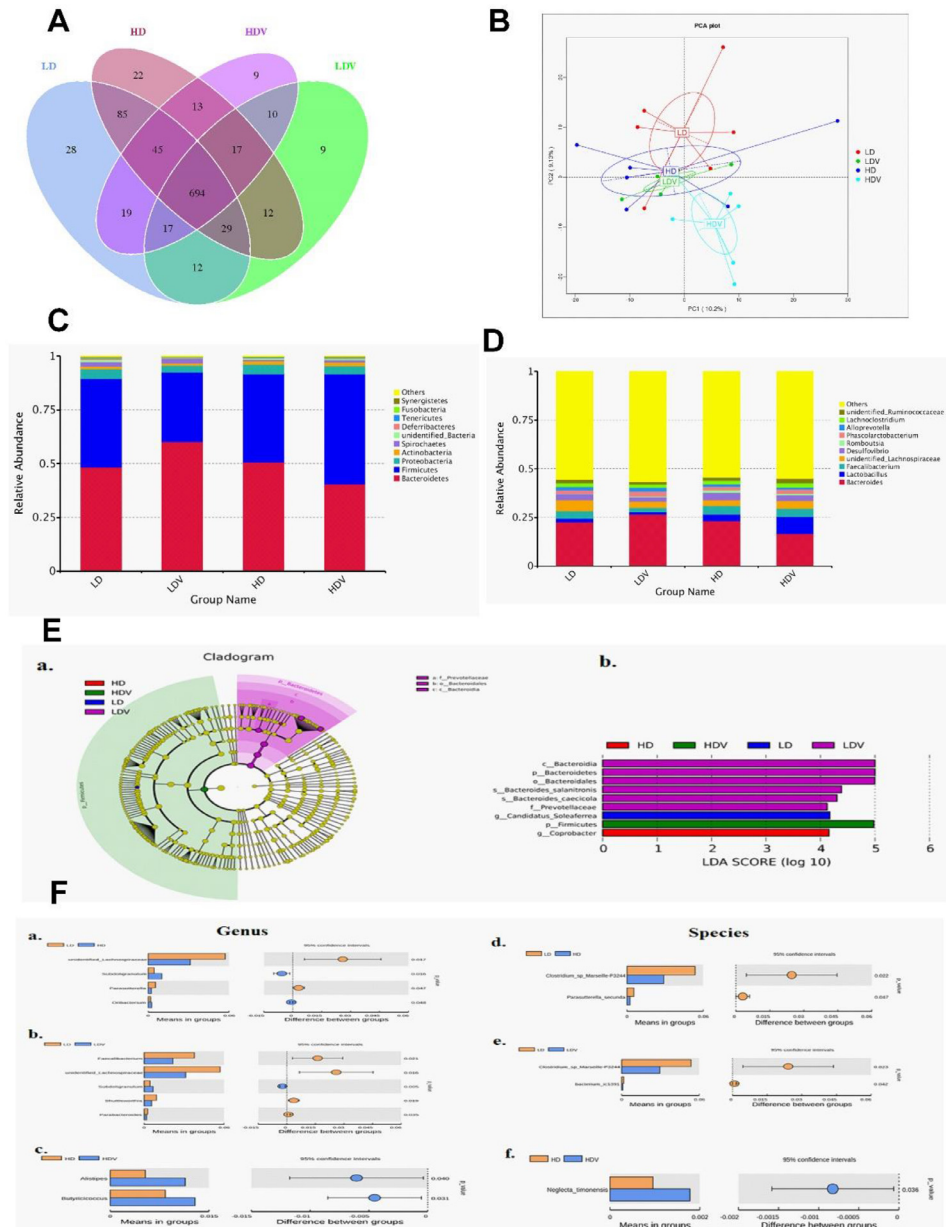


Figure 3. Effect of stocking density and dietary 25-OH-D₃ supplementation on cecal microbiota enrichment of layers. (A) The Venn diagram. (B) The principal coordinate analysis (PCA) of the cecal microbiota based on unweighted UniFrac metric. (C, D) The relative abundance of the top 10 phylum (C) and genus (D). (E) Linear discrimination analysis coupled with effect size (LEfSe) identified most differentially abundant taxa in the cecum with LDA significant threshold > 4 were shown. (F) Analysis of different species between groups at genus level (a) and species level (b). The difference N = 6. Statistical significance was evaluated by Students T-test, $P \leq 0.05$.

tight junction and mucus layer related genes and proteins has been considered a molecular evidence for impaired intestinal health (Moretó and Pérez-Bosque, 2009; Gilani et al., 2018). In this study, we found that HD led to down-regulation of intestinal barrier associated protein (claudin-1, mucin-1 and mucin 2). This result agrees with previous experiments that reported high stocking density decreased the expression of ZO-1 and JAM-2 in jejunal mucosa of broiler chickens (Goo et al., 2019). We also observed that 25-(OH)-D₃ supplementation improved the intestinal barrier function (higher gene expression of claudin-1, mucin-1 and mucin-2) of small intestine of layers regardless of the stocking density. Vitamin D plays a key role in immune system and also been involved in the intestinal mucosa

barrier homeostasis (Liu et al., 2013; Assa et al., 2014; Rodriguez-Lecompte et al., 2016). It has been evident that vitamin D ensures an appropriate level of antimicrobial peptides in the mucus and maintains epithelial integrity by reinforcing intercellular junctions (Kong et al., 2008; Fakhoury et al., 2020). Also, vitamin D and its receptor exhibited protective effect on intestinal structure and barrier function in colitis model by modulation the Th1, Th2 and Th17 response (Golan et al., 2015; Liu et al., 2013; Shi et al., 2020).

The gut microbiota has been implicated in a variety of stress-related conditions including stress, disease, and changed nutrient regimen on animal studies (Foster et al., 2017). The rearing system, including cage system and floor space were shown to alter the

Table 6. The relative abundance of the top 5 dominant microbiota ratio at phylum level in cecum.

Item		Bacteroidetes	Firmicutes	Proteobacteria	Actinomycete	Spirochaetes	Firmicutes/Bacteroidetes
Density	25-OH-D ₃						
Low	-	48.34 ^{bc}	41.19 ^a	4.53	1.20	2.11 ^a	0.85 ^b
Low	+	60.28 ^a	32.26 ^b	3.06	1.18	2.18 ^a	0.54 ^c
High	-	50.63 ^b	41.06 ^a	4.45	1.68	0.58 ^b	0.81 ^b
High	+	40.50 ^c	51.21 ^a	3.78	1.62	1.18 ^{ab}	1.26 ^a
SEM		4.60	3.93	0.9	0.43	0.61	0.11
P-value		<0.01	<0.01	0.32	0.47	0.02	<0.01
Main effect (<i>P</i> -Value)							
Density		0.04	0.04	0.14	0.62	<0.01	0.01
25-OH-D ₃		0.78	0.86	0.89	0.11	0.45	0.04
Density*25-OH-D ₃		<0.01	0.04	0.96	0.53	0.54	<0.01

^{a,b}Means with different superscripts within a column differ significantly ($P \leq 0.05$).

¹Each mean represents 10 replicates per treatment, with 1 layer per replicate.

Abbreviations: 25-OH-D₃, 25-hydroxyvitamin D; V/C, ratio of villus height to crypt depth.

microbiota composition in poultry (Wang et al., 2018; Zhu et al., 2019). In current study, HD also result in a reduction of the SCFA content in cecum and also decreased the cecal microbiota diversity, as indicated by Shannon, Chao1 and ACE indices. Interestingly, dietary supplementation with 25-OH-D₃ also reduced the ACE index under low stocking density. It has been suggested that vitamin D deficiencies result in less diverse, dysbiotic microbial communities and increased susceptibility to infection or injury of the gastrointestinal tract (Cantorna et al., 2019). Vitamin D-mediated regulation of the intestinal epithelium and mucosal immune system shape the microbial communities in the gut to maintain homeostasis. The reason why the result of 25-OH-D₃ on diversity is not consistent in different stocking density is not clear. Besides microbiota diversity, the phylogenetic composition of gut microbiota also shifts substantially between layers with different stocking density. SCFA, acetate, propionate and butyrate are the end products of microbiota (such as *Firmicutes* and *Bacteroidetes*) fermentation (Turnbaugh et al., 2006). We found that HD decreased the total SCFA (propionate and butyrate). Since SCFA accounts for dietary energy source for animals, this may suggest that HD had lower available energy for maintenance and performance. We also found that HD layers had lower proportion of *Bacteroidetes*, *Spirochaetes*, and higher enrichment of *Firmicutes* abundance and *Firmicutes/Bacteroidetes* ratio in cecum compared to LD layers at present study. However, Zhu et al. (2019) shown that heat stress decreased fecal *Firmicutes* and increased abundance of *Bacteroidetes* in layers. The discrepancy may result from the stress origin and animal species and physiological stage in different study. Also, the Lactobacilli was not affected by stocking density in current study, which is in accordance with the result of Harrow et al (2007), who also found that stocking density didn't change the numbers of *Lactobacillus salivarius* in ileal of broilers. Moreover, it also shown that 25-(OH)-D₃ increased the *Bacteroidetes* and decreased *Firmicutes* enrichment as well their ratio in the cecum of LD layers in current study. This observation suggests that *Firmicutes* are inhibited to a

larger extent by 25-OH-D₃, thus tilting the balance of gastrointestinal functionality and can be indicative of eubiosis conditions in the gastrointestinal tract. But this effect of 25-OH-D₃ on *Bacteroidetes* and *Firmicutes* enrichment in cecum was opposite in HD group, and the reason relied in this is not clear, which need to be further studied. At the same time, an in enrichment of *Coprobacter* and *Subdoligranulum*, and decreased *unidentified_Lachnospiraceae* (genus), *Clostridium_sp_Marseille-P3244* (Species) and *Parasulterilla_secunda* were found in cecum of HD layers in current study. The genus *Coprobacter*, first described in 2013, is described as a propionic and acetic acids producer (Shkoporov et al., 2013). Wang et al. (2018) described an increased cecal *Coprobacter* proportion in ducks with litter floor rearing system. Also, *Clostridium_sp_Marseille-P3244* belongs to *Firmicutes*, and is used as a probiotic candidate, which was decreased by HD. These observations suggest that the layers reared high stocking density enables its microbial assemblages within the cecum to response to the stress. Interestingly, we observed opposite results when using 25-OH-D₃ in alternation of *Bacteroidetes* and *Firmicutes* in cecum of layers with different stocking density (a rise of *Bacteroidetes* and lowing of *Firmicutes* in LDV), which may indicate the complex interaction among microbiome, nutrient diet regimen and environmental stressors. Moreover, we also found that supplementation 25-OH-D₃ in HD led to an increase in anti-inflammatory bacterial taxa, including *Bacteroidales*, *Butyricoccus* and *Alistipes* in current study. Wu et al. (2018) reported that high stocking density depleted these taxa in ducks. Also, studies suggested that patients with IBD and *Clostridium difficile* infection have a lower abundance of *Alistipe* than their healthy counterparts (Mancabelli et al., 2017). Few literatures can be found on the influence of vitamin D in microbiota of layers under different stocking density, but vitamin D was proved to alternate microbiome and have anti-inflammatory in mammals (Guillot et al., 2010; Cantorna et al., 2019; Fakhoury et al., 2020; Zhang et al., 2020b).

CONCLUSION

These results suggest that supplementing 25-OH-D₃ in diets may elevate gut health through the improvement of intestinal morphology, barrier function, antioxidant capacity and microbiota composition in laying hens with high stocking density.

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DISCLOSURES

No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors have been approved the manuscript that is enclosed.

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