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Polysaccharide from *Hericium erinaceus* improved laying performance of aged hens by promoting yolk precursor synthesis and follicle development via liver-blood-ovary axis

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ABSTRACT Little information is available on the effect of *Hericium erinaceus* polysaccharides (**HEP**) on laying hens, especially on improving liver and ovarian health and function. Therefore, this study was conducted to investigate the impacts of HEP on liver and ovarian function to delay the decline in the laying performance of aged hens. A total of 360 fifty-eight-wk-old laying hens were randomly allocated to 4 treatments, with 6 replicates of 15 birds each. After 2 wk of adaptation, the birds were fed basal diet (**CON**) or basal diets supplemented with 250, 500, and 750 mg/kg of HEP (HEP250, HEP500, and HEP 750, respectively) for 12 wk. The results showed that, compared with CON, hens fed HEP had significantly increased laying performance ($P < 0.05$) and promoted follicle development, as evidenced by the increased numbers of hierarchical follicles, small follicles, and total follicles ($P < 0.05$). Birds fed 500 mg/kg of HEP improved the liver function by increasing T-AOC activity ($P < 0.05$) and decreasing hepatic oxidative stress and inflammatory responses (inflammatory cell infiltration) caused by aging. The lipid metabolism was improved, and yolk precursor synthesis was promoted in the liver of HEP-treated laying hens by upregulating the mRNA expression of *FAS, MTTP, PPAR-α, APOVLDL-Ⅱ*, and *VTG-Ⅱ* ($P < 0.05$). In addition, HEP significantly decreased ovarian inflammation by regulating the mRNA levels of *NF-κB, IL-1β, IL-6*, and *TNF-α* ($P < 0.05$). As a result, the contents of E_2, LH, and FSH in serum and the gene expression of *ERα* of the liver and *FSHR* of the ovary increased in HEP-treated hens ($P < 0.05$). In conclusion, dietary HEP supplementation exhibited potential hepatic and ovarian protective effects, thereby increasing the laying performance of aged hens by enhancing reproductive hormone secretion hormone secretion and promoting yolk precursor synthesis and follicle development via the liver−blood−ovary axis. The optimal supplementation level of HEP in aged hens was 500 mg/kg.

Key words: *Hericium erinaceus* polysaccharides, aged hen, liver-blood-ovary axis, ovarian function

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INTRODUCTION

Oxidative stress and inflammatory responses generally occur in the liver and ovaries of laying hens after high-intensity metabolism during the peak laying production period, potentially compromising their function or resulting in dysfunction, and thus leading to declined liver and ovarian health with aging. (Dai et al., 2021). The main function of the liver is to participate in various key metabolic functions, immune functions, and detoxification processes (Zaefarian et al., 2019). The ovary plays vital roles in the development of ovarian follicles, and its dysfunction leads to a decline in follicle number and follicular atresia. In general, dysfunction of the liver and ovaries of aged hens accompanies by significant decreases in antioxidant capacity, reproductive hormone secretion, and follicular development, resulting in reduced synthesis of yolk precursors. This directly decreases egg production and quality, resulting in major economic losses for the poultry industry (Moradi et al., 2013; Zhang et al., 2019). Thus, alleviating liver and ovarian aging and maintaining their functions are essential to delay the decline in laying performance and maintain egg quality in aged hens.

Hericium erinaceus, also known as houtou, is a large edible and medicinal fungus belonging to the Basidiomycota (He et al., 2017), the total sugar (e.g.,

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polysaccharides, oligosaccharides) content of which reaches 61.3%. *Hericium erinaceus* polysaccharide (**HEP**) is the most important active substance, and its main representative components include β-glucan, α-glucan, glycoprotein complex, etc. (Rodrigues et al., 2015). Previous studies have shown that HEP has immunomodulatory (Kim et al., 2010), antioxidant activity (Zhang et al., 2012), hepatoprotective effect (Cui et al., 2016), anti-hyperglycemic, and hypolipidemic properties (Yang et al., 2003; Nie et al., 2019). The application of HEP in livestock and aquaculture has been reported previously. HEP can regulate the immunomodulatory activity of lymphocytes, macrophages, and dendritic cells in the spleen of grass carp, and thus confer disease resistance to *Aeromonas hydrophila* (Gou et al., 2018). HEP can decrease organ injury and improve the antioxidant capacity, serum protein levels, antibody levels, and complement levels to reduce immunosuppression and apoptosis in ducklings infected with duck reovirus (Liu et al., 2021).

However, little information is available on the effects of HEP on aged hens, particularly on improving liver and ovarian health and function. Accordingly, this study was conducted to evaluate whether HEP alleviates liver and ovarian aging and maintains health and function, thereby increasing the laying production of aged hens via the liver−blood−ovary axis.

MATERIALS AND METHODS

Animal Ethics Statement

All experimental procedures were approved by the Animal Care and Use Committee of Zhejiang University (Hangzhou, China, approval number ZJU20220310).

Birds and Experimental Design

A total of 360 58-wk-old Jingbai laying hens with similar laying rates were randomly allocated to 4 groups with 6 replicates of 15 birds each. After a 2-wk adaptation period, the birds received the basal diet (**CON**) or basal diets supplemented with 250 (HEP250), 500 (HEP500), or 750 (HEP750) mg/kg HEP for 12 wk. The basal corn-soybean meal diet for laying hens (Table 1) was formulated to meet or exceed the nutritional requirements as recommended by the National Research Council (National Research Council, 1994). HEP was isolated, extracted and purified in the laboratory to a final purified concentration of 40%. Birds were kept in staggered 3-layer cages with individual cages (dimensions: 45 × 45 × 40 cm) accommodating 3 laying hens. Five sets of adjacent cages, totaling 15 layers, were used for each replicate. The birds were housed in a facility maintained at a temperature of 24 ± 3°C, a relative humidity of 50 to 60%, and a lighting regimen of 16 hours of light followed by 8 hours of darkness. All birds were provided with food and water *ad libitum*.

Table 1. Ingredients and nutrient contents of the basal diet (air-dry basis).

Ingredients	Content, %	Nutrient levels[2]	Content
Corn, 8.0%CP	57.00	Metabolizable energy, Mcal/kg	2.63
Soybean meal, 46% CP	24.00	Crude protein, %	16.33
Wheat middling	5.0	Lysine, %	0.81
Emulsified fat powder, 50%Fat	1.50	Methionine, %	0.46
Limestone	9.00	Cysteine + methionine, %	0.73
Dicalcium phosphate	1.00	Calcium, %	3.62
Salt	0.30	Total phosphorus, %	0.65
DL-methionine	0.20	Available phosphorus, %	0.35
Premix [1]	2.00		
Total	100.00		

[1]The premix provided the following per kg of the diet: vitamin A, 12,500 IU; vitamin D_3, 4,000 IU; vitamin K_3, 2 mg; thiamine, 1 mg; riboflavin, 8.5 mg; calcium pantothenate, 50 mg; niacin acid, 32.5 mg; pyridoxine, 8 mg; folic acid, 5 mg; B_{12}, 5 mg; choline chloride, 500 mg; iron, 60 mg; copper, 10 mg; manganese, 80 mg; zinc 80 mg; iodine 0.3 mg; and selenium 0.30 mg.

[2]Metabolizable energy and available phosphorus in the nutrient levels were calculated values, while the others were measured values.

Sample Collection

Upon completion of the trial, 6 hens were randomly selected from each treatment group (one hen from each replicate) for jugular vein blood collection. The serum was obtained by centrifuging at 4°C (3,000 × *g* for 10 min) and subsequently preserved at -80°C for further analysis. Then the birds were euthanized by cervical dislocation. After euthanasia, ovarian tissue (comprising stroma and follicles) was collected and the liver was dissected in longitudinal section from the left lobe and a small piece of 25 x 25 x 2 mm was cut. A vernier caliper was employed to ensure the samples were collected from the same tissue areas. These samples were then fixed in 4% paraformaldehyde solution, while the remaining ovarian and liver tissues were frozen for subsequent experiments.

Laying Performance

Egg weight and numbers were recorded daily, while feed consumption was documented weekly on a per-replicate basis to determine the laying rate (**LR**), average egg weight (**AEW**), average daily feed intake (**ADFI**), and feed conversion ratio (**FCR**). LR, AEW, ADFI, and FCR were calculated at wk 1 to 4, 5 to 8, 9 to 12, and 1 to 12.

Histological Observation

Ovary, follicle and liver (n = 6) obtained from each group were fixed with 4% paraformaldehyde and paraffin-embedded. The paraffin-embedded samples were sectioned into 5 μm-thick slices and subsequently stained with hematoxylin and eosin (**H&E**) for histomorphology analysis (Liu et al., 2023). Each section underwent examination under a light microscope, with 6 H&E sections analyzed per tissue per treatment. Six fields of view per section were randomly selected for statistical

analysis. The NAFLD activity score (**NAS**) system was assessed in terms of 4 semiquantitative parameters: steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis as previously described (Yang et al., 2023).

Counting the Number of Follicles in the Ovary

The entire ovary was excised from the ovarian stromal root, thoroughly rinsed with PBS, and follicle diameters were measured using a straight edge to quantify the number of follicles, following the classification criteria outlined in a previous study (Lovell et al., 2003). These criteria included hierarchical follicles (F1 to F6, >8 mm in diameter), small yellow follicles (SYF, 6–8 mm in diameter), large white follicles (LWF, 4–6 mm in diameter), and small white follicles (SWF, 2–4 mm in diameter).

Assay of Antioxidant Indices in the Liver

Liver tissue homogenates were prepared by blending the liver tissue with ice-cold isotonic saline at a ratio of 1:9 (weight/volume) and subsequently centrifuging to obtain the supernatant. The activity of T-AOC, GSH-Px, and T-SOD, and MDA contents were determined using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China).

Serum Levels of Reproductive Hormones

Serum levels of FSH, LH, E_2, and P_4 were determined using ELISA kits (Jiancheng Bioengineering Institute, Nanjing, China). A double antibody sandwich method was applied and hormone levels in the serum samples were calculated from a standard curve.

Quantitative Real-Time PCR Analysis

RNA was extracted from liver and ovary tissues using a FreeZol Reagent kit (Vazyme, Nanjing, China). cDNA was synthesized using a cDNA Synthesis Kit (Vazyme, Nanjing, China). RT-qPCR analyses were conducted using the Taq Pro Universal SYBR qPCR Master Mix kit on the StepOne Plus Real-Time PCR system (Applied Biosystems, Carlsbad, CA). After normalization to the *β-actin* gene, fold change was calculated, and mRNA abundance was estimated using the $2^{-\Delta\Delta CT}$ method. The primer sequences for the target genes are listed in Table S1.

Statistical Analysis

Significant differences between groups were compared using ANOVA of SPSS 25.0 software. Multiple comparisons were performed using Duncan's method. The linear and quadratic effects of dietary HEP supplementation dose on each indicator were assessed using regression analysis. Different lowercase letters indicate statistical significance ($P < 0.05$). All figures were generated using GraphPad Prism software (version 8.0).

RESULTS

Laying Performance

There were linear and quadratic increases in the laying rate with increasing levels of HEP in hens during wk 9 to 12 (Table 2, $P < 0.05$). During wk 1 to 12, 500 mg/kg HEP supplementation significantly increased the laying rate compared to CON ($P < 0.05$). However, throughout wk 1 to 4, 5 to 8, 9 to 12 and 1 to 12 of the experiment, HEP addition had no significant effect on the AEW, ADFI, and FCR of aged hens ($P > 0.05$).

Table 2. Effect of HEP on laying performance of laying hens in the late laying period.[1]

Items	Dietary HEP levels (g/kg)				SEM[2]	*P*-value		
	0 (Control)	250	500	750		ANOVA	Linear	Quadratic
Laying rate, %								
Wk 1–4	84.29	85.08	85.04	85.12	0.40	0.879	0.521	0.676
Wk 5–8	82.66	83.29	85.40	84.84	0.52	0.212	0.068	0.560
Wk 9–12	78.49[b]	82.62[a]	83.93[a]	82.10[a]	0.66	0.014	0.022	0.013
Wk 1–12	81.81[b]	83.66[ab]	84.79[a]	84.02[ab]	0.44	0.090	0.043	0.118
Average egg weight, g								
Wk 1–4	61.36	62.19	62.39	61.91	0.17	0.159	0.215	0.056
Wk 5–8	61.49	62.58	61.95	62.58	0.21	0.200	0.167	0.575
Wk 9–12	62.25	61.63	61.94	61.65	0.20	0.667	0.414	0.684
Wk 1–12	61.69	62.14	62.09	62.05	0.13	0.630	0.396	0.374
Average daily feed intake, g/hen per day								
Wk 1–4	115.18	115.55	115.82	116.57	0.56	0.861	0.410	0.872
Wk 5–8	111.81	114.83	114.99	111.28	0.93	0.360	0.864	0.082
Wk 9–12	112.94	114.64	114.95	113.48	0.49	0.440	0.665	0.121
Wk 1–12	113.31	115.01	115.25	113.78	0.49	0.448	0.711	0.121
Feed conversion ratio, g/g								
Wk 1–4	2.23	2.18	2.18	2.21	0.01	0.633	0.745	0.216
Wk 5–8	2.20	2.20	2.18	2.10	0.02	0.332	0.106	0.395
Wk 9–12	2.31	2.25	2.23	2.25	0.02	0.487	0.213	0.362
Wk 1–12	2.25	2.21	2.20	2.18	0.01	0.412	0.107	0.705

[1]n = 6 replicates per treatment.
[2]SEM, standard error of mean.
[a-b]Values within a row with no common superscripts differ significantly ($P < 0.05$).

Counting the Number of Follicles in the Ovary

The pregrade and grade follicles in the ovaries of aged hens and their arrangement in developmental order are shown in Figure 1A. Statistical analysis of ovarian follicle counts indicated that HEP supplementation significantly increased hierarchical follicles, SYF, and the total number of follicles (TF, $P < 0.05$), with the greatest boosting effect observed at 500 mg/kg (Figure 1B). However, various concentrations of HEP supplementation did not significantly enhance the number of SWF and LWF in the ovaries during the late-laying period compared with CON ($P > 0.05$).

Hepatic Antioxidant Capacity

As shown in Figure 2, the T-AOC activity in both the HEP500 and HEP750 groups significantly increased ($P < 0.05$), whereas the MDA content in the HEP500 was significantly lower than that in CON ($P < 0.05$). However, HEP supplementation did not significantly affect the hepatic activities of GSH-Px and T-SOD in aged hens ($P > 0.05$).

Histological Observation

Hematoxylin and eosin-stained images of liver tissues revealed notable inflammatory cell infiltration, a substantial presence of lipid vacuoles (Figure 3A) and

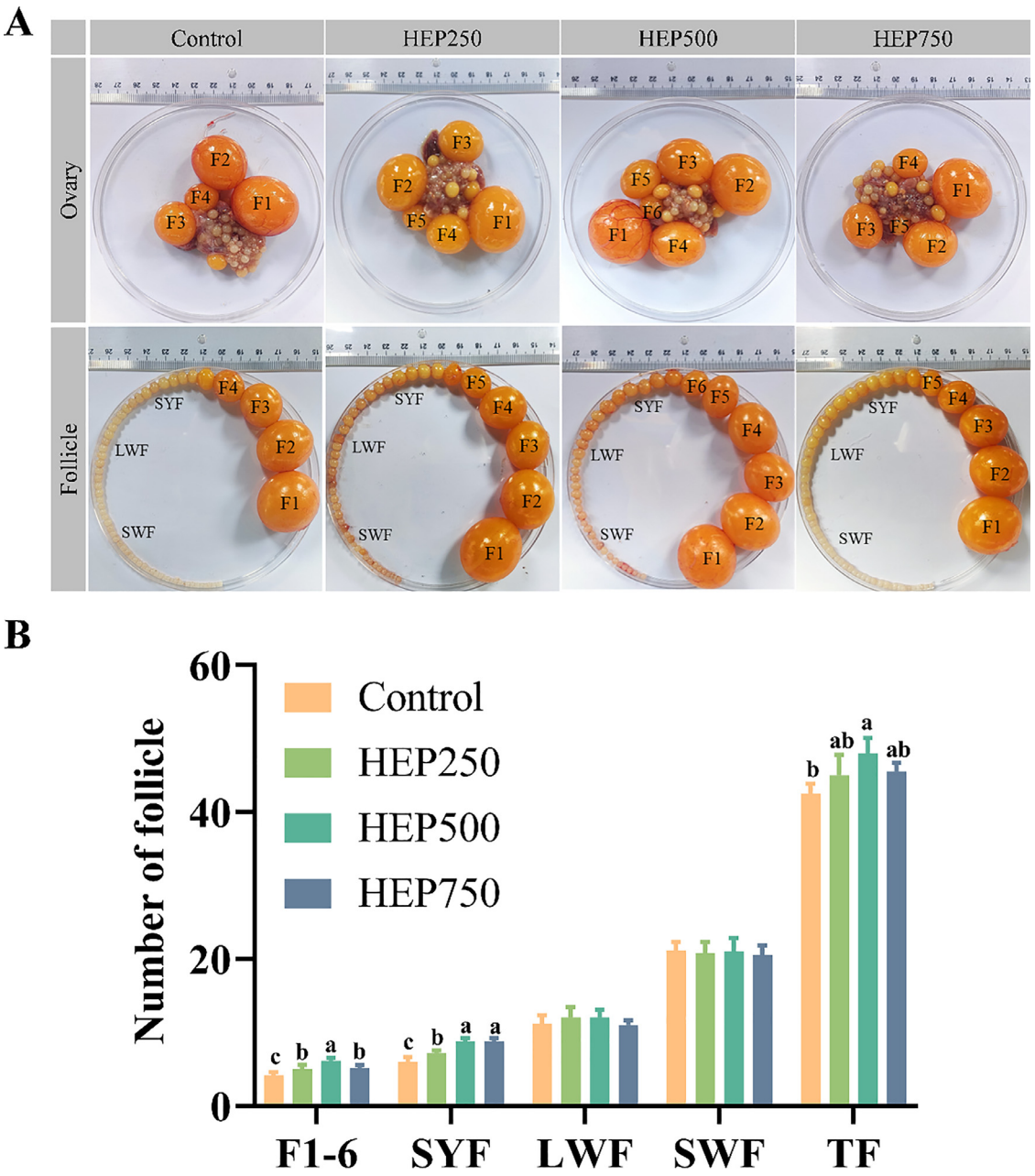

Figure 1. Hierarchical system of follicle development in ovaries of laying hens during the late laying period. (A) Pre-grade follicles and grade follicles in the ovaries of laying hens and arranged in developmental order. (B) Counting the number of follicles in the ovary. F1-6: hierarchical follicles; SWF: small white follicles; LWF: large white follicles; SWF: small white follicles; TF: total number of follicles. Data are presented as the mean ± SD; n = 6 hens per group. $^{a-c}$ Treatments with no common superscripts differ significantly ($P < 0.05$).

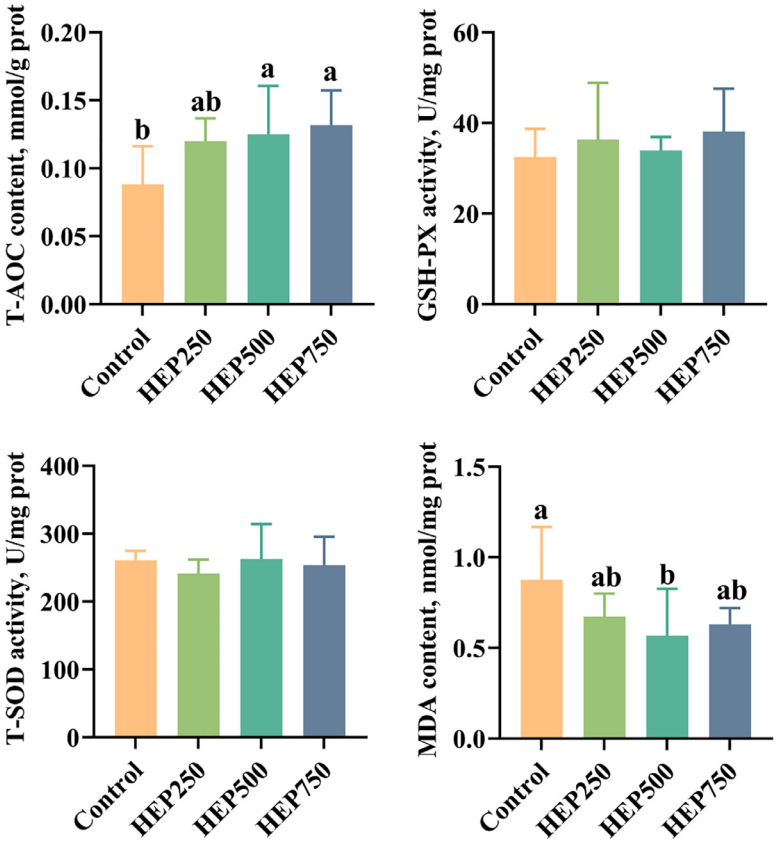

Figure 2. Effects of dietary supplementation with different levels of HEP on the hepatic antioxidant capacity of laying hens in the late laying period. T-AOC: total antioxidant capacity; T-SOD: total superoxide dismutase; GSH-Px: glutathione peroxidase; MDA: malondialdehyde. Data are presented as the mean ± SD; n = 6 hens per group. [a-b] Treatments with no common superscripts differ significantly ($P < 0.05$).

higher liver NAS score (Figure 3B) in the control group. However, these manifestations were ameliorated by HEP treatment. In the HEP group, laying hens exhibited diminished inflammatory cell infiltration, reduced inflammation, fewer or smaller cytoplasmic lipid vacuoles in their liver tissues (Figure 3A) and the liver NAS score was lower compared with the CON group (Figure 3B), with the greatest improvement observed at the HEP supplementation level of 500 mg/kg.

Expression of Genes Related to Lipid Metabolism and Yolk Precursor Synthesis

As shown in Figure 4A, compared with CON, HEP500 treatment upregulated the gene expression of *FAS, MTTP,* and *PPAR-α* of the liver ($P < 0.05$), but it did not have a significant effect on the gene expression of *ACC* and *PPAR-γ* ($P > 0.05$). HEP250 treatment significantly increased the gene expression of *PPAR-α*

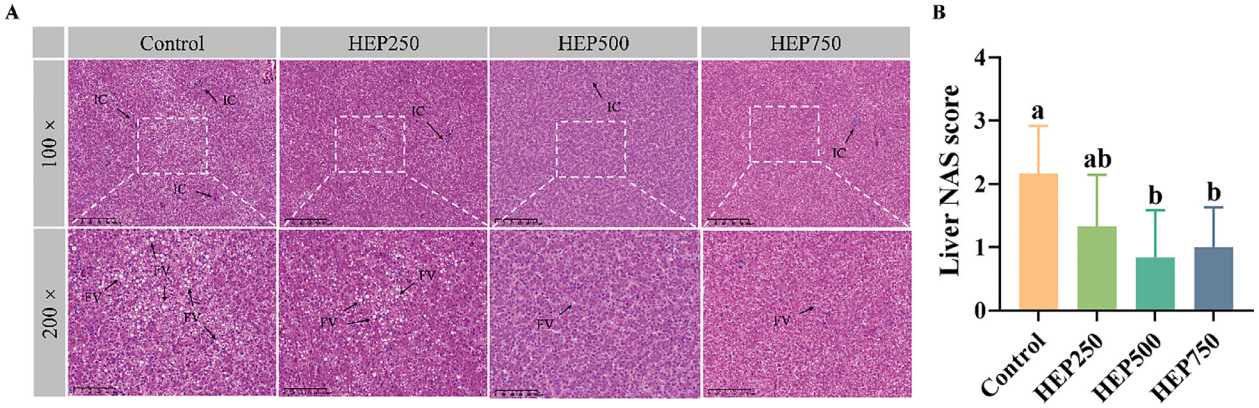

Figure 3. Effect of HEP on liver histology of laying hens in late laying period. (A) Representative photomicrographs of liver tissues with H&E staining. (B) Assessment of non-alcoholic fatty liver disease (NAFLD) activity score (NAS) of liver tissues based on histological sections. H&E staining was magnified 100 × and 200 × . IC, inflammatory cells; FV, fatty vacuoles. [a-b] Treatments with no common superscripts differ significantly ($P < 0.05$).

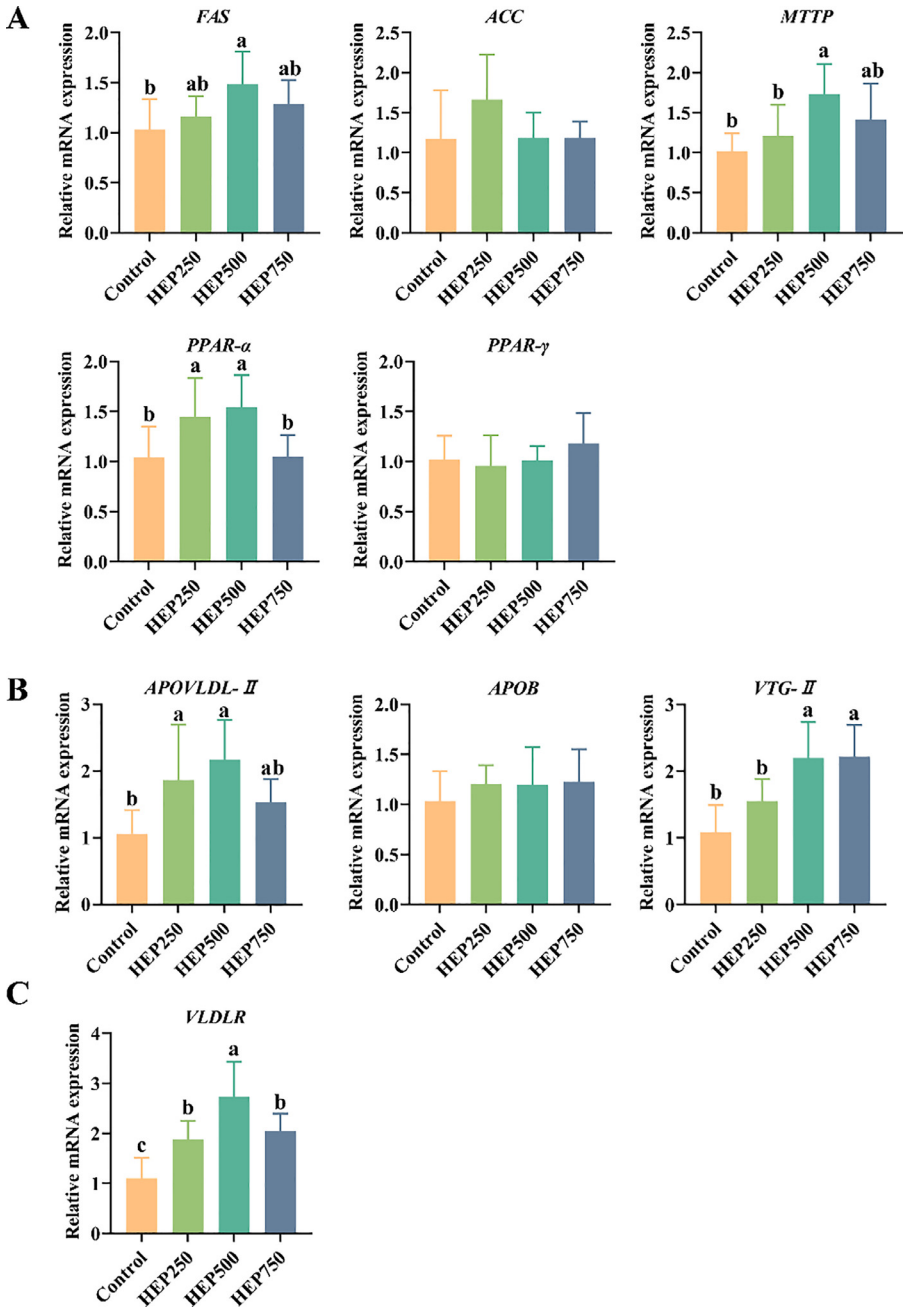

Figure 4. Effects of dietary supplementation with different levels of HEP on the expression of genes related to lipid metabolism and yolk precursor synthesis of laying hens in the late laying period. (A) The gene expression of *FAS, ACC, MTTP, PPAR-α* and *PPAR-β* of the liver. (B) The gene expression of *APOVLDL-II, APOB*, and *VTG-II* of the liver. (C) The gene expression of *VLDLR* of the ovary. FAS: fatty acid synthase; ACC: acetyl CoA carboxylase; MTTP: microsomal triglyceride transfer protein; PPAR-α: peroxisome proliferator activated α; PPAR-β: proliferator activated β; APOVLDL-II: very low-density apolipoprotein II; APOB: apolipoprotein B; VTG-II: vitellogenin II; VLDLR: very low-density lipoprotein receptor. Data are presented as the mean ± SD; n = 6 hens per group. [a-b] Treatments with no common superscripts differ significantly ($P < 0.05$).

($P < 0.05$), but it had no significant effect on the other genes ($P > 0.05$). In addition, Figures 4B and 4C demonstrate that compared with CON, hens receiving 500 mg/kg HEP showed significantly increased gene expression of *APOVLDL-Ⅱ* and *VTG-Ⅱ* in the liver as well as *VLDLR* in the ovary ($P < 0.05$), whereas the hens in HEP250 group only increased the gene expression of *APOVLDL-Ⅱ* ($P < 0.05$), and the HEP750 group only increased the gene expression of *VLDLR* ($P < 0.05$). Moreover, HEP supplementation, regardless of the inclusion level, showed no significant effect on the gene expression of *APOB* (Figure 4B, $P > 0.05$).

Reproductive Hormone Levels and Their Receptors

As shown in Figure 5A, dietary supplementation with HEP did not exert a significant effect on the content of P_4 in serum ($P > 0.05$). However, the levels of E_2 and FSH in HEP supplemented groups were higher than those in CON group ($P < 0.05$), and the content of LH in hens from both the HEP500 and HEP750 groups exhibited a significant increase ($P < 0.05$). Additionally, compared with CON, the mRNA expression of *ERα* in the liver was significantly upregulated in the HEP500

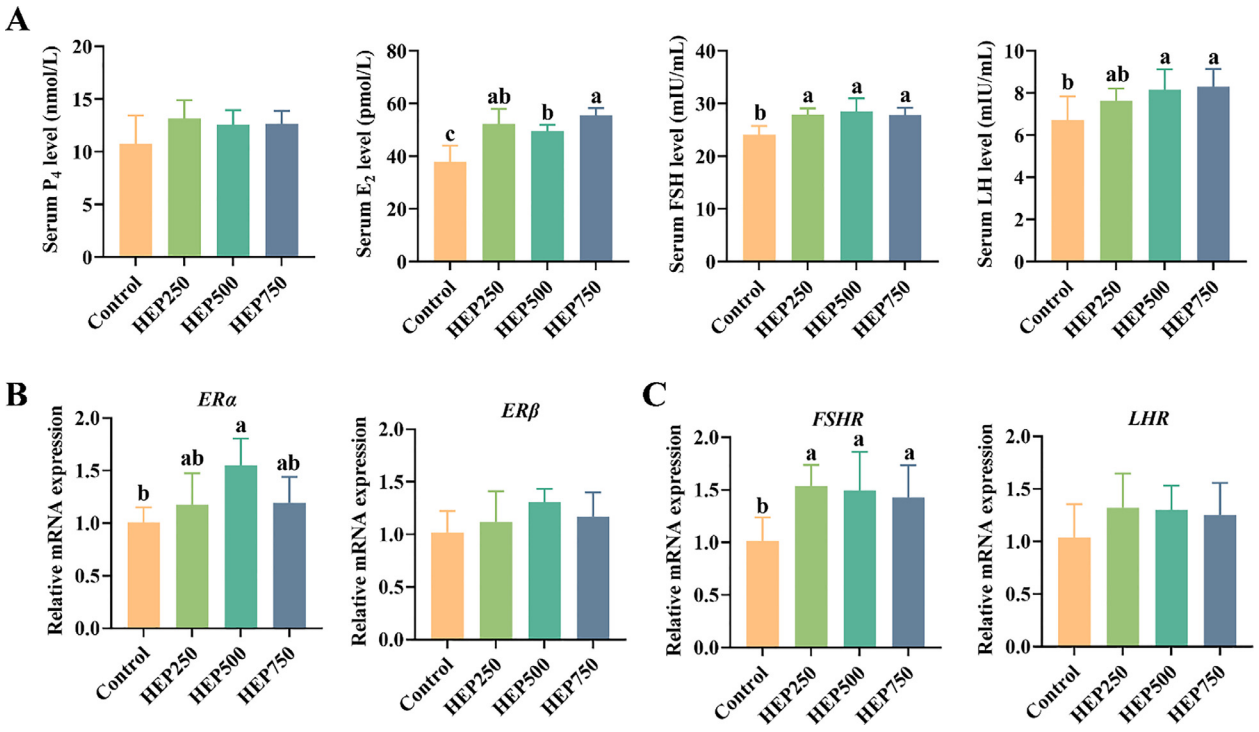

Figure 5. Effects of dietary supplementation with different levels of HEP on the hormone levels in the serum and related gene expression in the liver and ovary of laying hens in the late laying period. (A) The levels of hormones in the serum. (B) The gene expression of *ERα* and *ERβ* of the liver. (C) The gene expression of *FSHR* and *LHR* of the ovary. ERα: estrogen receptor α; ERβ: estrogen receptor β; FSHR: follicle stimulating hormone receptor; LHR: luteinizing hormone receptor. Data are presented as the mean ± SD; n = 6 hens per group. [a-c] Treatments with no common superscripts differ significantly ($P < 0.05$).

group ($P < 0.05$;) but had no significant effect on the gene expression of ERβ ($P > 0.05$, Figure 5B). In ovarian tissue, compared with the control group, HEP supplementation increased the gene expression of *FSHR* ($P < 0.05$) but had no significant effect on the gene expression of *LHR* ($P > 0.05$, Figure 5C).

Ovarian Histomorphology and Immune Response

The ovarian structure and morphology appeared normal in all groups, with varying numbers of follicular structures observed (Figure 6A). However, all HEP-supplemented hens exhibited higher numbers of primary follicles (**PF**) and secondary follicles (**SF**) with denser medullary structures (**M**) than CON. In contrast, birds in CON group showed a higher incidence of atretic follicles (**AF**). Additionally, observation of the follicular membranous and granulosa layers revealed that hens in the control group had thinner layers, and the demarcation between the inner and outer membranous layers was distinct. In contrast, HEP-supplemented hens displayed tightly structured follicular granulosa and membrane layers, characterized by thicker membrane layers and granulosa cells arranged in multiple nearby layers. As shown in Figure 6B, compared with CON, HEP500 treatment could significantly decreased the genes expression of *IL-1β, TNF-α* and *NF-κB* ($P < 0.05$), while HEP750 treatment significantly decreased the genes expression of *IL-6* and *NF-κB* ($P < 0.05$).

DISCUSSION

Increasing evidence suggests that the decreased functions of the liver and ovaries in aged hens is accompanied by significant decreases in antioxidant level, reproductive hormone secretion, and follicular development, leading to reduced yolk precursor synthesis, thus directly affecting egg production (Zhang et al., 2019). A recent study reported that *flammulina* stems can improve the antioxidant capacity of aged hens and regulate ovarian apoptosis, hepatic lipid metabolism, and transport, thus improving the egg production of aged hens (Wu et al., 2023). HEP is derived from *Hericium erinaceus*, a fungus that has the similar medicinal and food homology as *flammulina*. In this study, the addition of HEP effectively improved the laying rate of aged laying hens, and 500 mg/kg HEP supplementation optimized their productive performance. The improved laying performance is probably due to improved liver and ovary function, which promotes yolk precursor synthesis and follicle development via the liver-blood-ovary axis.

The liver, being the primary organ responsible for fat synthesis in poultry, becomes crucial as the ovaries of laying hens lack the capability to synthesize fat and provide necessary lipids for egg production, necessitating reliance solely on liver fat synthesis (Trott et al., 2014). Oxidative stress is an imbalance between the production and removal of ROS from the body, which is a primary cause of aging (Miyamoto et al., 2010). Accumulation of ROS easily exceeds the antioxidant capacity of hepatocytes in aging animals (Amevor et al., 2021), resulting in

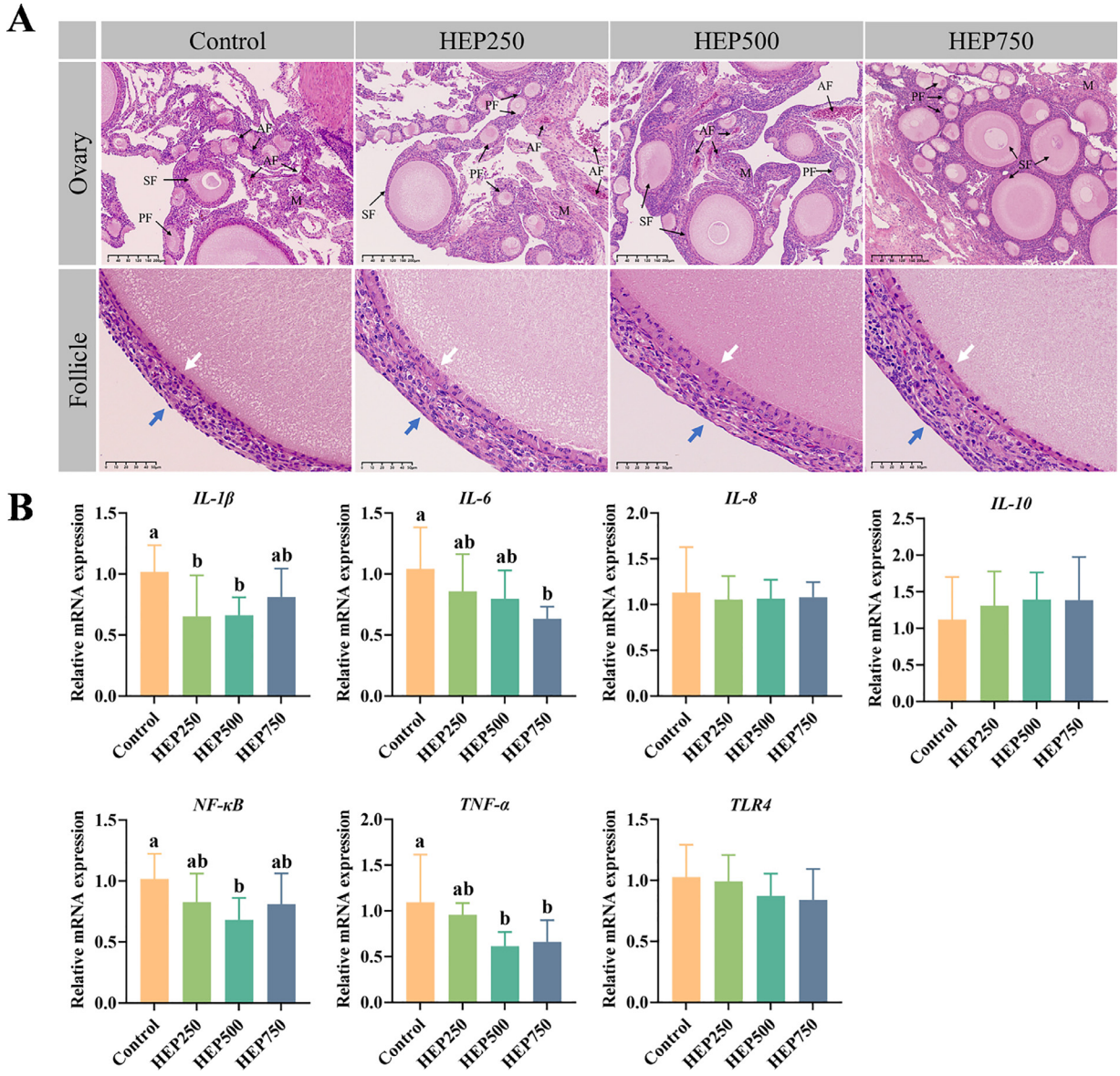

Figure 6. Effects of dietary supplementation with different levels of HEP on the ovary and follicle histomorphology and expression of genes related to the immune response of laying hens in the late laying period. (A) Ovarian histomorphology. (B) Expression of genes related to immune response. The blue arrows indicate the follicular membrane layer and the white arrows indicate the follicular granular layer. AF, atretic follicles; PF, primary follicles; SF, secondary follicles; M, medullary structures; IL-1β, interleukin 1β; IL-6, interleukin 6; IL-8, interleukin 6; IL-10, interleukin 10; NF-κB, nuclear factor kappa-B. TNF-α, tumor necrosis factor alpha; TLR4, toll-like receptor 4. Data are presented as the mean ± SD; n = 6 hens per group. [a-b] Treatments with no common superscripts differ significantly ($P < 0.05$).

serious damages in the liver (Wang et al., 2022). T-AOC activity reflects the scavenging ability of the antioxidant system on oxygen free radicals, and the free radicals act on lipids to cause peroxidation; the oxidation end product is MDA, which can cause the cross-linked polymerization of life macromolecules such as proteins and nucleic acids (Haridevamuthu et al., 2024). In the present study, HEP supplementation in aged hens significantly increased T-AOC activity and reduced MDA content in the liver, which is consistent with the results of a previous study on broilers (Shang et al., 2014).

Hepatic oxidative stress ultimately affects various liver functions such as lipid metabolism and yolk precursor production. The fat components in hens' liver are mainly glycerol A-phosphate and acyl-coenzyme A, which enter the blood and synthesize TG (Ho et al., 2019). However, TG can only exist in the form of synthetic lipoproteins in the blood (Ho et al., 2019). When the TG synthesized in the liver exceeds the ability of the body to synthesize lipoproteins, a large number of TG molecules are stored in the liver in the form of fat droplets, which further damages the liver and leads to the degeneration of liver cells, thus affecting the health of laying hens (Benzertiha et al., 2019). In the present study, multitudinous fat droplets accumulated in the liver, accompanied by inflammatory responses. However, this phenomenon was partially reversed upon the addition of HEP. A previous study reported that HEP reduced fat deposition and promoted cholesterol metabolism in broilers (Shang et al., 2015). These results can

be explained by the fact that HEP increases the activity of HMG-CoA reductase, an enzyme that catalyzes the conversion of HMG-CoA to mevalonic acid and participates in the biosynthesis of cholesterol, thus reducing total cholesterol, low-density lipoprotein cholesterol, and triglycerides in the plasma (Yang et al., 2003). To further investigate the effects of HEP on lipid synthesis in the liver, the genes related to lipid synthesis were identified. It is well known that Acetyl-CoA carboxylase (**ACC**) is a rate-limiting enzyme that catalyzes animal fat production (Goedeke et al., 2018), fatty acid synthetase (**FAS**) can affect fat production, and the fat content in poultry liver is positively correlated with the activity of FAS (Mu et al., 2020). Interestingly, in the present study, HEP significantly increased *FAS* gene expression in the liver of laying hens. However, HE results showed that lipid droplets in the liver were reduced compared with the control group, which may be related to the gene expression of *MTTP* and *PPAR-α* increased with HEP treatment. *MTTP* is a lipolysis-related gene and *PPAR-α* is a lipid transport-related gene, both of them contribute to the synthesis of yolk precursor substances (Amevor et al., 2021).

The ovary is the main organ in the reproductive system that produces follicles, and increasing the number of follicles is a key measure to improve the laying rate of aged hens. In this study, dietary supplementation with 500 mg/kg HEP significantly increased the number of graded follicles, small yellow follicles, and total follicles. These results are consistent with our previous observation that the HEP500 group showed significantly increased egg production during the late-laying period. Previous studies have reported that follicular atresia occurs, and the number of original follicles decreases in the ovaries during aging (Manolagas, 2010). In the present study, HEP effectively reduced the occurrence of atretic follicles and increased the number of primary and secondary follicles in aged hens. Ovarian granulosa cells are important mediators of follicular production and estradiol secretion (Rivas et al., 2016). However, the ovaries of aged hens have thin granular cells and a loose ovarian matrix (Manolagas, 2010). Our results indicate that HEP supplementation tightly structured the follicular granulosa and membrane layers and densified the ovarian matrix, thus increasing estradiol secretion. Inflammation of

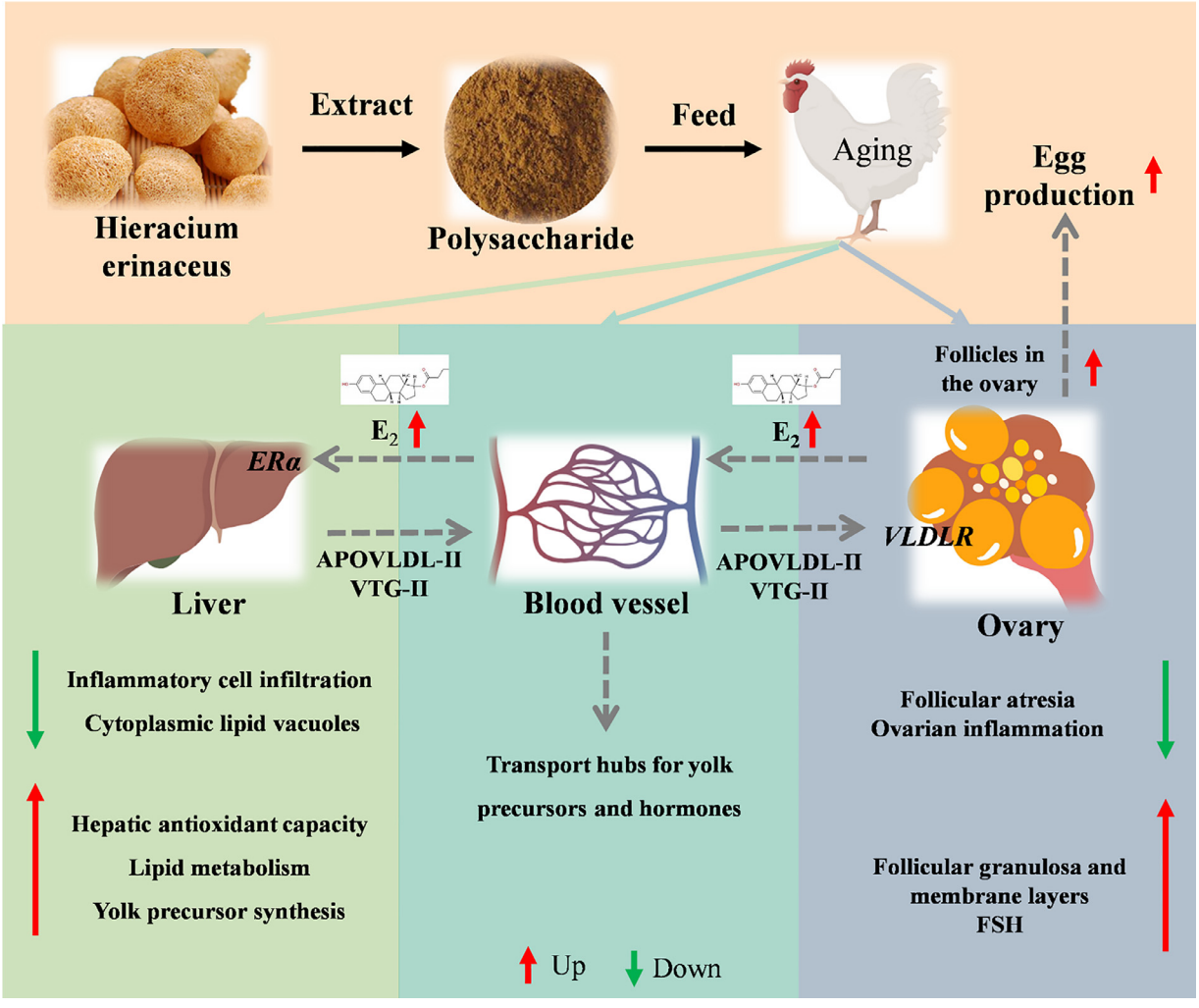

Figure 7. Graphical summary of the effect of HEP on the egg performance of aged laying hens through on the liver-blood-ovary axis.

the ovaries of hens during aging also compromises ovarian function. NF-κB is a primary transcription factor that rapidly regulates cellular responses, and the Tolly-like receptor, as a special recognition molecule, can activate the NF-κB pathway, thereby inducing the production of inflammatory factors IL-1β, IL-6 and TNF-α (Baker et al., 2011). Previous studies have shown that HEP has anti-inflammatory and immunomodulatory activities (Zhang et al., 2017). The anti-inflammatory activity was confirmed in this study, where HEP supplementation decreased the pro-inflammatory cytokines expression in the ovary.

The reason for the decrease in egg production rate in aged hens is not only the decrease in ovarian function, oocyte quality, and the number of follicles entering the graded developmental stage, but also the decrease in yolk production and deposition ability (Lillpers and Wilhelmson, 1993). E_2 secreted by the ovary is transported to the liver through the blood, bound to the estrogen receptor (**ER**) in the liver, induces the synthesis of APOVLDL-Ⅱ in the liver, and promotes the synthesis of ApoB in the liver, which then combines with triglycerides, total cholesterol, and phospholipids synthesized by the liver through the endoplasmic reticulum and Golgi body assembly and is processed into VLDL, which is different from the normal VLDL that targets egg yolk production (Li et al., 2014). In the present study, HEP increased E_2 level in the serum and the gene expression of *APOVLDL-Ⅱ* and *ERα* in the liver. In addition, VTG II, the most important subtype of VTG synthesized during egg production, is transported to the ovary through the blood with VLDL and deposited in the follicle after binding with VLDLR on the egg membrane cells to meet the nutritional needs of the rapid development and maturation of the follicle (Schneider, 2009). In this study, HEP treatment significantly increased the expression of *VTG Ⅱ* in the liver and *VLDLR* in the ovaries. These results suggested that estrogen increases mRNA expression of *APOVLDL-II* and *VTG-II* mainly by acting on ERα, and HEP might promote this process. FSH and LH are anterior pituitary hormones that control gonadal function. FSH stimulates the fractional growth of tiny follicles and induces progesterone synthesis in granulosa cells, whereas LH stimulates the production and secretion of steroid hormones (Oduwole et al., 2021). In the present study, HEP treatment significantly increased serum FSH levels and ovarian *FSHR* gene expression. Overall, HEP supplementation promoted follicle development and ovulation, thereby improving the egg production performance of aged hens. HEP promotes the production and transport of yolk precursors as well as follicular development by increasing the reproductive hormones levels and yolk deposition-associated receptors, thereby increasing the follicles number in the ovary.

CONCLUSIONS

Dietary supplementation with 500 mg/kg HEP showed optimal effects in improving the laying performance of aged hens owing to its protective effects on the liver and ovaries. HEP supplementation enhanced the antioxidant capacity of the liver and reduced hepatic lipid deposition by regulating lipid metabolism. In addition, HEP supplementation alleviated ovarian inflammation, maintained hormone levels, and increased the expression of yolk deposition-associated receptors. The improved hepatic and ovarian functions promoted the synthesis of yolk precursor substances, facilitated the transport and exchange of various substances via the liver−blood−ovary axis, promoted follicular development and ovulation processes, and thus increased laying performance in aged hens (Figure 7).

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103810.

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