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Pu-erh tea theabrownin improves the ovarian function and gut microbiota in laying hens

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ABSTRACT Studies have reported that theabrownin can moderate the lipid metabolism and intestinal microbiota, thereby affecting the health of humans and model animals, however the research on laying hens is scarce. The present study aimed to investigate the effects of dietary theabrownin supplementation on lipid metabolism, microbial composition and ovarian function in laying hens. A total of 80 laying hens (25 wk of age) were fed with normal diet (**CON**) and normal diet +100 mg/kg theabrownin (PT group) for 12 wk. The results showed that the addition of theabrownin enhanced villus height of duodenum and decreased crypt depth of jejunum ($P < 0.05$). At the same time, compared with CON, the concentration of IL-6 and the mRNA expression of *IL-1β* and *IL-6* were decreased significantly in PT group ($P < 0.05$). Dietary theabrownin reduced the concentration of total cholesterol and glycerol, while decreased lipid droplet optical density in liver ($P < 0.05$). Compared with CON group, the mRNA expression of *PPARγ, HMG-CoAS, ACC* were down-regulated and the mRNA expression of *CYP8B1* was up-regulated in PT group ($P < 0.05$). The ACE, Chao1 and Observed_species indexes in cecum microbiota were increased by PT group intervention ($P < 0.05$). Dietary PT supplementation enhanced the relative abundance of *Firmicutes* (phylum), *Lactobacillus* (genus) and the *Firmicutes* to *Bacteroidetes* ratio, and reduced the relative abundance of *Bacteroidetes* (phylum) in cecum ($P < 0.05$). The organic acids and its derivatives were up-regulated by theabrownin intervention in serum metabolites ($P < 0.05$). Dietary theabrownin supplementation resulted in higher mRNA expression of *Bcl-2* and *SIRT1* in ovary and increased the concentration of estradiol in serum ($P < 0.05$). These discovering indicated that dietary theabrownin supplementation enhanced the intestinal function and influenced serum metabolism by improving intestinal morphology, microbiota community structure and reducing the concentration and expression of inflammatory cytokines in intestine. Dietary theabrownin reduced hepatic lipid deposition and it also decreased the cell apoptosis rate to improve ovarian function and egg weight which were associated with the SIRT1 pathway.

Key words: laying hens, theabrownin, gut microbiota;ovarian function, metabolite

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INTRODUCTION

Pu-erh tea, a celebrated traditional Chinese tea produced in Yunnan province, is mainly fermented by *Aspergillus niger*, yeast and other microorganisms (Liang et al., 2005). It has been reported that Pu-erh tea possess multiple beneficial effects including attenuation or reversal of hypercholesterolemia, hyperlipidemia, obesity, steatohepatitis, and hyperglycemia (Du et al., 2012; Zhang et al., 2012). Theabrownin is the most bioactive and ample compounds in Pu-erh tea, a complex water-soluble polyphenolic substance produced with the fermentation of Pu-erh tea. The polyphenols and products of oxidative polymerization of polyphenols with caffeine, proteins, sugars, and amino acids are considered the primary constituents of theabrownin (Tan et al., 2012; Liu et al., 2017). Previous studies had revealed that the main functions of theabrownin are anti-inflammatory, antioxidant, lipid metabolism-regulating and regulation in intestinal microbiota (Kuang et al., 2020; Deng et al., 2021). Also, we found that dietary theabrownin was found to improve production and egg quality in the previous study (Zhang et al., 2022).

The ovary is an important organ that determines the performance of laying hens. The apoptosis as well as atresia of follicles and secretion of hormone are the mainly influencing factors of ovarian function. Atresia of follicles

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is mainly regulated by the mitochondrial pathway, which is mainly regulated by the interaction of pro-apoptotic and anti-apoptotic factors (Fu et al., 2013). There are previous studies have manifested that pituitary gonadotropins, steroid hormones and other local regulators in the ovary precisely regulate follicular growth through autocrine, endocrine and paracrine (Ginther et al., 2011; Ginther et al., 2012). Therefore, how to reduce follicular atresia, turn more follicles into dominant follicles, promote the development and maturity of follicles, and improve the production performance of laying hens has become the focus of animal husbandry workers. The gut microbiota is now recognized as an indispensable metabolic "organ" that facilitates the transformation of nutrients and produces countless metabolites to maintain a balance of host metabolism. Studies have shown that the correlation between microbiota and body health is closely. For example, fecal microbiota transplantation could rebalance the gut microbiota and attenuated myocarditis, protected gut barrier in inflammatory bowel diseases colitis (Hu et al., 2019; Zhen et al., 2020; Cao et al., 2023). On the other hand, there were studies have demonstrated that the gut microbiota was altered and changed bile acids metabolism in liver of mice by theabrownin intervention (Yue et al., 2019; Kuang et al., 2020). Therefore, it is worthy to investigate the correlation between gut microbiota and layers health under the intervention of theabrownin.

Thus, this study aimed to investigate the positive effect of theabrownin intervention on ovarian function, gut microbiota and serum metabolome in layer model.

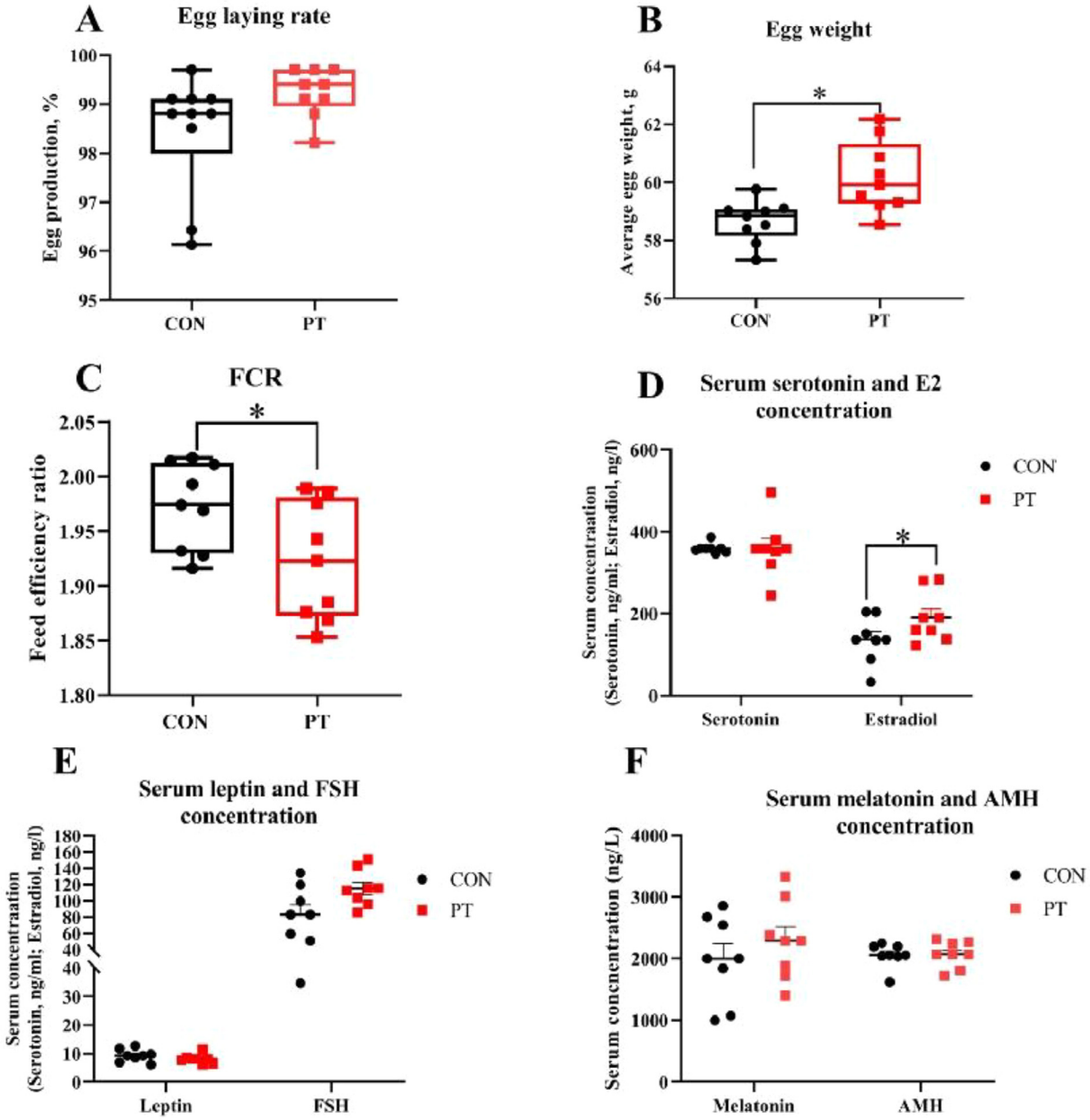


Figure 1. Dietary theabrownin supplementation improved laying production. Data are means and SEM represented by vertical bars or plot individual values. (A−C) Egg production performance (laying rate, egg weight, and FCR). (D−F) Serum hormone levels. Abbreviations: CON, control; PT, 100 mg/kg theabrownin; FCR, feed conversion ratio; FSH, follicle-stimulating hormone; AMH, anti-Mǘllerian hormone. Statistical significance was evaluated by the Independent-Samples *t*-test, * $P < 0.05$.

MATERIALS AND METHODS

Extraction of Theabrownin

Theabrownin was obtained from Yunnan Tangren Biotechnology Co., Ltd (Kunming, Yunnan, China), with the purity of 83%. Theabrownin extraction was performed following these details (Huang et al., 2019).

Briefly, Pu-erh tea powder (3,500 *g*) was suspended into a tenfold volume of absolute ethyl alcohol, mixed for twelve hours, and then vacuum strained. The residuum was leached with a tenfold volume of boiled distilled water, retain at 83 °C for 20 min with unremitting stirring, and then vacuum strained. Repeating the same extraction method 3 times, combining the extracts, and then the volume was decreased to one-fifth by vacuum evaporating. A series of liquid-liquid extraction processes were adopted to extract the concentrated solution, that obtained before, respectively, involving same volume extraction with chloroform, ethyl acetate, and n-butanol for 2, 3, and 4 times. The complete volume of the water layers was evaporated to one-quarter and then absolute ethyl alcohol was added to a final proportion of 85% to immerse the PT coarse extracts.

Animals, Diets, and Design

The experimental protocol used in the study was approved by the Animal Care and Use Committee of the Sichuan Agricultural University (SYXK2020-067). Eighty Lohman laying hens (25 wk of age) were randomly allocated into two treatments. For each treatment, there were 10 replicates, and each replicate had 4 birds, which were raised in a cage (45 × 60 cm^2). One group fed with basal diet (control group, **CON**), the other one fed with basal diet +100 mg/kg threabrownin (**PT**). All hens were housed in an environmentally controlled room (22 ± 2°C temperature; lighting cycle, 16 h/d; 05:00 am to 09:00 pm for light) and supplied with water ad libitum and same amount (110 g/d) of complete feeding mixture in mash form (Supplementary Table 1).

Sample Collection and Measurements

The total egg weight was recorded every day for each replicate. At the end of 12th wk, 20 hens (n = 10) were individually weighted, and blood samples were collected from the jugular vein into a sterile syringe. Samples were then centrifuged at 3,000 × *g* for 15 min, and then, serum was stored at -20°C pending analysis. The same

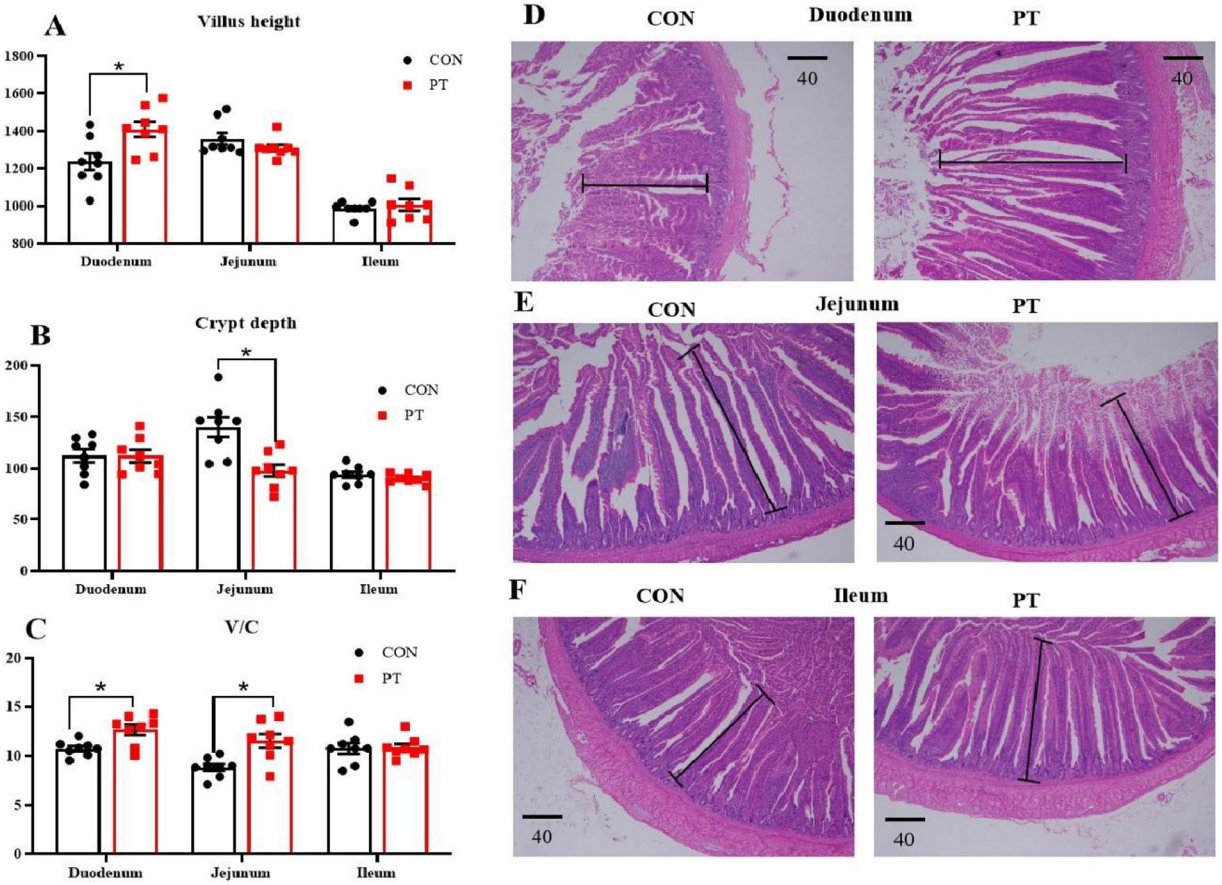


Figure 2. Dietary theabrownin supplementation improved intestinal health. Data are means and SEM represented by vertical bars or plot individual values. (A) Villus height of duodenum, jejunum and ileum. (B) Crypt depth of duodenum, jejunum and ileum. (C) The ratio of villus height/crypt depth (V/C) of duodenum, jejunum and ileum. (D–F) Light microscopy of the cross-sections of the duodenum, jejunum and ileum.Statistical significance was evaluated by the Independent-Samples *t*-test, * $P < 0.05$.

hens were then sacrificed by CO_2 suffocation, the ovary, liver and middle of intestinal tracts (duodenum, jejunum, and ileum) were immediately harvested and placed into 4% paraformaldehyde (pH = 7.2) fixation and paraffin for intestinal morphology (**H&E**), hepatic lipid droplet optical density and cell apoptosis (**TUNEL**) analysis. The ovary, liver, intestinal mucosa of jejunum and cecum contents were collected and then stored at -80°C till analysis.

Serum Reproductive Hormone and Jejunum Inflammatory Cytokines Assay

End of the animal trail, blood samples were collected (n = 10) from the jugular vein after 12 h of fasting and the serum were separated by incubation at 4°C for 30 min and subsequent centrifugation at 1,500 × *g* for 20 min. Serum concentration of serotonin, anti-Műllerian hormone (**AMH**), follicle stimulating hormone (**FSH**), estradiol (**E2**), leptin, melatonin and interleukin-6 (**IL-6**) of jejunum were examined by Commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (ELISAGenie, Nanjing, Jiangsu, China).

Ovary Function and Inflammatory Cytokines Related mRNA Expression by RT-PCR

The total RNA and real-time RT-PCR were carried out as described previously (Wang et al., 2021b). In

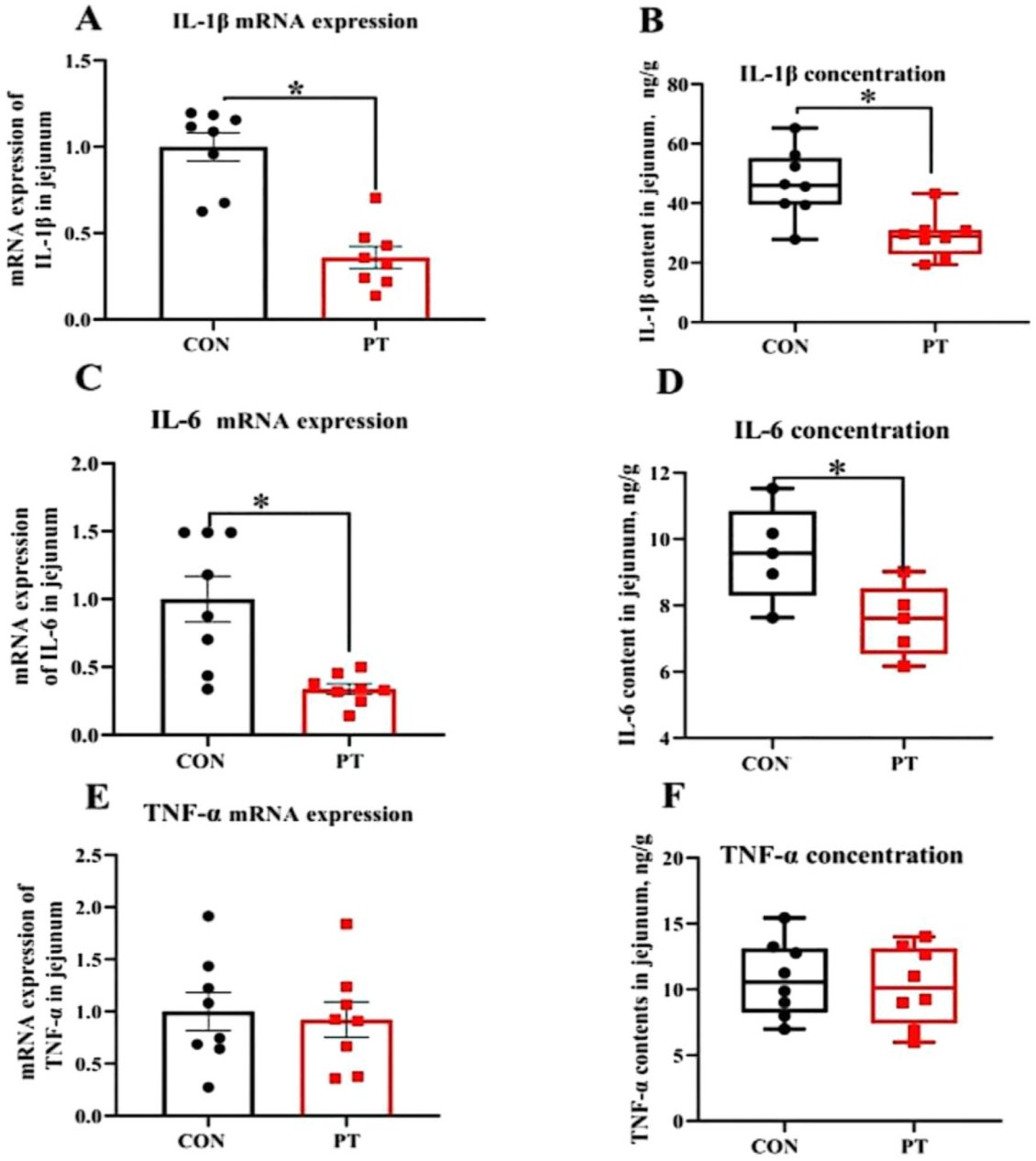


Figure 3. Dietary theabrownin supplementation reduced inflammatory cytokines secretion in jejunum. (A−F) The mRNA expression and test levels of IL-1β, IL-6 and TNF-α in jejunum mucosa. Abbreviations: CON, control; PT, 100 mg/kg theabrownin; L-1β, interleukin 1β; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α. Statistical significance was evaluated by the Independent-Samples *t*-test, * $P < 0.05$.

short, total RNA were extracted with Trizol followed by DNase1 treatment to remove genomic DNA. Gene expression of *caspase-3, Bax*, ***FoxO1*** (forkhead box O1), *cytochrome-c, Bcl-2* (B-cell lymphoma-2), *Bcl-xl*, ***IL-1β*** (interleukin 1β), ***IL-6*** (interleukin 6) and ***TNF-α*** (tumor necrosis factor-α) were determined by quantitative real-time PCR in the ovary of layers by ABI 7900 Real-Time PCR system (ABI Biotechnology, Eldersburg, MD). The primer information for all the genes is listed in Supplementary Table 2. The $2^{-\Delta\Delta CT}$ method was used to calculate target gene expression, and mRNA expression in CON was used as baseline relative to treatment groups.

Gut Microbiota and Metabolic Profiling Analysis

Microbial profile in the cecum digesta (n = 10) was evaluated by the sequencing and clustering of 16S rRNA gene with high-throughput pyrosequencing, the sequencing and bioinformatics analysis were performed by Novogene Bioinformatics Technology Co (Tianjin, China). The serum samples (n = 8) were taken to analyze the effect of theabrownin on serum metabolic and biochemical alternation in laying hens. The method was performed by Novogene Bioinformatics Technology Co. (Tianjin, Tianjin, China). Discrepancy were manifested

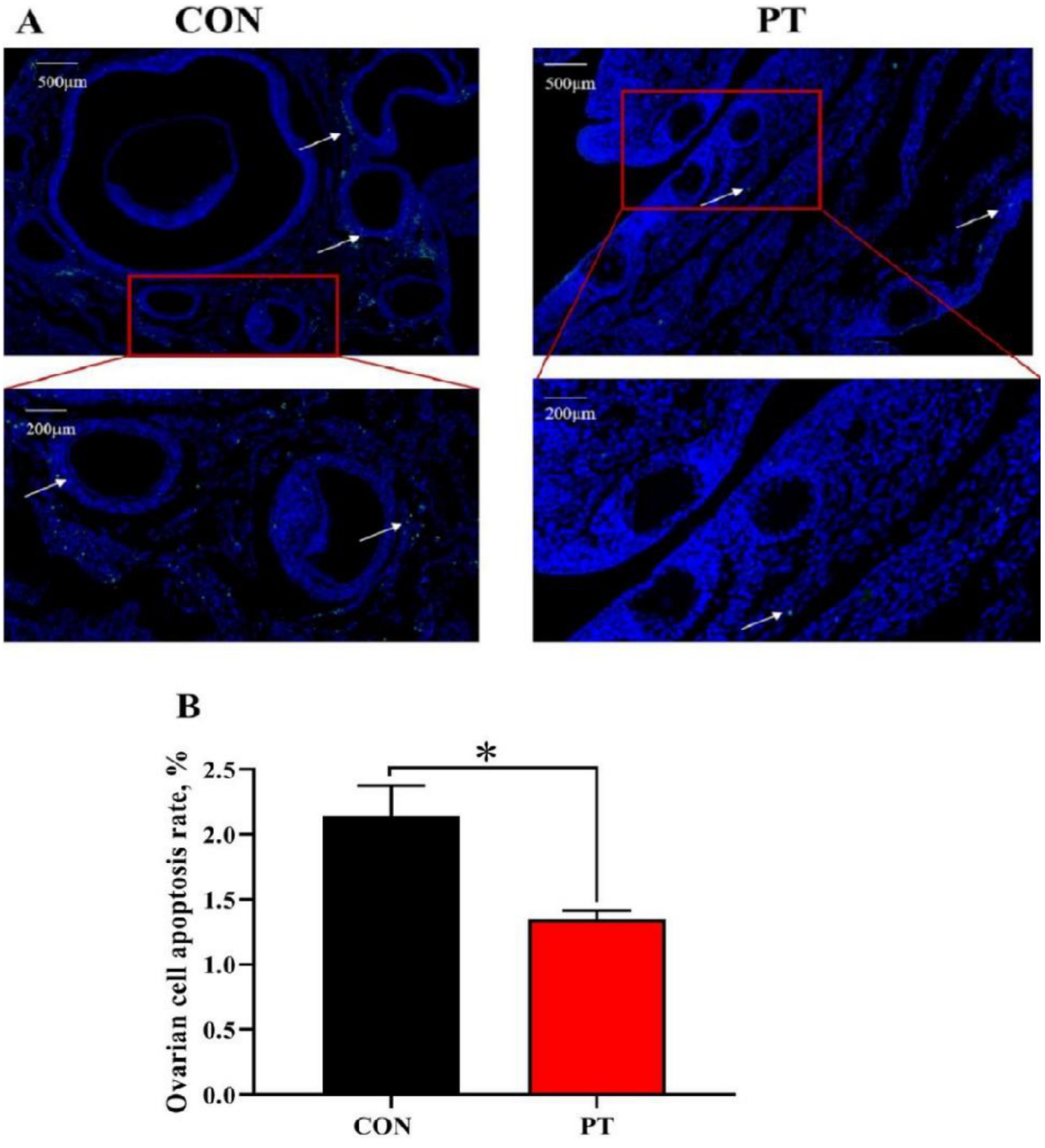


Figure 4. Effect of dietary theabrownin supplementation on cell apoptosis of ovary. (A) TUNEL analysis for cell apoptosis in ovary; (B) The immunofluorescence results of TUNEL with the green color presents the positive cells. Abbreviations: CON, control; PT, 100 mg/kg theabrownin. Statistical significance was evaluated by the Independent-Samples t-test, * $P < 0.05$.

when p-value was <0.05, VIP (Variable Importance in the Projection) > 1, and only fold changes >1.5 were considered.

Hepatic Health

The hepatic lipid droplet optical density was analyzed by Yonkers Bio-Technology Co., LTD (Chengdu, Sichuan, China). The analysis of mRNA expression of ***PPARα*** (peroxisome proliferator activated receptor α), ***PPARγ*** (peroxisome proliferator activated receptor γ), ***HMG-CoAS*** (3-hydroxy-3methyl glutaryl coenzyme a synthetase), *apoB-100, apoVLDL-Ⅱ*, ***ACC*** (Acetyl-CoA carboxylase), ***FAS*** (fatty acid synthetase), ***CYP7A1***, ***CYP8B1***, ***CYP27A1***, ***CYP7B1***, ***LPL*** (lipoprotein lipase) and *HSL* (Hormone sensitive lipase) in liver. The concentration of total cholesterol (**TC**) and total glycerol (**TG**) in liver was analyzed by Commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (ELISAGenie, Nanjing, Jiangsu, China).

TUNEL Assay

At the end of experiment, the ovary tissue (n = 10) was quickly removed and placed immediately into methylaldehyde; then a TUNEL assay was performed, using an In Situ Cell Apoptosis Detection Kit I (POD), according to the manufacturer's protocol (Roche Group, Basel, Switzerland). Using BA200Digital (Mike Audi

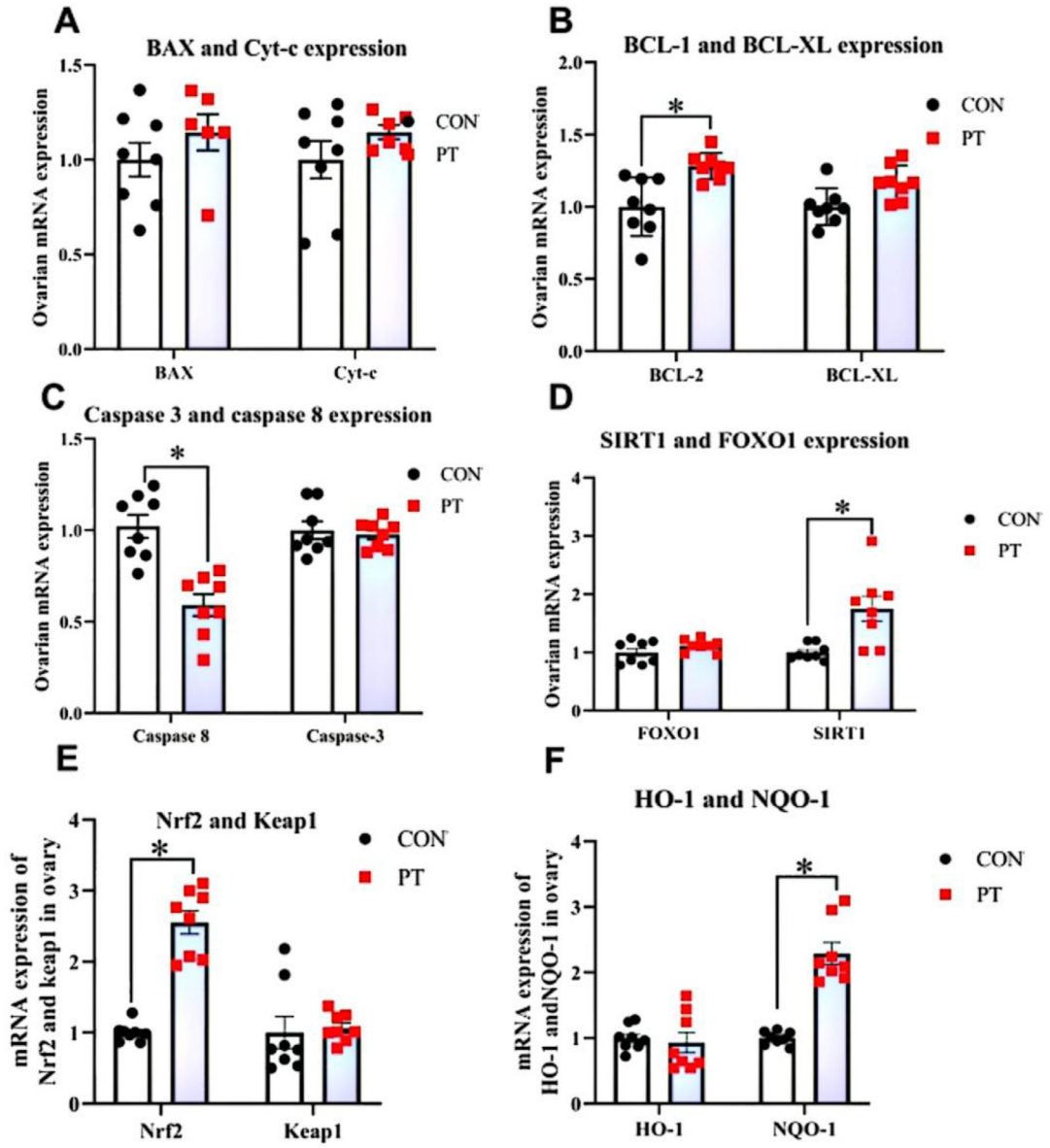


Figure 5. Dietary theabrownin supplementation improved ovarian function. Data are means and SEM represented by vertical bars or plot individual values. (A−C) The mRNA expression of apoptosis related genes (*BAX, Cyt-c, BCL-2, BCL-XL, caspase 3* and *caspase 8*) in ovary. (D−F) The mRNA expression of antioxidant response signaling pathway related genes (*SIRT1, FOXO1, Nrf2, Keap1, HO-1* and *NQO-1*) in ovary. Abbreviations: CON, control; PT, 100 mg/kg theabrownin; BAX,; Cyt-c, cytochrome-c; BCL-2, B-cell lymphoma-2; FOXO1, forkhead box O1; SIRT1, sirtuin 1; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO-1, NAD(P)H:quinone oxidoreductase 1. Statistical significance was evaluated by the Independent-Samples t-test, * $P < 0.05$.

Industrial Group Co., Ltd., Xiamen, Fujian, China) for image acquisition. The diaminobenzidine reacted with the labeled sample to generate a light green signal, while blue-green signified nonapoptotic cells.

Statistical Analysis

Data were analyzed by Independent-Samples T-Test of SPSS 13.0 (SPSS 13.0 for Windows, SPSS, Inc., Chicago, IL). The results are presented as mean and SEM. Differences were considered significant at a probability value of ≤ 0.05.

RESULTS

Production Performance and Serum Hormone Concentration

It has shown that dietary supplementation with theabrownin increased egg weight and feed efficiency (lower FCR) than CON group in current study (Figure 1A-C, $P < 0.05$). Compared to CON group, layers fed with theabrownin had higher concentration of E2 of serum (Figure 1D-F; $P < 0.05$). The egg laying rate, serum serotonin, leptin, FSH, melatonin and AMH concentration didn't differ between two group ($P > 0.05$).

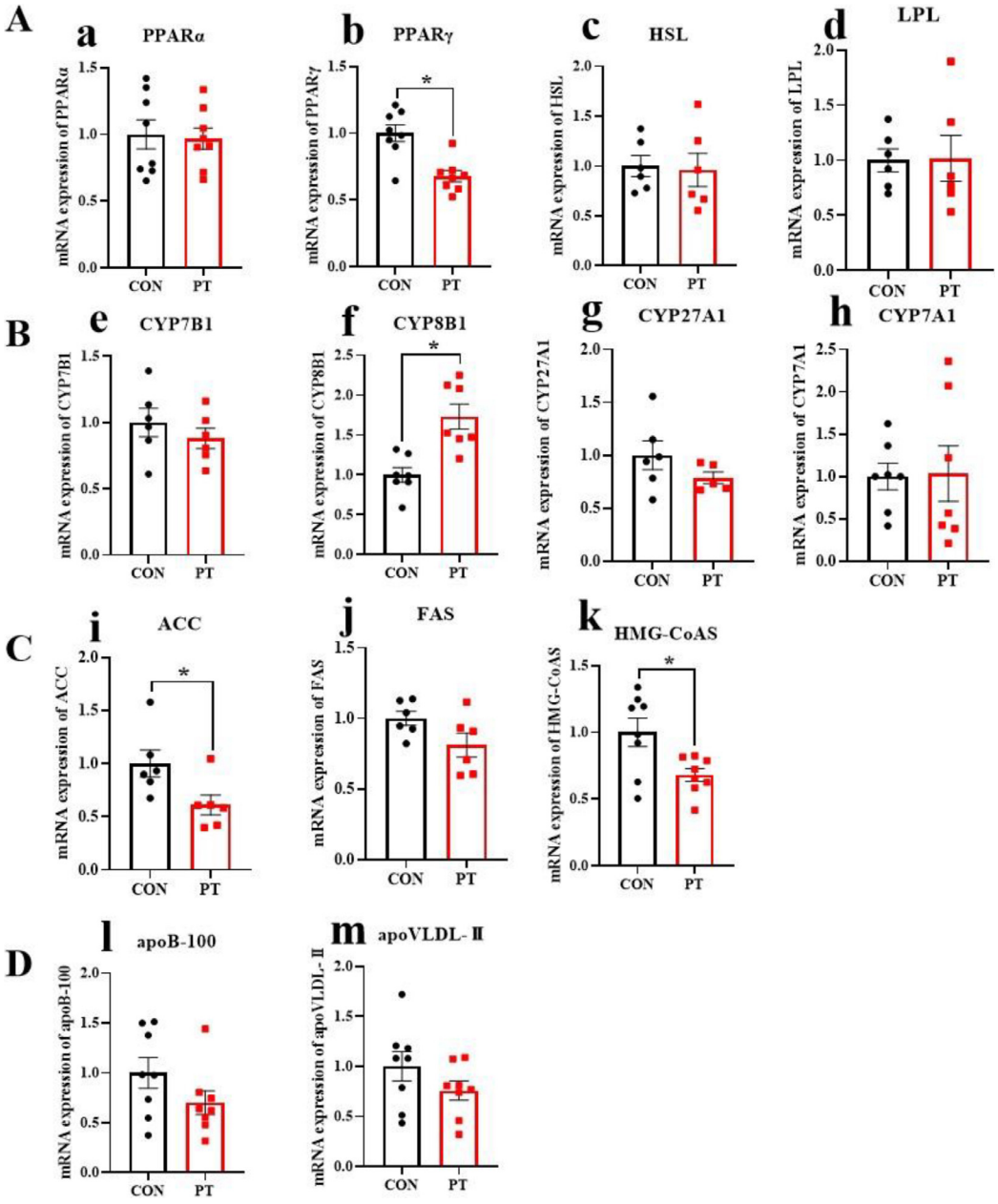


Figure 6. Effect of dietary theabrownin supplementation on expression of lipid metabolism related-genes in liver. Data are means and SEM represented by vertical bars or plot individual values. (A) The mRNA expressions of lipid oxygenolysis related-genes (*PPARα, PPARγ, HSL* and *LPL*) in liver. (B) The mRNA expressions of cholesterol decomposition related-genes (*CYP7B1, CYP8B1, CYP27A1,* and *CYP7A1*) in liver. (C) The mRNA expressions of lipid synthesis related-genes (*ACC, FAS,* and *HMG-CoA*S) in liver. (D) The mRNA expressions of lipid transportation related-genes (apoB-100 and apoVLDL-Ⅱ) in liver. Abbreviations: CON, control; PT, 100 mg/kg theabrownin. Statistical significance was evaluated by the Independent-Samples *t*-test, * $P < 0.05$.

Intestinal Morphology and Inflammatory Cytokines

Theabrownin were found to increase villus height of duodenum and decreased crypt depth of jejunum significantly (Figure 2; $P < 0.05$). Meanwhile, intervention of theabrownin in laying hens enhanced the ratio of villus height and crypt depth of duodenum as well as jejunum ($P < 0.05$). Nevertheless, dietary theabrownin supplementation did not influence the morphology of ileum ($P > 0.05$). Also, addition of theabrownin down-regulated gene expression and secretion levels of pro-inflammatory factors IL-1β and IL-6 in jejunum (Figures 3A−3D; $P < 0.05$).

Ovarian Function

Compared to CON group, the ovarian cell apoptosis rate were lower in PT group (Figure 4; $P < 0.05$), while PT group also up-regulated the anti-apoptosis factor *BCL-2,* and antioxidant capacity related genes *(SIRT1, Nrf2* and *NQO-1)* mRNA expression in ovary (Figures 5A−5F; $P < 0.05$).

Hepatic Lipid Metabolism

As shown in Figure 6, the mRNA expression of *PPARγ, HMG-CoAS,* and *ACC* were down-regulated in PT than in CON group ($P < 0.05$). On the other hand, the mRNA expression of *CYP8B1* was up-regulated by theabrownin intervention (Figure 6 $P < 0.01$). The addition of theabrownin significantly reduced the concentration of TC, TG and decreased lipid droplet optical density in liver (Figures 7A and 7B; $P < 0.01$).

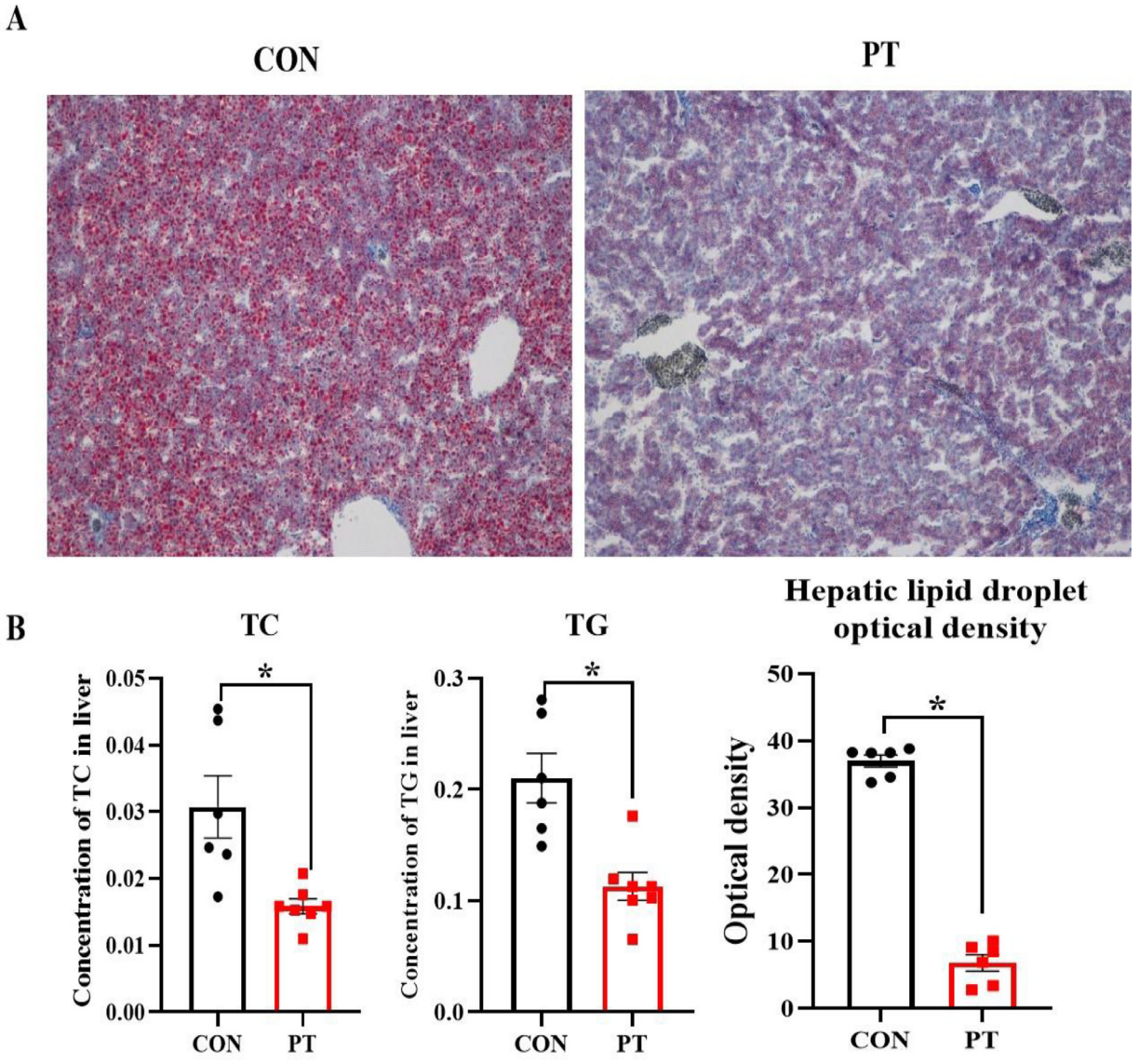


Figure 7. Effect of dietary theabrownin supplementation on lipid concentration in liver. Data are means and SEM represented by vertical bars or plot individual values. (A) The figures of oil red staining in liver. (B) The concentration of TC, TG and lipid droplet optical density in liver. Abbreviations: CON, control; PT, 100 mg/kg theabrownin; TC, total cholesterol; TG, total glycerol. Statistical significance was evaluated by the Independent-Samples t-test, * $P < 0.05$.

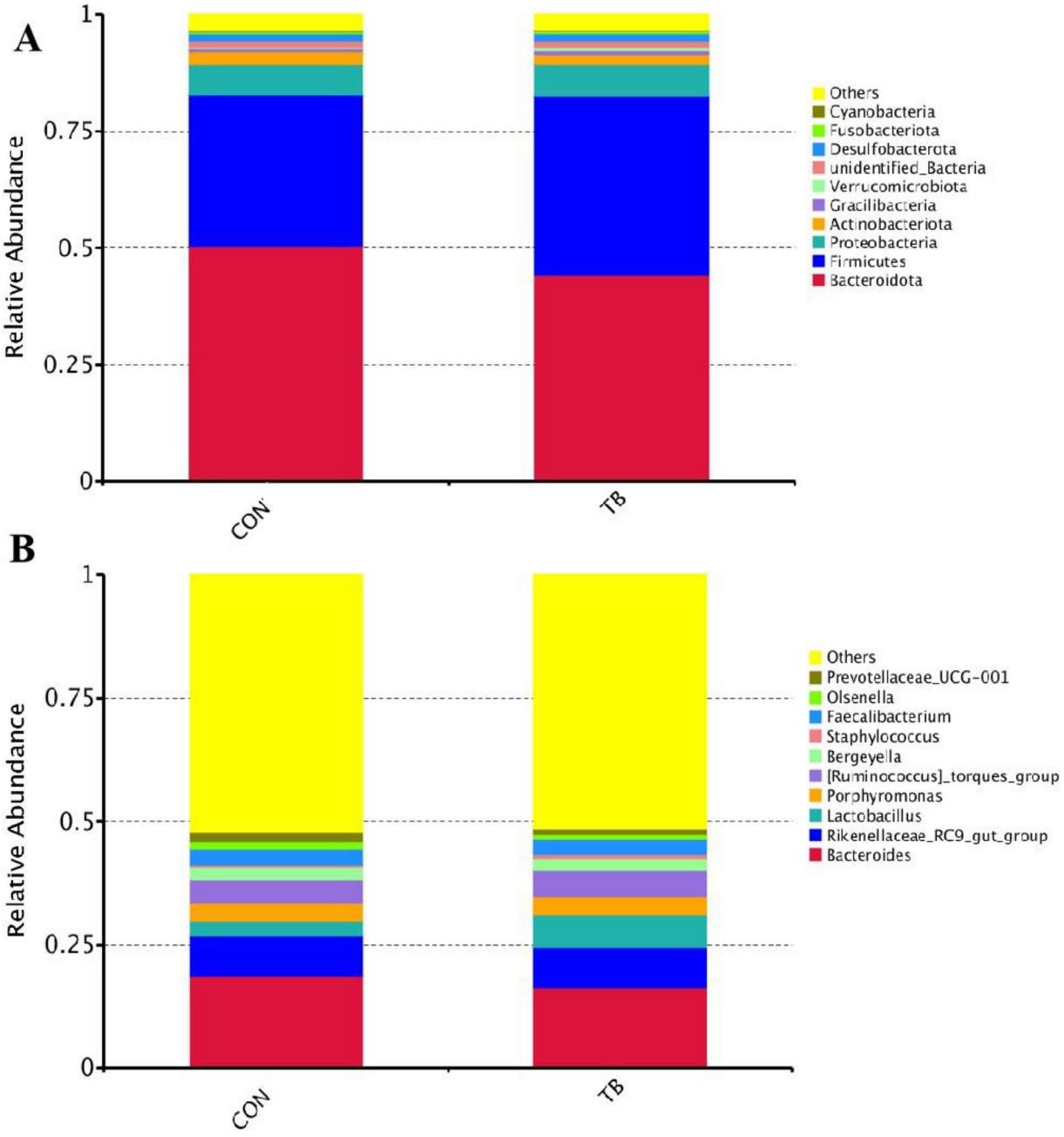


Figure 8. Dietary theabrownin supplementation changed microbiota diversity. (A) The relative abundance of the top 10 phylum, and (B) the relative abundance of the top 10 genus from samples. Abbreviations: CON, control; PT, 100 mg/kg theabrownin. Statistical significance was evaluated by the Independent-Samples t-test, * $P < 0.05$.

Cecum Microbiota

Theabrownin group induced changes in the community structure of cecum microbes in laying hens as revealed by the PCoA weighted UniFrac metric (Supplementary Figure 1; $P < 0.05$). The Observed species, ACE and Chao1indexes were increased significantly by PT intervention (Figure 9; $P < 0.05$). As analyzed by Adonis and LEfSe (Figure 10; log10 LDA > 3), PT intervention significantly increased the relative abundance of *Firmicutes* (phylum), *Desulfobacterota* (phylum), *Lactobacillus* (genus) and *Bacilli* (class), while it led to a reduction in the enrichment of *Bacteroides* (genus), *Prevotellaceae_ UCG-001* (genus), *Bacteroidetes* (phylum) (Figure 8 and 10; $P < 0.05$). The *Firmicutes* to *Bacteroidetes* ratio was higher in the PT group compared with the CON group ($P < 0.01$).

Serum Metabolomic Profile

In present study, in total 665 metabolites were identified; the layers in the PT group showed a remarkable metabolite alteration as compared to the CON group, as showed by PLSDA analysis (Figure 11A). We observed 25 metabolites were altered by theabrownin

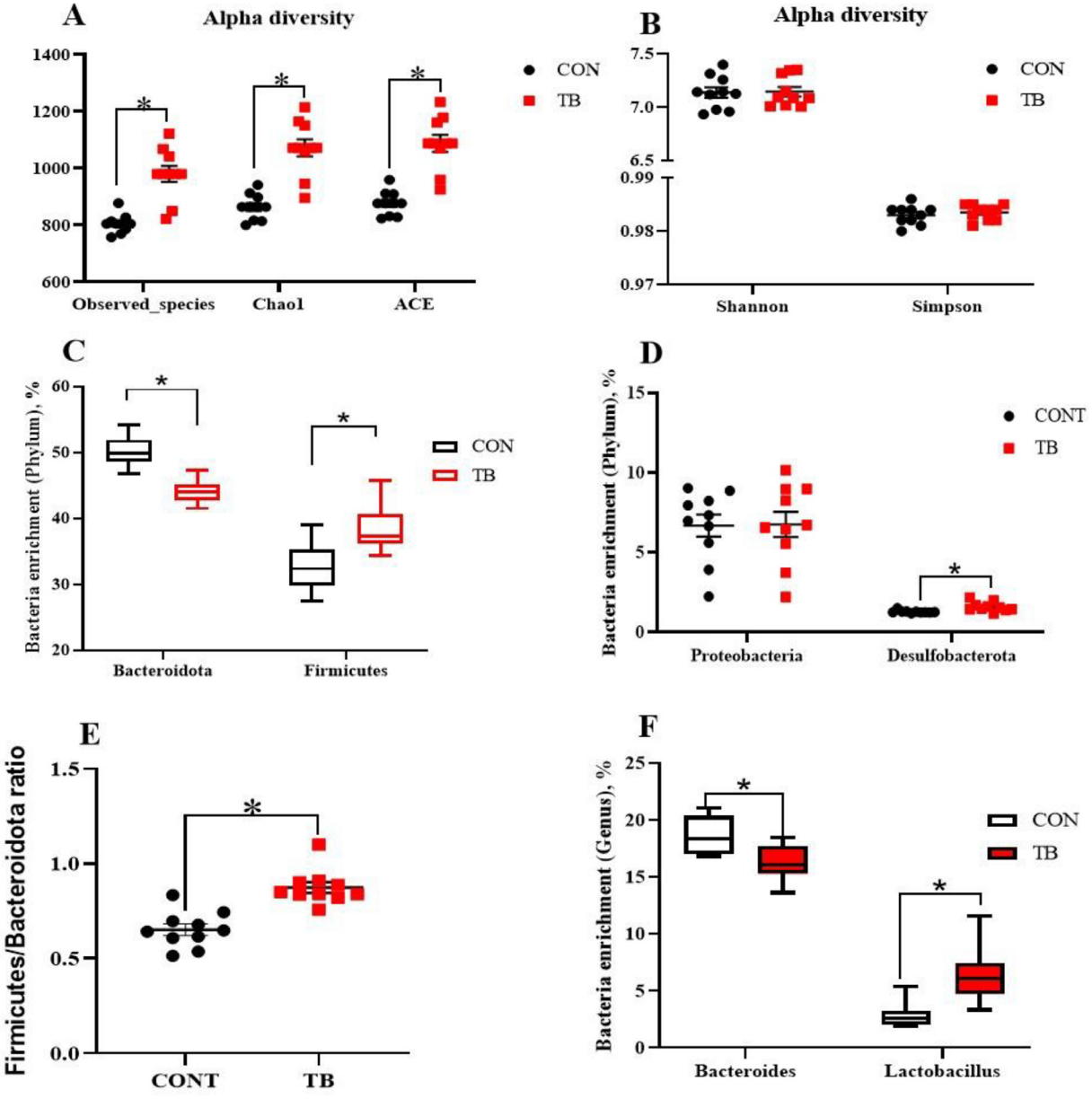


Figure 9. Dietary theabrownin supplementation changed microbiota diversity. Data are means and SEM represented by vertical bars or plot individual values. (A, B) Alpha diversity of cecum microbiota, with Observed species, Chao 1, ACE (A) and Shannon and Simpson index (B). (C −F) The relative abundance of microbiota in cecum, with Bacteroidota and Firmicutes in phylum (C), Proteobacteria and Desulfobacterota in phylum (D), Firmicutes/Bacteroidota ratio (E), Bacteroides and lactobacillus in genus (F). Abbreviations: CON, control; PT, 100 mg/kg theabrownin. Statistical significance was evaluated by the Independent-Samples *t*-test, * $P < 0.05$.

supplementation, which 10 of them were upregulated, and the rest 15 were down-regulated (Figure 11B; $P < 0.05$, VIP>1). The significantly altered metabolites were distributed as organic acids, benzoic acids, amino acids, hormones, bile acids, nucleotides, benzenes, and polyamines. The concentration of differential metabolites in the corresponding metabolic pathways of propanoate metabolism, purine metabolism, valine, leucine, and isoleucine metabolism, steroid hormone biosynthesis, ovarian steroidogenesis, steroid degradation, propanoate metabolism, progesterone androgen, and estrogen receptor agonists/antagonist, D-alanine metabolism, arachidonic acid metabolism (Figure 12, $P < 0.05$).

Correlation Between Microbiotas and Metabolites

Relationship between metabolites and microbiota with prominent differences between the two groups were acquired via Spearman's correlation analysis. As demonstrated in Figure 13, we discovered that the *Odoribacter, Parabacteroides, Succinatimonas, Lactobacillus, Anaerofustis, Escherichia-Shigella* and *Bacteroides* were most closely related to the changed metabolites in the serum of PT group ($P < 0.05$). Especially, bacteria of *Odoribacter* genera were negatively correlated to the quinoline-4 carboxylic acid, (R)-3-hydroxyisobutyric acid, testosterone and 2-phenylethylamine and positively correlated to the o-anisic

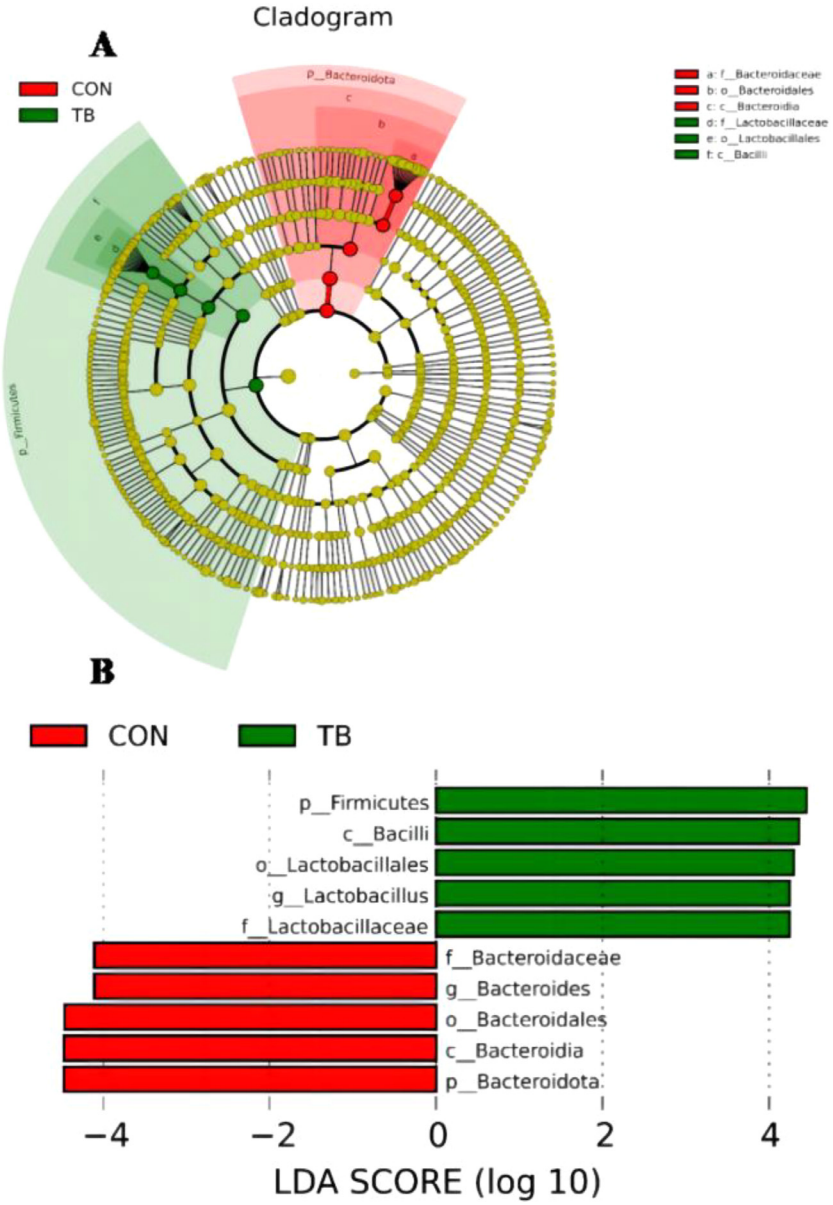


Figure 10. Dietary theabrownin supplementation changed microbiota enrichment. Data are means and SEM represented by vertical bars or plot individual values. (A, B) Linear discrimination analysis coupled with effect size (LEfSe) identified most differentially abundant taxa in the cecum with LDA significant threshold > 3 were shown. (Red) CON enriched taxa; (Green) PT enriched taxa. Abbreviations: CON, control; PT, 100 mg/kg theabrownin. Statistical significance was evaluated by the Independent-Samples *t*-test, $P < 0.05$.

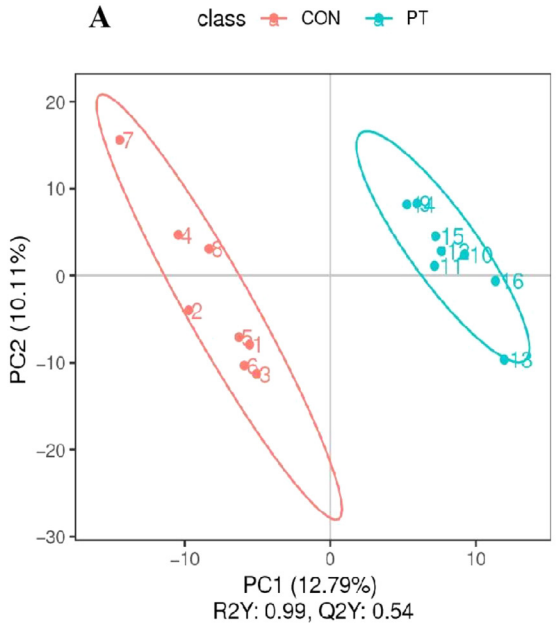
acid, dehydrocholic acid, 2-hydroxy-6-aminopurine, 11-ketoetiocholanolone, oxymetazoline, guanine, and 2-phenylglycine ($P < 0.05$). Meanwhile, the correlation between bacteria of *Bacteroides* and metabolites was similarly to *Odoribacter*. However, the bacteria of *Lactobacillus* genera were positively correlated to quinoline-4 carboxylic acid, (R)-3-hydroxyisobutyric acid, testosterone and 2-phenylethylamine and negatively correlated o-anisic acid, dehydrocholic acid, 2-hydroxy-6-aminopurine, 11-ketoetiocholanolone, oxymetazoline, guanine, and 2-phenylglycine ($P < 0.05$).

Correlations Between Cecum Microbiota and Egg Weight as well as Jejunum IL-6

The potential link between alterations of intestinal microbiota composition and the egg weight as well as jejunum expression of IL-6 in laying hens was assessed via Spearman correlation analysis (Figure 14). The genera *Lactobacillus, Akkermansia, UCG.005* and *Alistipes* were positively correlated to egg weight ($r = 0.47$, 0.49, 0.51, 0.48; $P < 0.05$), while genera *Fournierella, Prevotellaceae_ UCG.001, Olsenella* and *Bacteroides* were negatively correlated to egg weight (r = -0.48, -0.45, -0.58, -0.53; $P < 0.05$). At the same time, the genera *Lactobacillus* and *Akkermansia* were negatively correlated to jejunum expression of IL-6 (r = -0.54, -0.65), while genera *CHCK1001* and *Alloprevotella* were positively correlated to jejunum expression of IL-6 ($r = 0.49$, 0.55).

DISCUSSION

It has been widely accepted that intestinal track is a crucial part of digestion, absorption and immunity in



B

Serum different metabolites in PT group compared to controls

Name	VIP	FC	Up . Down
O-Anisic Acid	2.61	0.66	down
Prostaglandin G2	1.57	0.64	down
L-aspartic Acid	2.04	0.61	down
Guanine	1.88	0.47	down
2-Phenylglycine	1.82	0.48	down
Inosine	1.75	0.61	down
Dehydrocholic acid	1.96	0.56	down
2-Hydroxy-6-Aminopurine	1.94	0.49	down
Isobutyrylglycine	1.87	0.52	down
Oxymetazoline	1.87	0.54	down
2-Phenylethylamine	1.92	1.78	up
Aminomalonic acid	1.79	1.59	up
Citramalate	1.79	1.56	up
Quinic acid	1.78	1.62	up
Ethylmalonate	1.60	1.63	up
2-Hydroxybutyric acid	2.07	1.87	up
L-Threonine	2.00	1.66	up
Testosterone	1.95	1.57	up

Figure 11. Dietary theabrownin supplementation changed the serum metabolites. (A) The principal component analysis (PCA) of the serum metabolites. (B) Serum different metabolites in PT group compared to controls, $P < 0.05$. Abbreviations: CON, control; PT, 100 mg/kg theabrownin.

the body and first parclose against the invasion of germs. The villus height, crypt depth and V/C are vital indexes of intestinal absorption. In present study, we observed that the villus height of duodenum was enhanced and crypt depth of jejunum was decreased, meanwhile, the ratio of V/C of duodenum and jejunum were increased via theabrownin intervention. In the meantime, we found that PT decreased the pro-inflammatory factors (TNF-α, IL-1β, IL-6) expressions in jejunum. A previous study showed that the mRNA expression of IL-6 and TNF-α were higher in jejunum of low performance breeders (Mao et al., 2021). In this study, dietary PT supplementation significantly decreased the concentration IL-1β and IL-6 in jejunum.

Lipid metabolism mainly carried out in the liver, which was very important for laying hen due to the egg formation (Zhang et al., 2022). ACC and FAS play key roles during lipid synthesis, meanwhile, LPL and HSL

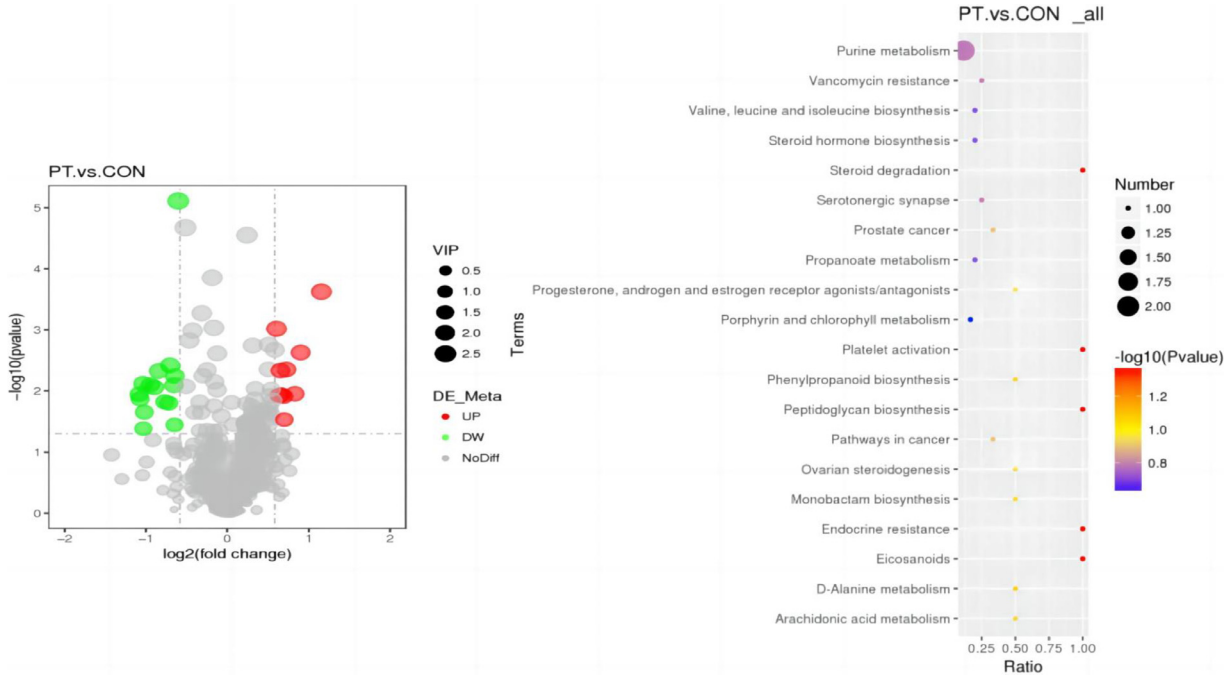


Figure 12. KEGG pathway enrichment of target metabolites by dietary theabrownin supplementation. (A) Description of the different metabolites between PT and CON group by volcano plot. (B) KEGG pathway enrichment of target metabolites. Abbreviations: CON, control; PT, 100 mg/kg theabrownin.

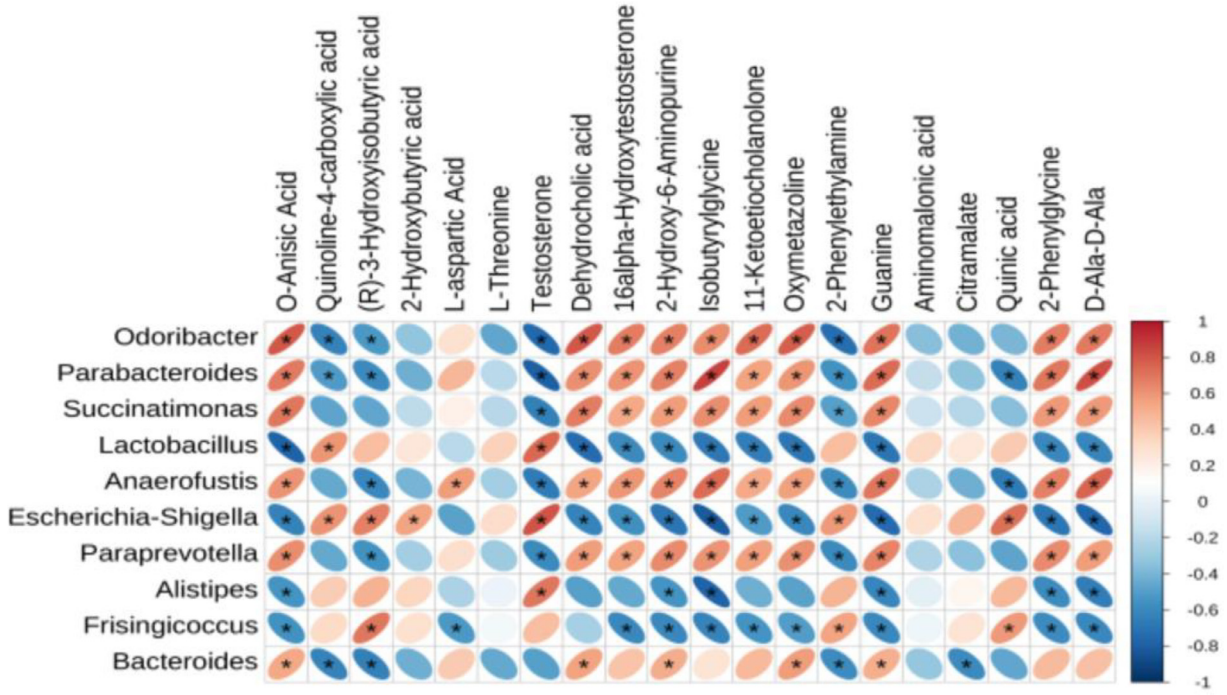


Figure 13. Spearsman correlation between metabolites and microbiota, $^{*}p < 0.05$ and $^{**}p < 0.01$. Abbreviations: CON, control; PT, 100 mg/kg theabrownin.

play key roles during lipid decomposition. Study had shown that in the process of fatty liver hemorrhage syndrome formation, the expression of HMG-CoAS and PPARγ was up-regulated, while the expression of PPARα was down-regulated (Chen, 2013). At the same time, apoB-100 and apoVLDL-Ⅱ play an important role in lipid transportation of laying hens. It had been demonstrated that theabrownin reduces TC levels in liver via increasing bile acids synthesis in the alternative pathway and reverse transporting of TC into liver (Yue et al., 2019; Kuang et al., 2020). In this experiment, dietary PT significantly down-regulated the expression of UCP-1, HMG-CoAS, ACC and PPARγ and up-regulated the expression of CYP8B1 in liver. In the meantime, dietary theabrownin significantly reduced the concentration of TC, TG and decreased the lipid droplet optical density in liver. These results manifested that theabrownin decreases the lipid synthesis by reducing the expression of lipid-forming signals HMG-CoAS, and PPARγ and the key factor of lipid synthesis regulation ACC in liver. Meanwhile, theabrownin reduces the concentration of TC in liver by increasing the bile acids synthesis in the classic pathway.

Hormones play a vital role in the regulation of ovarian growth. It had been proved that FSH and LH have the effect of regulating the growth, development and maturation of the ovary, promoting the secretion of E2 in the ovary and causing ovulation (Lewis et al., 2005). Meanwhile, the Bcl-2 family is including anti-apoptotic (*Bcl-2* and *Bcl-xl*) and pro-apoptotic (*Bax* and *Bak*) factors, whose play a crucial role in the regulation of follicle atresia and granulosa cell apoptosis (Matsuda et al., 2012). In our experiment, addition of theabrownin significantly increased the concentration of E2 in serum and decreased the cell apoptosis rate in ovary. Estradiol and FSH are known to activate antioxidant defense systems scavenging ROS in many organs and systems, especially in the ovary (Shen et al., 2017). As observed in current study, the mRNA expression of antiapoptotic-related gene Bcl-2 and antioxidant capacity related genes expression (*SIRT1, Nrf2* and NQO-1) in ovary were up-regulated by PT supplementation. In our previous study, we have indicated that SIRT1 and Nrf2 were found to involved in the aging or stress-related antioxidant capability in lay hens (Wang et al., 2022). These may also explain a better egg weight in PT group in present study and also proved in our previous study (Zhang et al., 2022).

It had been demonstrated that intestinal microbiotas were vital for maintaining gastrointestinal and immune function and normal digestion of nutrients (Neu et al., 2007; Zhang et al., 2022). Microbial balance is critical for nutrient digestion, absorption and utilization in poultry (Nguyen et al., 2022). There were previous studies showed that anaerobic bacteria such as *Lactobacillus* could improve intestinal barrier function and intestinal mucosal immunity, therefore improving the production performance and egg quality in laying hens (Shimazu et al., 2012; Liu et al., 2023). In our experiment, the abundance of *Lactobacillus* in PT group was increased significantly compared to CON group. At the same time, the intestinal morphology was improved by PT intervention. The Firmicutes to Bacteroidetes ratio is an important biomarker of gastrointestinal functionality and can be used as an indicator of eubiosis conditions in the gastrointestinal tract (Pereira et al., 2016). Wang et al. (2021a) reported that oxidative stress could cause the disturbance of the microbial flora and decrease the

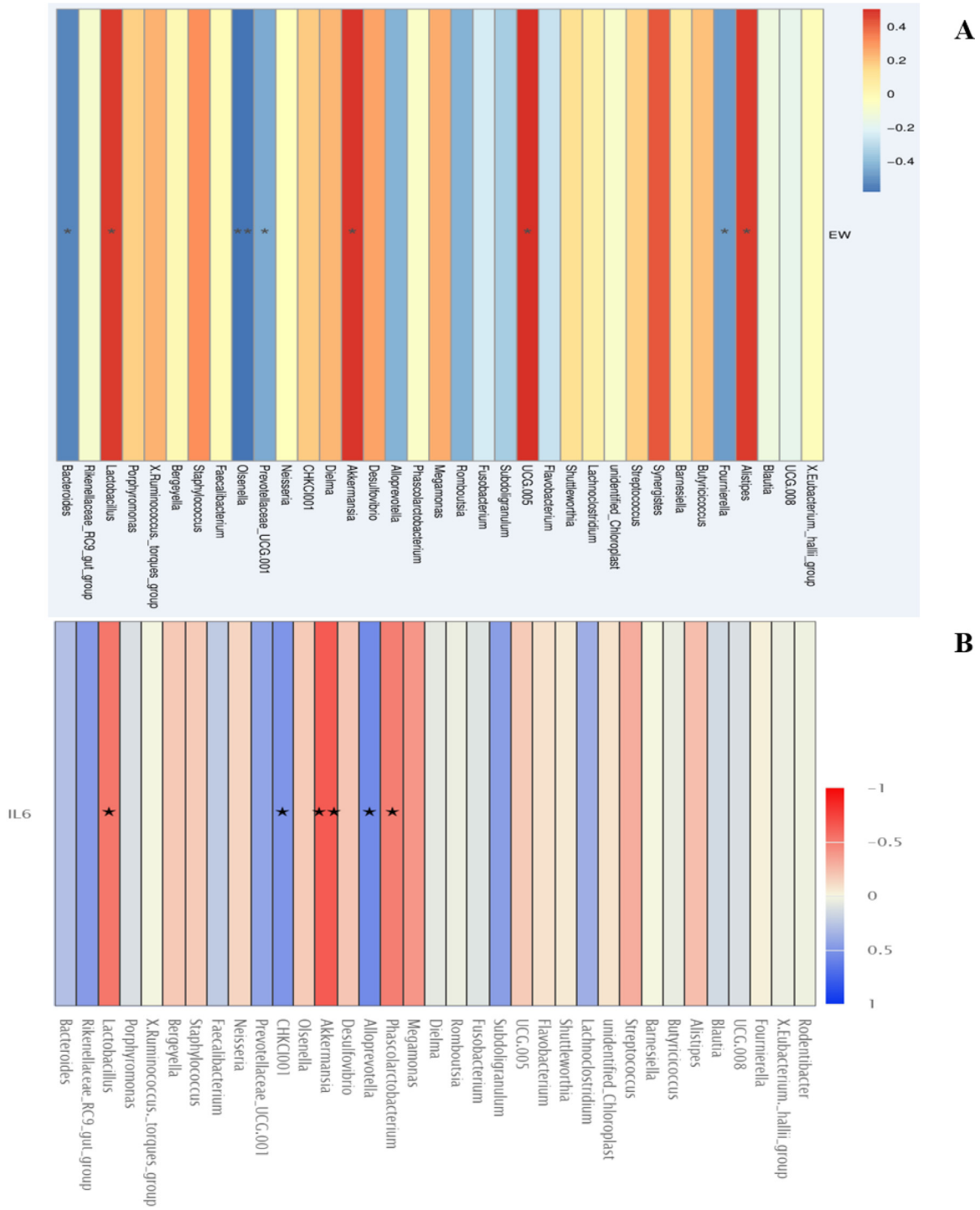


Figure 14. Heatmap of Spearman correlations between the gut microbiota significantly modified by different egg weight and IL-6 at genus level (Top 35). (A) Red indicates positive correlation, and blue indicates negative correlation. (B) Red indicates negative correlation, and blue indicates positive correlation. The color is darker, the correlation is higher. $^{*}P < 0.05$ and $^{**}P < 0.01$. Each mean represents 10 replicates, with a replicate. Abbreviations: CON, control; PT, 100 mg/kg theabrownin; EW, egg weight.

Firmicutes to *Bacteroidetes* ratio in laying hens. There were studies proved that *Firmicutes* participate in the metabolism of energy substances and play an important role in the digestion of food (Krajmalnik-Brown et al., 2012; Ma et al., 2018; Rowland et al., 2018). Our experimental results of cecal microbiota showed that there were decreased relative abundance of *Bacteroidetes* phylum as well as increased relative abundance of *Firmicutes* phylum and *Firmicutes* to *Bacteroidetes* ratio in laying hens with PT treatment. *Akkermansia*, one type of anaerobic and non-spore-forming oval bacterium, are connected with intestinal health. It had been demonstrated that increased *Akkermansia* intestinal numbers were effective in alleviating colitis (Earley et al. 2019; Wang et al. 2020). *Akkermansia* could protect intestinal health from diseases by modulating immunological barriers (Asquith et al. 2016; Ansaldo et al. 2019). In our experiment, the genera *Akkermansia* were positively

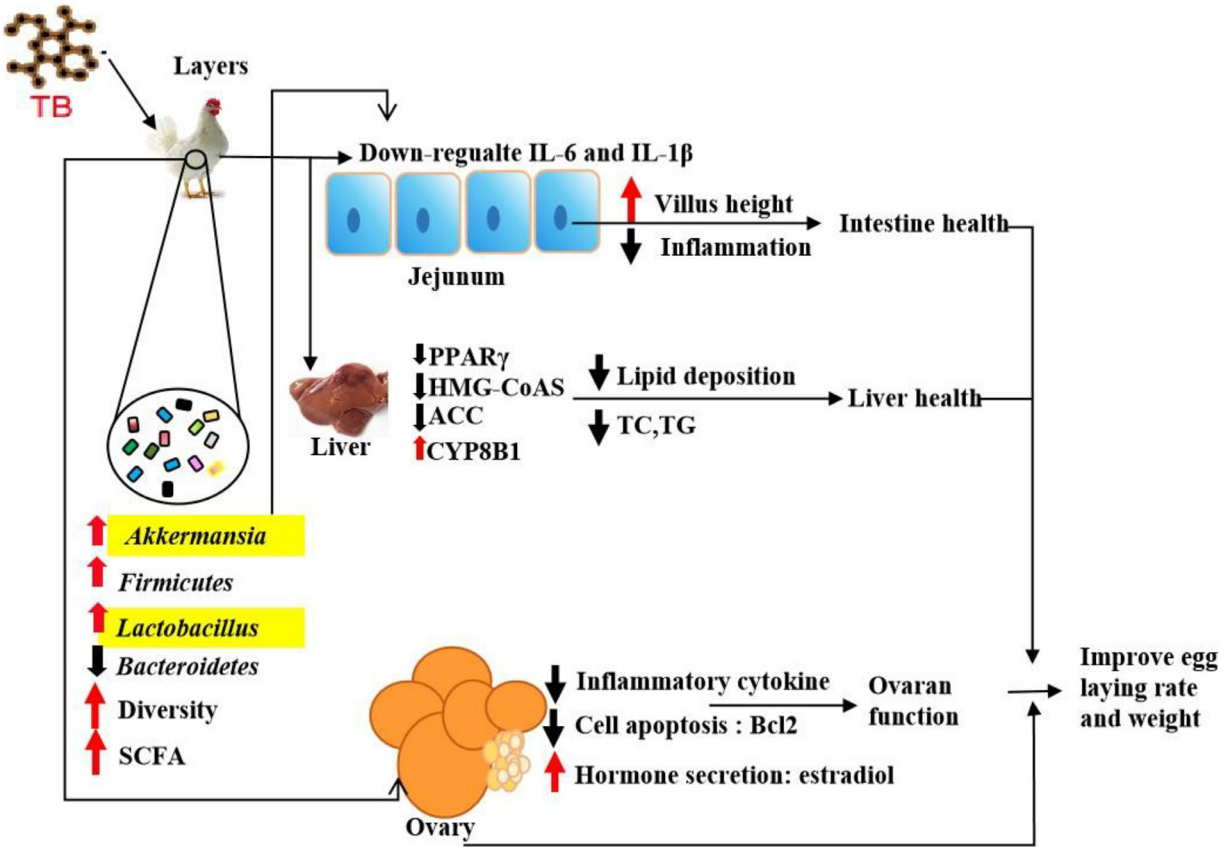


Figure 15. Proposed mechanism for theabrownin influence the health of laying hens. Theabrownin promoted intestinal health by simultaneous improvement of intestinal morphology, inhibit the expression of intestinal inflammatory cytokines, increase the relative abundance of *Firmicutes* and *Lactobacillus*, decrease the relative abundance of *Bacteroidetes*. At the same time, theabrownin and *Akkermansia* work together to inhibit intestinal inflammation. Theabrownin improved the ovarian function by promoting secretion of estradiol and mRNA expression of BCL-2 in ovary. In the meantime, theabrownin reduced lipid deposition by decreasing lipid synthesis and enhancing the bile acids synthesis in the classic pathway. Ultimately, egg weight of laying hens was increased via this mechanism.

correlated to egg weight and negatively correlated to expression of IL-6 in jejunum. Furthermore, intestinal health is connected with ovarian function. This result revealed that *Akkermansia* had positive influence to decrease intestinal inflammation and increase egg weight. Moreover, the mRNA expression of inflammatory cytokines was down-regulated in jejunum via PT intervention. Therefore, we hypothesize that addition of PT in diet is beneficial to *Akkermansia* and they work together to alleviate intestinal inflammation and promote intestinal health, ultimately, the health of laying hens was improved. It has been indicated that Pu'er tea extract can restore intestinal barrier function, maintaining gut microbiota, and reducing inflammation, thereby improving the intestinal health of animals in previous studies (Zhang et al., 2021; Zhou et al., 2023). Alpha diversity indices, including the Chao1, ACE, Simpson and Shannon index, were used to reflect the richness and uniformity of the samples. The larger the Chao1 or ACE index, the more abundant the relative abundance of the microflora. In present study, the PT group had higher observed species and greater Chao1 and ACE indexes than CON group. These results revealed that dietary PT supplementation suppressed the relative abundance of *Bacteroidetes* phylum and accelerated the relative abundances of *Firmicutes* phylum and *Lactobacillus* genera and so on, so that improved intestinal health and microorganism community richness of laying hens.

Organic acids, a sort of weak acids which have a carboxylic acid group (**R-COOH**), intermediates in the degradation pathways of carbohydrates, amino acids and fats, and have nutritional value and antimicrobial effects in animal diets (Caneschi et al., 2023). It has been proved that organic acids could maintain gut barrier cellular integrity, modulate intestinal microbiota, improve digestion and nutrient absorption rate and production performance (Nguyen and Kim, 2020; Dai et al., 2021). A previous study showed that supplementation of butyric acid and its salts could modulate gut microbiota and keep poultry intestinal health (Yadav and Jha, 2019). The concentration of organic acids such as (R)-3-hydroxyisobutyric acid, 2-hydroxybutyric acid, citramalate and quinoline-4-carboxylic acid were up-regulated by PT intervention. Meanwhile, the analysis of correlation between microbiotas and metabolites indicated that the bacteria of *Lactobacillus* genera were positively correlated to (R)-3-hydroxyisobutyric acid and quinoline-4-carboxylic. These results manifested that theabrownin increased the abundance of butyric acids-producing microbiotas, which in turn increased the concentration of butyric acid in laying hens and improved intestinal health.

CONCLUSIONS

Overall, our findings indicated that dietary theabrownin supplementation enhanced the intestinal function and influenced serum metabolism by improving intestinal morphology, microbiota community structure and reducing the concentration and expression of inflammatory cytokines. Dietary theabrownin reduced hepatic lipid deposition by decreasing the lipid synthesis and promoting lipid metabolism. Finally, the SIRT1-related apoptosis were found to involved in the process of improving ovarian function and egg weight in current study (Figure 15).

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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.2024.103795.

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