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RESEARCH ARTICLE

Effect of *Broussonetia papyrifera* Leaves on Serum Antioxidants and Metabolites in Broilers

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ABSTRACT

This study investigated the effects of dietary supplementation with different levels of *Broussonetia papyrifera* (BP) leave on growth performance, serum antioxidant status, and serum metabolites in broilers. A total of 240 one-day-old broilers were randomly allocated to four treatments with three replicates of 20 birds each in a single-factor design. Birds in the control group were fed a basal diet, while those in groups B1, B2, and B3 were fed diets in which soybean meal was replaced with 4, 8 and 12% BP leaves, respectively. After a 42-day feeding trial, blood samples were collected from the subclavian vein for serum analysis. Results showed that 4% BP leaves had no adverse effect on broiler growth performance and improved immune organ indices compared with the control, whereas higher inclusion levels reduced growth performance. The 4% BP leaf group exhibited significantly increased serum T-AOC, GSH-PX, and CAT levels. Both 4% and 8% BP leaf groups presented higher SOD activity and lower MDA content than the control. Overall, 4% and 8% BP leaf supplementation (B1, B2) exerted favorable antioxidant and anti-damage effects and were considered optimal levels. LC-MS/MS-based untargeted metabolomics revealed that α-ketoglutarate, L-lysine and other key differential metabolites were notably enriched in pathways including alanine, aspartate and glutamate metabolism, and the tricarboxylic acid cycle. Metabolic regulation was relatively weak in the 4% BP leaf group, whereas the 8% group showed the most significant pathway enrichment and optimal metabolic balance among energy generation, amino acid utilization, and antioxidant capacity. Conversely, metabolic pathway disturbance was observed in the 12% group.

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INTRODUCTION

The search for safe, sustainable, and high-quality alternative protein feedstuffs has become a key research priority in modern broiler production, especially with the phasing out of antibiotic growth promoters and rising pressures on conventional protein sources such as soybean meal (Park *et al*., 2018; Altmann *et al*., 2020; Onbaşılar *et al*., 2023). Woody feed resources are widely distributed and rich in protein and phytochemicals, making them promising candidates to partially replace traditional protein feeds and improve animal health and metabolic status (El-Aziz *et al*., 2025; Wang *et al*., 2025; Ma *et al*., 2026).

Broussonetia papyrifera (BP), a fast-growing deciduous tree of the Moraceae family, is abundant in East and Southeast Asia and has been officially listed as a feed raw material in China (Hao *et al*., 2020). BP leaves are characterized by high crude protein content, balanced amino acid profiles, and rich bioactive components including flavonoids, polyphenols, and polysaccharides (Han *et al*., 2016; Jiang *et al*., 2025; Zheng *et al*., 2025). These components endow BP leaves with notable antioxidant, anti-inflammatory, and immunomodulatory functions, which can enhance redox balance and regulate nutrient metabolism in livestock and poultry (Guo *et al*., 2023; Zhang *et al*., 2021; Xu *et al*., 2022).

Previous studies have demonstrated that moderate dietary inclusion of BP leaves can improve growth performance, meat quality, and intestinal health in broilers (Liang *et al*., 2023; Niu *et al*., 2023; Liang *et al*., 2026). However, high levels of BP leaves may reduce growth efficiency due to elevated fiber and anti-nutritional factors (Fang *et al*., 2023; Dong *et al*., 2025). However, studies investigating the antioxidant properties and metabolic influence of BP leaves supplementation in broilers are scarce.

Therefore, the present study evaluated the effects of graded levels of BP leaves (replacing soybean meal) on growth performance, serum antioxidant enzyme activities and serum metabolomics in broilers. The results aim to clarify the optimal dietary inclusion rate of BP leaves and reveal its underlying metabolic regulatory mechanisms, providing a scientific basis for the rational application of BP leaves in broiler feed formulations.

MATERIALS AND METHODS

Ethical statement: The Animal Ethics Committee of Yunnan Agricultural University, China approved all protocols involving animals (Approval No. 202103032).

Experimental design, diet composition and bird management: BP leaves were obtained from Hebei Changjiu Chinese Medicinal Materials Co., Ltd. and Arbor Acres broilers were purchased from Zhumadian Jintaiyang Animal Husbandry Co., Ltd. In a single-factor design, 240 one-day broilers were allocated randomly to four groups, each group with three replicates of 20 broilers. Broilers were raised in a three-tier cage system with ad libitum access to feed and water and routine vaccinations were applied. The feeding trial lasted 42 days. Diets were formulated according to the national broiler feeding standard GB/T 5916-2020 and divided into two phases: 1-21 d and 22-42 d. All diets were iso-caloric and iso-nitrogenous. Group C was fed a basal diet, while Groups B1, B2 and B3 were fed diets with soybean meal replaced by 4, 8 and 12% *BP* leaves, respectively. The dietary composition and nutrient levels (Table 1).

Growth performance: Broilers were fed and water was provided *ad-libitum* during the experiment. The values of final body weight (FW), total weight (TW), average daily feed intake (ADFI), average daily gain (ADG) and feed conversion ratio (F/G) were computed for the stage of 1-21 d, 22-42 d and 1-42 d. A 24-h lighting scheme was applied (6 W/m² for the first 3 d, gradually reduced to 1.5 W/m²). Environmental conditions were strictly controlled: 30-35°C (decreasing 2-3°C weekly) and 60-70% RH for 1-21 d; 20-25°C and 50-60% RH for 22-42 d. Ventilation, cleaning, and disinfection were performed routinely. All groups were raised under environmentally controlled management conditions.

Table 1: Ingredients and chemical composition of the experimental basal diets

Ingredients (%)	1-21 days				22-42 days			
	C	B_1	B_2	B_3	C	B_1	B_2	B_3
Corn	53.7	53.4	51.5	52.4	40.1	39.9	40.5	39.5
Wheat bran	7.00	5.27	5.00	1.47	26.2	22.7	20.6	19.9
Soybean meal	27.5	23.6	19.6	15.6	23.2	19.2	15.2	11.2
Corn gluten meal	5.00	7.18	9.39	11.9	3.51	7.10	8.44	10.6
BP Leaves powder	0.00	4	8	12	0.00	4.00	8	12
Calcium hydro-phosphate	3.02	2.98	2.86	2.84	2.01	2.17	2.31	2.14
Soybean oil	0.10	0.00	0.00	0.00	0.53	1.01	0.74	0.41
Limestone powder	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Lysine	0.28	0.32	0.38	0.44	0.35	0.25	0.44	0.50
Methionine	0.31	0.29	0.32	0.29	0.51	0.45	0.70	0.70
Threonine	0.07	0.06	0.05	0.07	0.60	0.29	0.08	0.07
Sodium chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
*Premixed	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Total	100	100	100	100	100	100	100	100
Formulated Nutrients (%)								
Metabolic energy (MJ/kg)	13.1	13.1	13.1	13.1	12.4	12.3	12.3	12.3
Crude protein	21.23	21.15	21.19	21.15	20.32	20.77	20.23	20.3
Calcium	0.82	0.82	0.84	0.88	0.78	1.05	1.02	0.9
Phosphorus	0.63	0.63	0.62	0.62	0.48	0.51	0.54	0.52
Lysine	1.21	1.21	1.22	1.23	1.18	1.05	1.18	1.20
Methionine	0.65	0.63	0.66	0.65	0.81	0.78	1.02	1.02
Threonine	0.84	0.84	0.83	0.85	1.32	1.02	0.81	0.80
Crude fiber	3.56	3.73	3.91	4.03	4.72	4.74	4.91	5.15

*The premix provided; VA 6500 IU, VD_3 1625 IU, VE 8 IU, VK 0.8 mg, VB_1 2 mg, VB_{12} 0.08 mg, VB_9 0.4 mg, VB_7 0.04 mg, VB_3 8 mg, VB_5 4 mg, Mn 57.5 mg, Cu 9 mg, Fe 80 mg, Zn 70 mg, Se 0.15 mg, I 0.35 mg.

Antioxidant activity: After six weeks of feeding, one broiler was randomly chosen from each replicate for blood sample via venipuncture to prepare serum. Blood was drawn into 10 mL clot activator vacuum tubes (Sinopharm Group Co., Ltd., Beijing, China). Assay kits from Nanjing Jiancheng Bioengineering Institute (China) were employed to evaluate serum antioxidant indices: total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT).

Metabolic pathways: Metabolites extraction reagents used during the experiment included methanol (Thermo Fisher Scientific Inc.), acetonitrile (Thermo Fisher Scientific Inc.), 2-chloro-L-phenylalanine (Shanghai Aladdin Biochemical Technology Co., Ltd.), formic acid (Tokyo Chemical Industry Co., Ltd.), ammonium formate (Sigma-Aldrich Corporation), and H_2O (Millipore Corporation). The metabolite extraction instruments used in the experiment included a refrigerated centrifuge (Xiangyi H1850-R), a vortex mixer (BE-2600), a vacuum concentrator (5305) and filter membranes (0.22μm Polytetrafluoroethylene).

Non-targeted metabolomics and metabolic pathways: The original mass spectrometry data were transformed into mz XML format with the MS Convert tool from the ProteoWizard software (version 3.0.8789). Utilizing the R XCMS software, a data matrix containing metabolite characteristic information was obtained. Subsequently, normalization processing was conducted to generate standardized data suitable for comparative analysis. Multivariate statistical analysis was performed in R language, and metabolites with significant differences were screened using the threshold of P<0.05 and VIP>1. Metabolic pathways were identified on the KEGG database（https://www.kegg.jp/）.

Statistical analysis: Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test in IBM SPSS Statistics 27.0. During the experiment, chemometric principles and two multivariate statistical analysis methods, PCA analysis and PLS-DA analysis (using the Ropls package in R language) were applied to the metabolomics data analysis to perform dimensionality reduction and classification analysis on the collected multidimensional data.

RESULTS

Effect of BP leaves levels on the growth performance of broilers: As shown in Table 2, from 1-21 days, FW, ADG and ADFI of broilers in B1 group did not differ significantly compared with group C (P<0.05). With increasing added levels of BP leaves, FW, ADG and ADFI of broilers gradually decreased (P<0.05). The F/G of B3 group was increased significantly (P<0.05). From 21-42 days, FW, ADG and ADFI of broilers for B1 group have significant difference compared with C group (P<0.05). FW and ADG of group B3 were lower (P<0.05) while F/G was higher than other groups (P<0.05). With increasing added levels of BP leaves, FW, ADG and ADFI decreased, and F/G increased significantly (P<0.05).

Table 2: Effect of *BP* Leaves Supplementation in Feed on the Growth Performance and immune organ index of Broilers

Parameters	Level of BP Leaves Supplementation			
	C (0%)	B1 (4%)	B2 (8%)	B3 (12%)
1-21d				
Initial weight (g)	39.47±0.29	39.25±0.23	39.44±0.14	39.74±0.17
FW (g)	494.58±10.04^{a}	485.01±9.70^{ab}	468.76±9.69^{b}	430.92±4.87^{c}
ADG (g/d)	21.67±0.49^{a}	21.23±0.46^{ab}	20.44±0.46^{b}	18.63±0.24^{c}
ADFI (g/d)	31.80±0.53^{a}	31.44±0.19^{a}	30.33±1.00^{b}	29.49±0.15^{b}
F/G	1.47±0.04^{b}	1.48±0.22^{b}	1.48±0.24^{b}	1.58±0.24^{a}
22-42 d				
FW (g)	6.76±22.81^{a}	2288.65±16.48^{a}	8.36±22.98^{b}	860.01±41.29^{c}
ADG (g/d)	86.85±1.08^{a}	85.88±0.78^{a}	76.17±1.09^{b}	68.07±1.97^{c}
ADFI (g/d)	146.88±3.05^{ab}	149.93±4.39^{a}	142.12±1.13^{b}	140.22±3.30^{b}
F/G	1.70±0.04^{c}	1.75±0.06^{c}	1.86±0.02^{b}	2.06±0.04^{a}
1-42 d				
TW (g)	7.28±22.93^{a}	2249.40±16.64^{a}	028.92±22.87^{b}	820.28±41.42^{c}
ADG (g/d)	53.98±0.54^{a}	53.56±0.41^{a}	48.31±0.54^{b}	43.33±0.99^{c}
ADFI (g/d)	89.34±1.39^{ab}	90.68±2.13^{a}	86.24±0.57^{bc}	84.75±0.87^{c}
F/G	1.66±0.03^{c}	1.69±0.05^{c}	1.79±0.02^{b}	1.96±0.03^{a}

Values with different superscripts in the same row differ significantly (P<0.05), while those without any mark indicate non-significant differences (P>0.05).C=control; B_1=4% BP leaves; B_2=8% *BP* leaves; B_3=12% *BP* leaves.

The FW, ADG and ADFI of AA broilers in B1 group did not differ significantly compared with C group (P>0.05) from 1-42 days. TW and ADG of group B3 were lower while F/G was higher than other groups (P<0.05). With increasing added levels of BP leaves TW, ADG and ADFI decreased, F/G increased significantly (P<0.05), Whereas ADFI showed an initial increase followed by a decrease.

According to Table 3, liver index and Thymus index of B1 group were higher than C group (P<0.05), B1 and B2 group significantly increased Thymus index (P<0.05) spleen index of the B2 group was significantly higher than that of the other groups. Appropriate addition can increase the immune organ index.

Table 3: Immune organ index of each treatment group at 42 days of age

Organs index	C	B1	B2	B3
Liver index (g/kg)	36.36±1.56^{b}	41.78±1.67^{a}	38.82±1.35^{b}	25.25±0.42^{c}
Bursa of Fabricius	3.04±0.18^{c}	4.24±0.04^{b}	5.19±0.28^{a}	2.81±0.19^{c}
Thymus index (g/kg)	2.26±0.14^{b}	3.34±0.29^{a}	1.95±0.06^{bc}	1.83±0.11^{c}
Spleen index (g/kg)	1.46±0.12^{b}	1.72±0.08^{a}	1.39±0.06^{b}	1.33±0.29^{b}

Different letters within the same row indicate significant differences (P<0.05), while those without any mark indicate non-significant differences (P>0.05). C=control; B1=4% BP; B2=8% BP; B3=12% BP

Effect of BP leaves levels on the antioxidants' activity of broilers: Serum Antioxidant Capacity can be seen from Fig. 1. Supplementation with BP leaves significantly affected antioxidant indicators (P<0.05). Group B1 significantly increased the contents of T-AOC, GSH-PX, and CAT (P<0.05). Groups B1 and B2 significantly increased the content of SOD (P<0.05). The addition level of BP leaves in groups B1 and B2 significantly decreased the content of MDA (P<0.05). Overall, the addition levels of B1 and B2 are preferable, and the BP leaf addition groups exhibit significant antioxidant and anti-damage effects.

Effect of BP leaves levels on the non-targeted metabolomics and metabolic pathways of broilers

Data quality control and reliability assessment: In this study, non-targeted metabolomic analysis based on LC-MS/MS was performed to acquire metabolite signals from 12 serum samples of white-feathered broilers under both positive and negative ionization modes. Raw data were preprocessed by format conversion using ProteoWizard, peak identification and alignment with XCMS, and normalization to the total peak area, followed by systematic quality control.

The base peak chromatograms (BPC) showed symmetric chromatographic peaks, stable retention times, and highly consistent peak profiles among groups, indicating good repeatability of sample preparation and instrumental analysis (Fig. 2). Principal component analysis (PCA) score plots revealed that quality control (QC) samples clustered tightly, and all biological samples fell within the 95% Hotelling's T^2 confidence ellipse with no outliers, demonstrating excellent overall data stability (Fig. 3). Quality assurance results showed that more than 70% of metabolic features in QC samples had relative standard deviations (RSD) < 30%, meeting the quality control criteria for non-targeted metabolomics. Thus, the dataset was reliable and suitable for subsequent statistical analysis.

Multivariate statistical analysis: Supervised OPLS-DA was performed on the normalized data to filter out orthogonal variation unrelated to grouping and improve the resolution of inter-group differences. Model fitting indicators showed that R^2Y=0.999, indicating an extremely high degree of explanation for inter-group variation by the model. Q^2 ranged from 0.4 to 0.5, suggesting relatively limited predictive power of the model, which may be attributed to the small sample size or large within-group biological variation. The model was

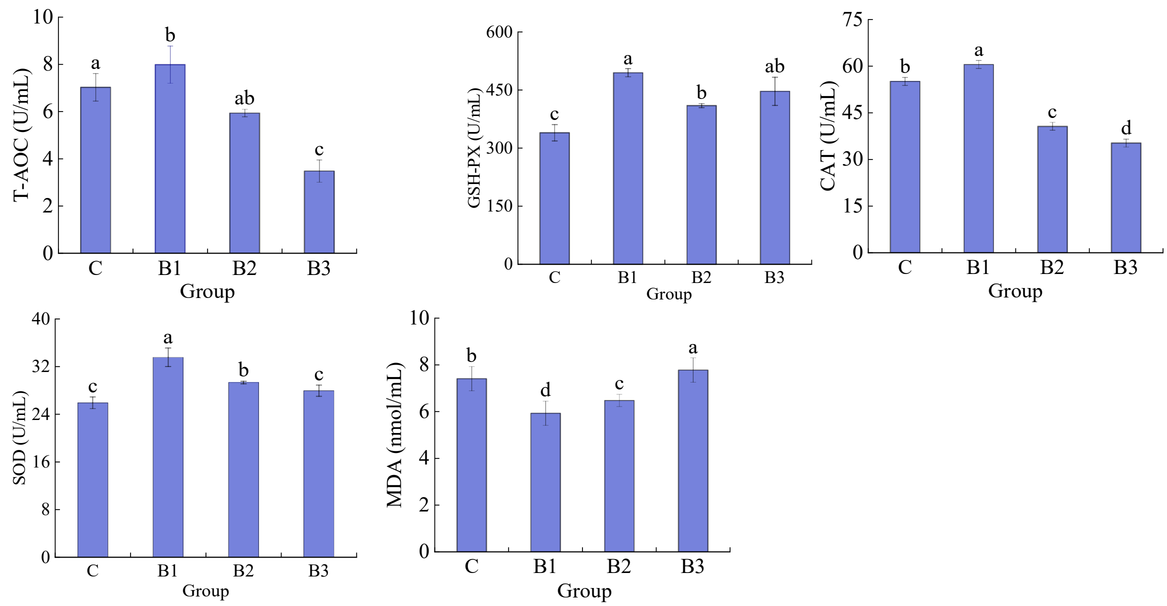

Fig. 1: Effect of levels of BP leaves on the antioxidant function of broilers. Note: With different letters were significantly different (P<0.05), while those without any mark indicated non-significant differences (P>0.05).

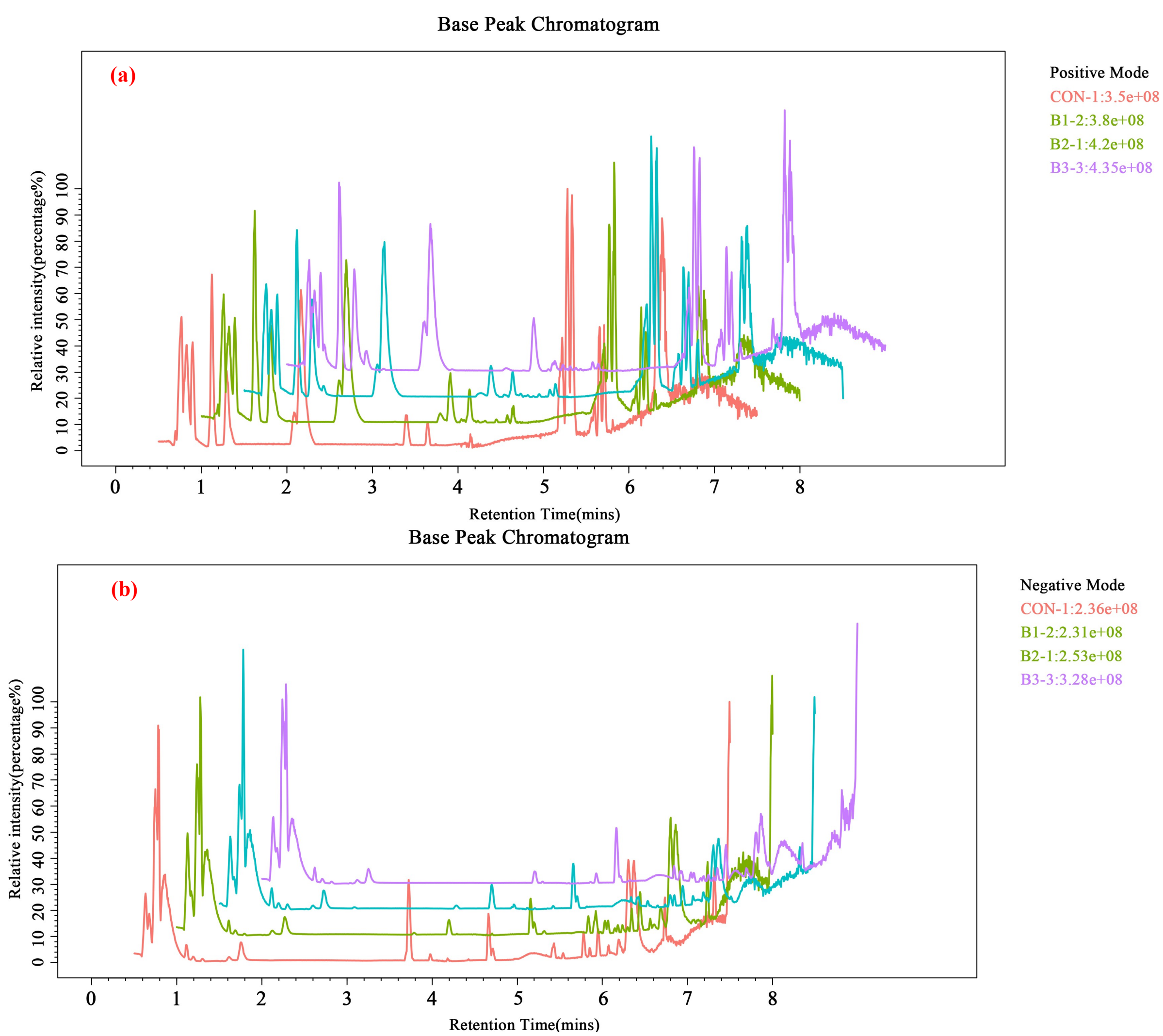

Fig. 2: Base peak chromatogram of a representative sample; a: Positive ion mode; b: Negative ion mode.

verified to be free of overfitting by 200 permutation tests (Fig. 4, 5). Metabolites with variable importance in the projection (VIP) > 1 were extracted based on the OPLS-DA model and used as the candidate set for subsequent differential metabolites.

Screening and identification of differential metabolites: Pairwise comparisons were conducted among all groups to screen for significantly differential metabolites, using the criteria of VIP>1 and P<0.05. Volcano plots intuitively illustrated the distribution of significant differential metabolites in each group, with a clear distinction between upregulated and downregulated metabolites (Fig. 6). Structural annotation was performed by matching accurate molecular weights (mass error<30 ppm) and comparing MS/MS fragmentation patterns against the HMDB, MassBank, LipidMaps and mzCloud databases. After excluding exogenous drugs, artificial compounds, and low-confidence substances, several endogenous differential metabolites were finally identified, including α-ketoglutarate, L-lysine, benzoyl-CoA, 3-chlorocatechol, and pyrazinemethanethiol.

Venn diagram analysis demonstrated that differential metabolites across different comparison groups possessed both shared and unique features (Fig. 7). Specifically, 11 common metabolites were detected, suggesting that different inclusion levels of BP leaves exert conserved regulatory effects on broiler metabolism. Additionally, 46, 60, and 65 unique metabolites were identified in each group, respectively, indicating that different dietary addition levels also exert specific regulatory effects.

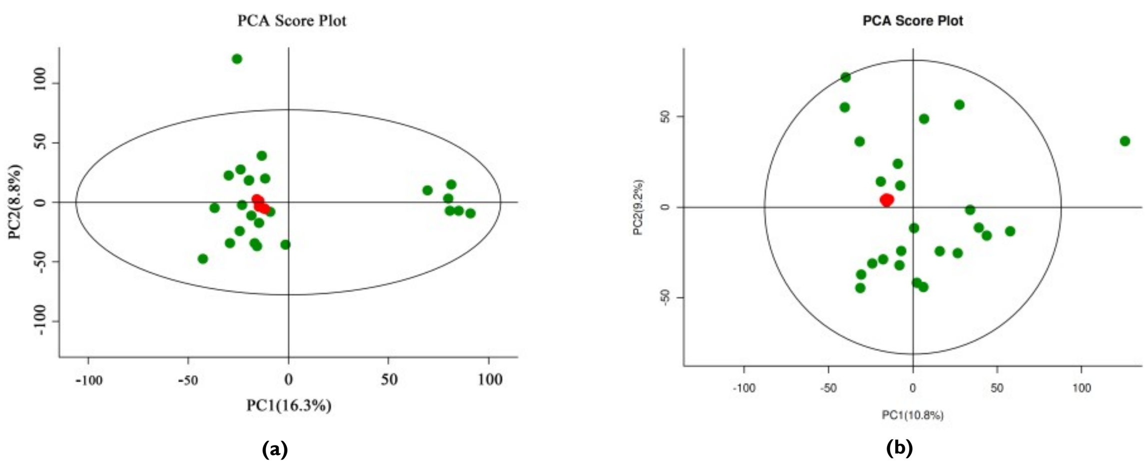

Fig. 3: PCA score plot of all samples. a. Positive ion mode; b. Negative ion mode.

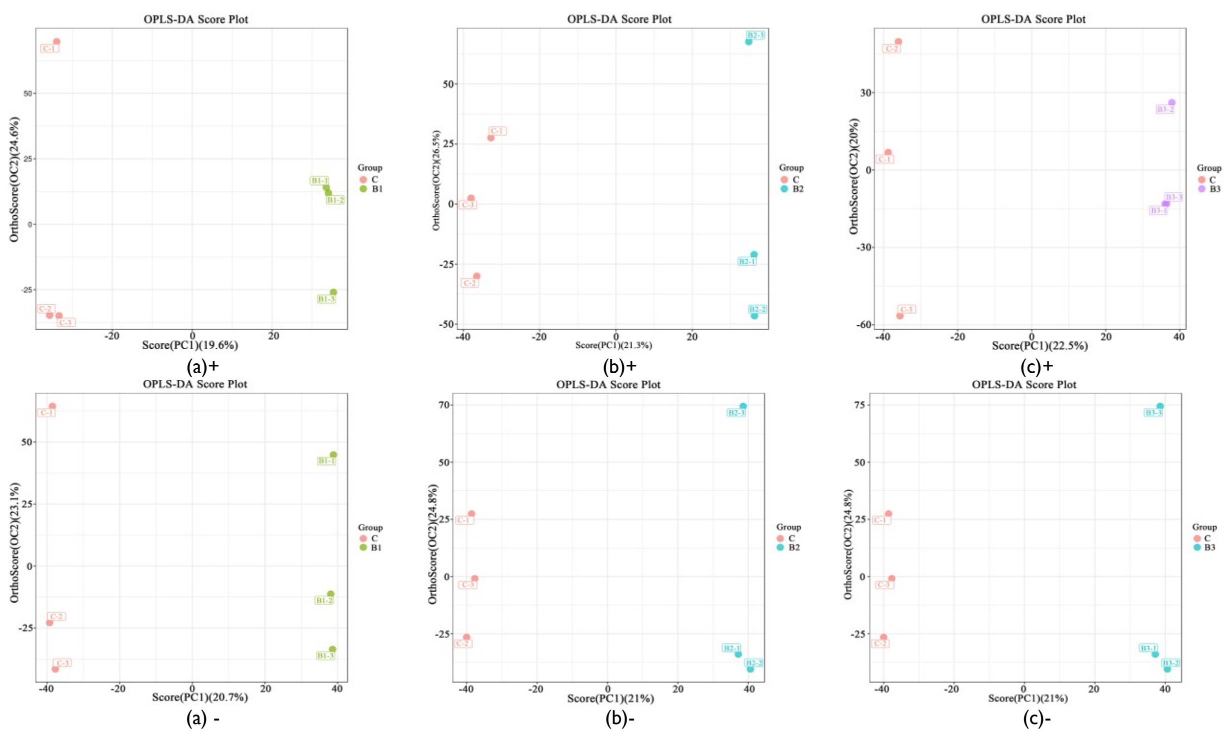

Fig. 4: A: OPLS-DA score plot in positive and negative ion mode; a :C-B1, b : C-B2, c: C-B3. The same as below.

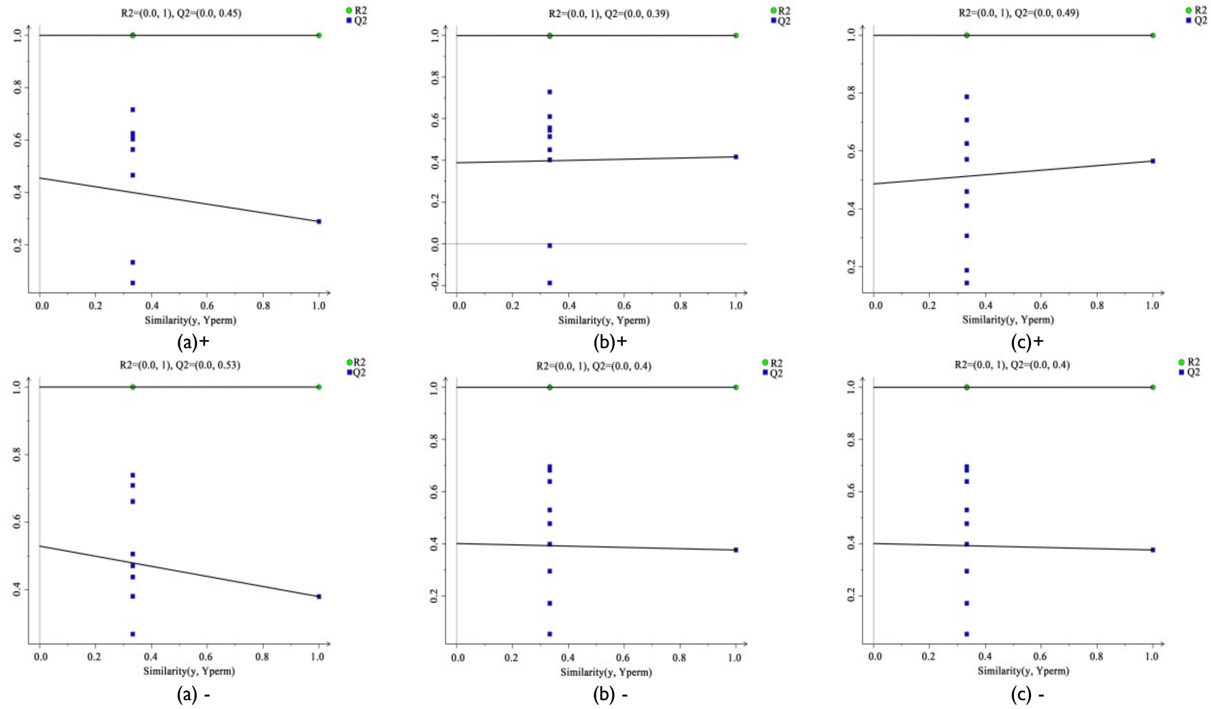

Fig. 5: A: Permutation test plot of the OPLS-DA model in positive and negative ion mode.

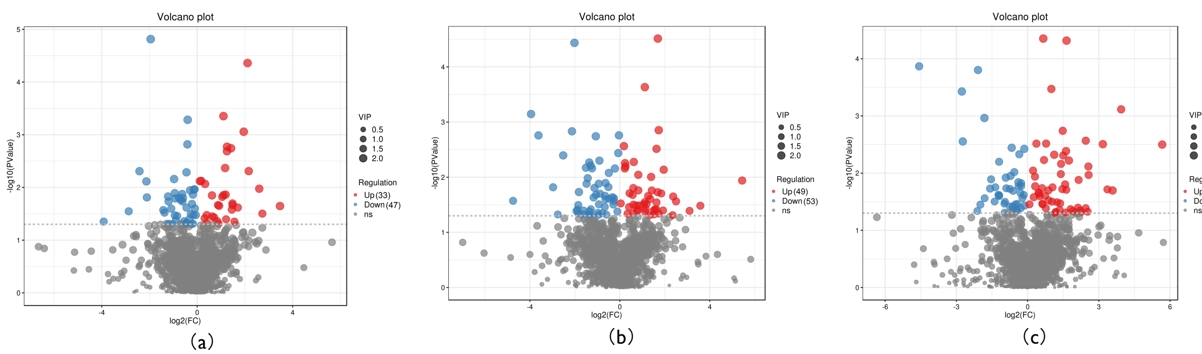

Fig. 6: Volcano plots of serum differential metabolites.

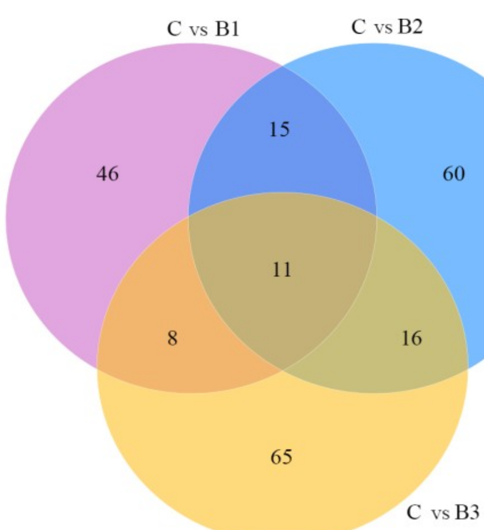

Fig. 7: Venn diagram of serum differential metabolites.

Expression pattern and cluster analysis of differential metabolites: A hierarchical clustering heatmap demonstrated that the expression patterns of differential metabolites were highly consistent within each group but significantly distinct between groups, which allowed for clear discrimination of different experimental groups (Fig. 8). Z-score normalization analysis revealed that the relative abundances of key differential metabolites differed significantly among groups, with regular and distinguishable expression trends (Fig. 9). Pearson correlation analysis showed that there were significant synergistic or antagonistic regulatory relationships among differential metabolites. Specifically, amino acids and organic acids exhibited a strong positive correlation ($r>0.8$, $P<0.05$), suggesting that these two classes of metabolites are jointly involved in the processes of energy metabolism and nitrogen metabolism in broilers (Fig. 10).

Pathway enrichment and network analysis of differential metabolites: The identified differential metabolites were mapped to the KEGG database, and

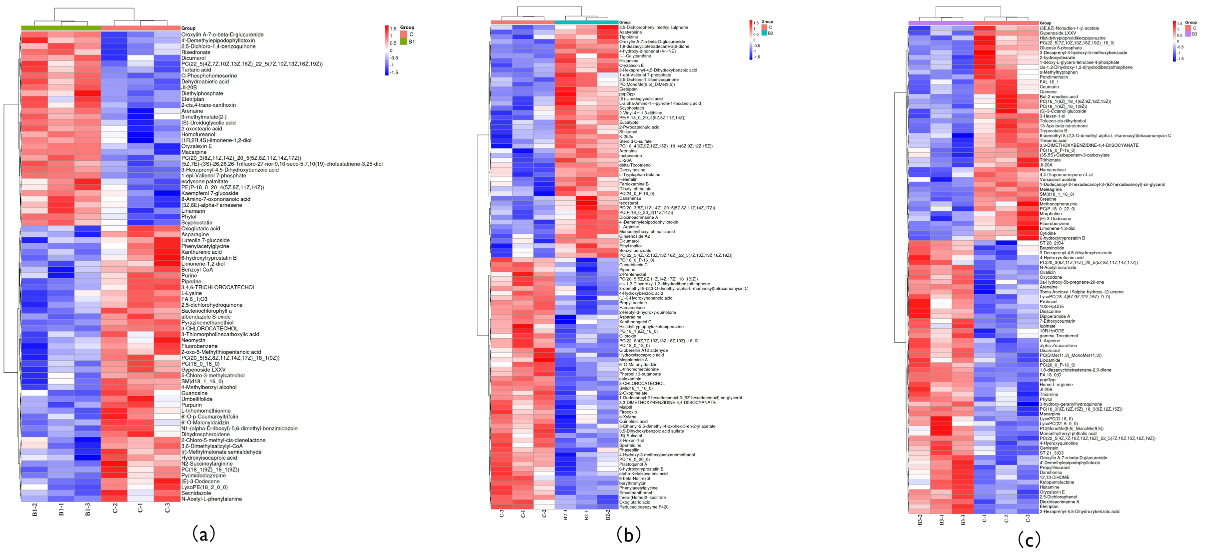

Fig. 8: Hierarchical clustering heatmap of differential metabolites.

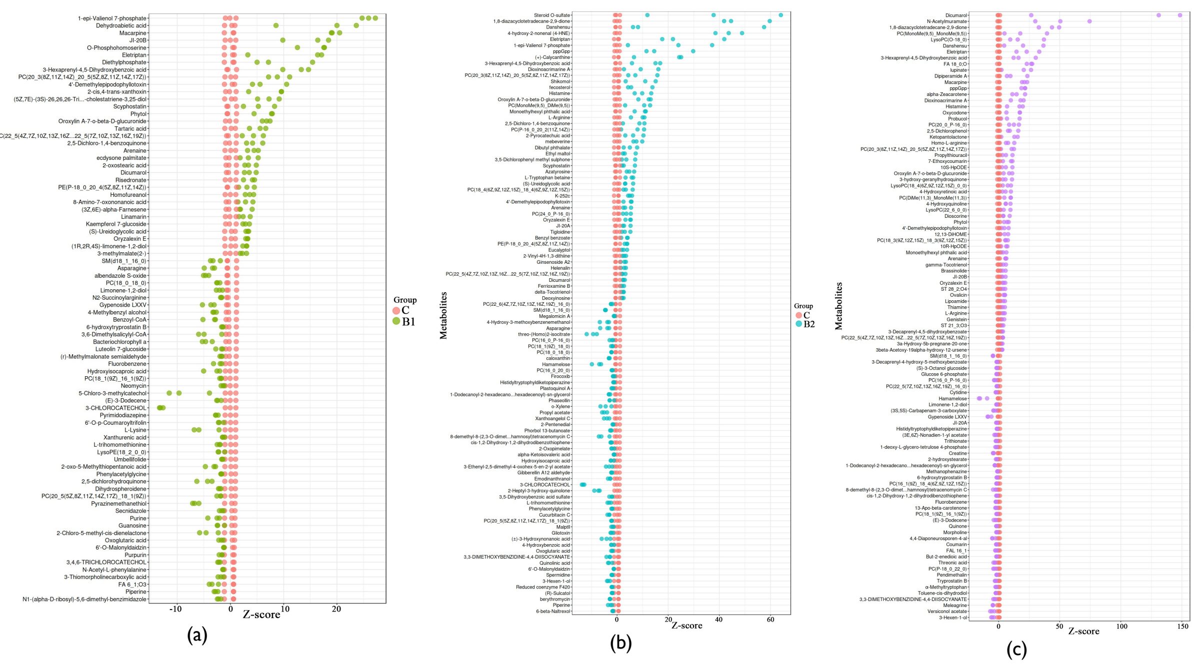

Fig. 9: Z-score plot of differential metabolites.

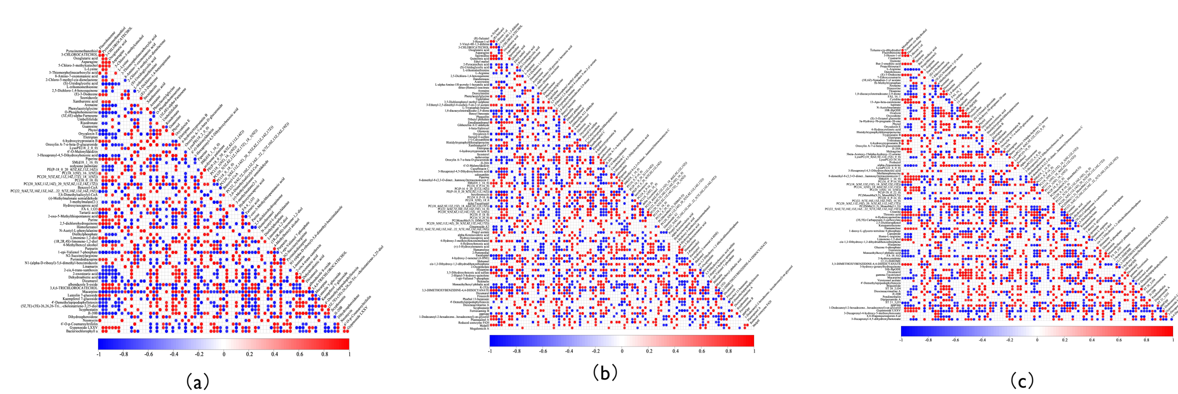

Fig. 10: Correlation heatmap of differential metabolites.

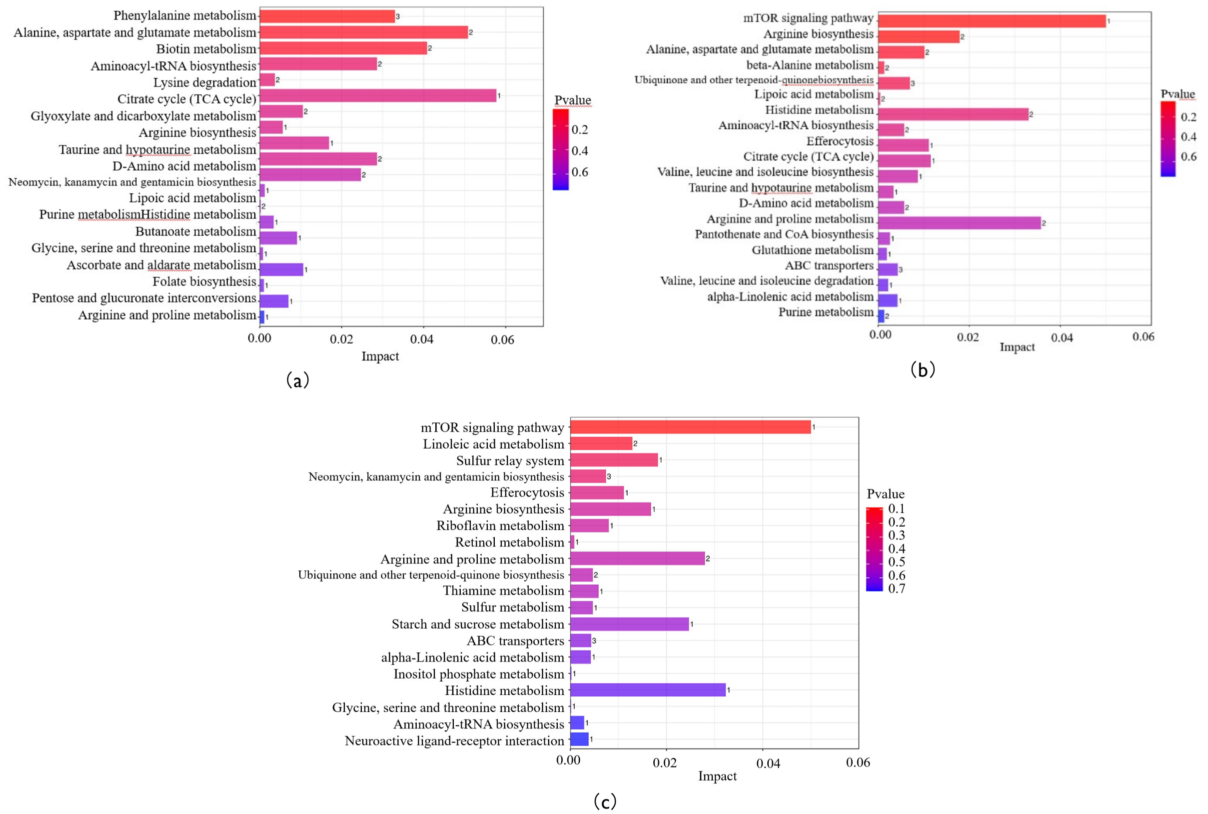

Fig. 11: Bar graph of metabolic pathway influencing factors.

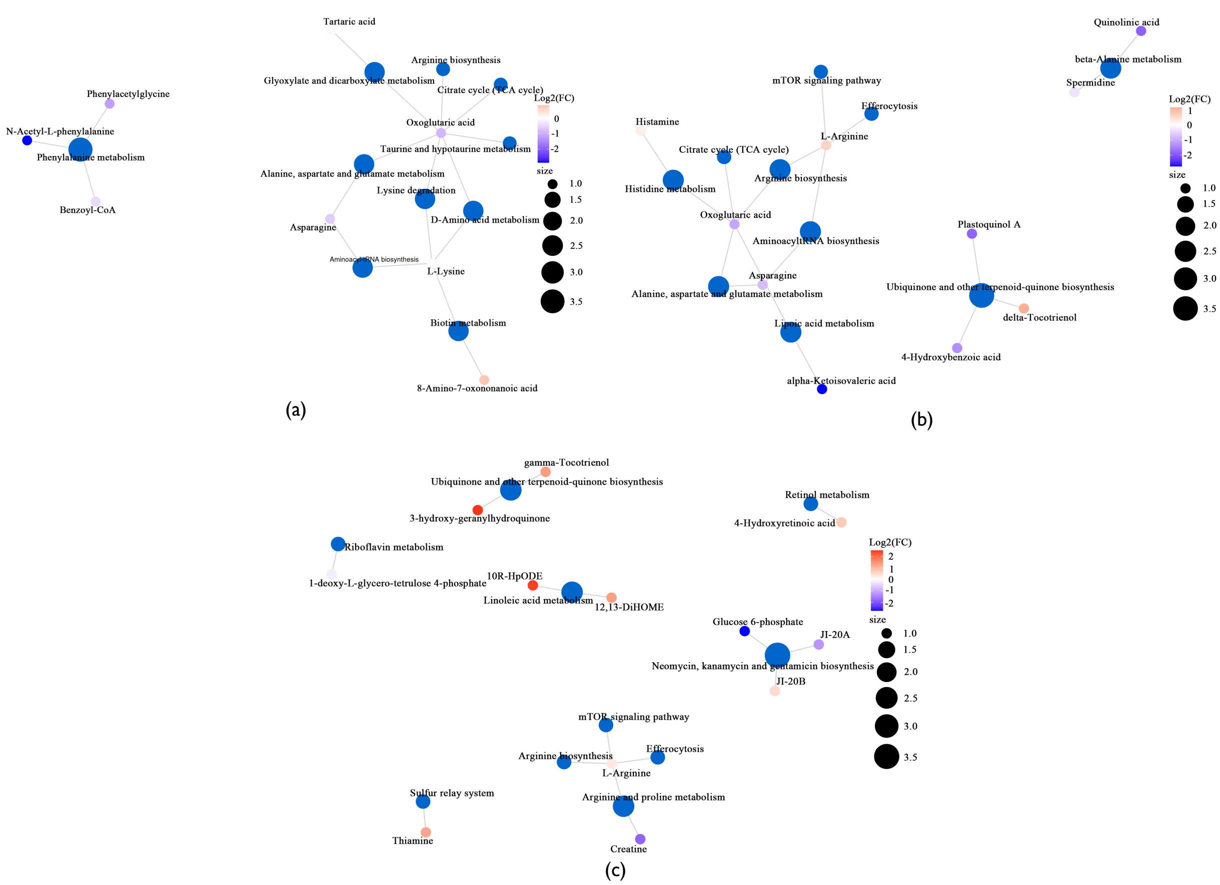

Fig. 12: Metabolic network correlation diagram.

pathway enrichment analysis was performed based on hypergeometric distribution test ($P<0.05$). A total of 20 significantly enriched metabolic pathways were identified, among which the key pathways included phenylalanine metabolism, alanine, aspartate and glutamate metabolism, biotin metabolism, aminoacyl-tRNA biosynthesis, tricarboxylic acid (TCA) cycle, arginine biosynthesis, and taurine and hypotaurine metabolism. According to comprehensive evaluation of pathway significance and impact value, alanine, aspartate and glutamate metabolism, phenylalanine metabolism, and biotin metabolism were determined as the crucial regulatory pathways (Fig. 11). The metabolic correlation network showed that metabolites such as α-ketoglutaric acid (AKG), L-lysine (L-Lys) and phenylalanine (Phe) linked multiple core pathways, playing pivotal hub roles in energy metabolism, amino acid synthesis, and redox homeostasis in broilers (Fig. 12).

DISCUSSION

Effects of BP leaves on growth performance of broilers: China is confronted with a shortage of protein feedstuffs. Notably, soybean meal, the most common protein feed, is mainly supplied through imports. Therefore, to mitigate this challenge, extensive research has focused on low-protein diets and novel protein feeds, yielding significant positive results (Wang *et al*., 2018). However, limited studies have focused on reducing soybean meal usage by using roughage resources. In the present study, three supplemental levels of BP were selected to replace soybean meal in the diet. Our results demonstrated that the 4% BP leaves level had no detrimental impact on growth performance. Balanced supply of essential nutrients is fundamental to maintaining animal health and ensuring optimal productivity. (Ganguly *et al.,* 2021).

Recognizing the critical role of nutrient intake in determining growth of performance animal (Zhang *et al.,* 2022). The 4% BP leaves added level group had similar growth performance with C group, that consistent with previous studies (Zhang *et al.,* 2022). Previous studies have shown that lower quantities of BP did not significantly affect the ADG of animals (Chen *et al.,* 2021; Hu *et al.,* 2022). Analysis indicated that excessive inclusion of BP leaves reduced feed intake and consequently impaired broiler growth performance, that be attributed to the immature digestive system of chicks during the brooding stage. The effect of feeding these leaves solely during the grower phase warrants further evaluation. Through experiments, it was found that adding 8 and 12% BP Leaves diet AA broilers diets can reduce their ADG and improve F/G. While 4% BP leaves were included in the diet, no reduction in growth performance of broilers. As content of BP leaves was added, the growth performance of broilers decreased, especially in 8 and 12% groups. However, the growth performance of broilers with 4% BP leaves added to their diet was similar to that of the control group and did not affect growth performance.

Effects of BP leaves on antioxidant capacity of broilers: Broussonetia papyrifera was rich in bioactive components such as polysaccharides, polyphenols, and flavonoids, making it a natural antioxidant (Li *et al*., 2025). Studies have demonstrated that bioflavonoids, as natural antioxidants, can influence antioxidant capacity indices, including superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) (Ali *et al*., 2016), such as by increasing the activities of SOD and GSH-Px. Flavonoids from plant leaves can enhance cell viability and reduce malondialdehyde (MDA) content (Zhang *et al*., 2014). Malondialdehyde (MDA) content, a key product of lipid peroxidation, reflects the degree of oxidative stress damage. Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) are important endogenous antioxidant enzymes within the antioxidant system and serve as critical defenses against oxidative stress (Xu *et al*., 2021). The present study confirmed that dietary supplementation with 4% paper mulberry leaves increased serum T-AOC, GSH-Px, SOD, and CAT levels while decreasing MDA content in broilers, which is consistent with the findings of the aforementioned studies. This may be attributed to the fact that paper mulberry leaves are rich in flavonoid antioxidants, which can stimulate the activity of antioxidant enzymes, promptly scavenge generated free radicals, and inhibit lipid peroxidation.

Effects of BP leaves supplementation levels on serum metabolites in broilers: In the present study, non-targeted LC-MS/MS metabolomics was employed to systematically investigate the effects of dietary supplementation with graded levels of BP leaves (4, 8 and 12%) on the serum metabolic profiles of white-feathered broilers. The results demonstrated that BP leaves supplementation significantly altered multiple key endogenous serum metabolites, including α-ketoglutarate (AKG), L-lysine (L-Lys), benzoyl-CoA, 3-chlorocatechol and pyrazinemethanethiol, which were mainly involved in organic acid, amino acid, and acyl metabolic pathways.

As a crucial intermediate in the tricarboxylic acid (TCA) cycle, AKG links energy metabolism and amino acid metabolism (Hou *et al*., 2011; Li *et al*., 2020). Additionally, as a precursor of glutamate, AKG participates in glutamine synthesis, providing nitrogen for nucleotide biosynthesis and antioxidant defense. In this study, AKG content was significantly upregulated in the 8% BP leaves group ($P<0.05$), suggesting that BP leaves enhance energy production and nitrogen utilization efficiency. L-Lys is an essential amino acid for broilers, participating in protein synthesis and energy metabolism (Shi *et al*., 2021). In the present study, serum L-Lys levels were significantly increased in BP leaves supplemented groups, with the most coordinated expression observed in the 8% group, indicating that BP leaves improves lysine utilization in broilers.

Benzoyl-CoA (HMDB0002252) is an intermediate in phenylalanine (Phe) metabolism and participates in the catabolism of aromatic amino acids and detoxification of xenobiotics. Its abundance is closely associated with hepatic detoxification and antioxidant capacity. Benzoyl-CoA was moderately upregulated in the 8% BP leaves group but excessively accumulated in the 12% group, implying that appropriate BP leaves supplementation enhances antioxidant and anti-stress capacity, whereas excessive inclusion may overload detoxification pathways. 3-Chlorocatechol and pyrazinemethanethiol are sulfur- and chlorine-containing metabolites; the former may be related to intestinal microbial metabolism or xenobiotic transformation, while the latter is involved in methionine metabolism and antioxidant defense. Their significant alterations in the 12% group suggest that high-level BP leaves may disturb intestinal microbial homeostasis or redox balance, which warrants further verification combined with 16S rRNA sequencing.

Hierarchical clustering and correlation analysis revealed strong positive correlations between amino acid and organic acid metabolism, indicating that BP leaves functions by coordinately regulating energy and amino acid metabolism. This finding is consistent with the serum metabolomic results reported by Wang *et al*. (2024) in Wumeng black-bone chickens. Metabolic network analysis further demonstrated that AKG, L-Lys, and Phe serve as hub metabolites connecting the TCA cycle, amino acid biosynthesis, nitrogen metabolism and antioxidant pathways. The coordinated changes among these metabolites suggest that BP leaves achieve global metabolic remodeling by modulating multiple metabolic hubs.

KEGG pathway enrichment analysis showed that differential metabolites were significantly enriched in alanine, aspartate and glutamate metabolism; phenylalanine metabolism; biotin metabolism; as well as the TCA cycle, aminoacyl-tRNA biosynthesis, arginine biosynthesis, and taurine and hypotaurine metabolism. Alanine, aspartate and glutamate metabolism acts as a central bridge connecting carbon and nitrogen metabolism. As an amino donor, glutamate participates in transamination to generate alanine and aspartate, providing substrates for protein synthesis. The upregulation of this pathway by BP leaves indicates promoted nitrogen turnover and reduced toxic ammonia accumulation. Phenylalanine metabolism: Phe is an aromatic amino acid whose metabolites include tyrosine, dopamine, and melanin, which are involved in neurotransmitter synthesis, pigment formation, and antioxidant defense. Regulation of this pathway by BP leaves may improve anti-stress capacity and meat quality in broilers. Biotin metabolism: Biotin is a cofactor for carboxylases and participates in fatty acid synthesis, gluconeogenesis, and amino acid catabolism. Upregulation of biotin metabolism by BP leaves suggests enhanced energy metabolism efficiency, which is particularly important during the high-energy-demand growth stage.

The TCA cycle and aminoacyl-tRNA biosynthesis: The TCA cycle is the core of energy metabolism, and aminoacyl-tRNA synthesis is a key step in protein synthesis. The simultaneous enrichment of both pathways indicates that BP leaves not only support energy supply but also promote protein synthesis efficiency, thereby benefiting growth performance. Li *et al*. (2022) reported that the apparent utilization rate of crude protein from BP leaves reached 70.96% and the metabolic rate of gross energy was 44.08% in medium-growing yellow-feathered broilers, confirming the favorable energy and protein utilization efficiency of BP leaves.

Taurine and hypotaurine metabolism: Taurine exerts antioxidant effects, regulates osmotic pressure, and stabilizes cell membranes. Enrichment of this pathway suggests that BP leaves may alleviate oxidative stress and improve health status by enhancing taurine synthesis. Collectively, these pathways coordinately contribute to nitrogen turnover, protein synthesis, energy supply, and antioxidant processes, representing the core mechanisms underlying the nutritional regulatory effects of BP leaves.

Regarding inclusion levels, the 4% BP leaves group exhibited fewer differential metabolites, lower pathway enrichment, and non-significant changes in key metabolites, indicating weak metabolic regulatory effects at a low dose, which is consistent with the findings of Fang *et al*. (2023). The 8% BP leaves group showed a moderate number of differential metabolites, the most significant enrichment of core metabolic pathways (e.g., alanine, aspartate and glutamate metabolism, TCA cycle), and the most coordinated expression of hub metabolites (AKG, L-Lys), without excessive disturbance of metabolic homeostasis. This group achieved optimal metabolic balance in energy production, amino acid utilization, and antioxidant capacity. This result is in line with the commonly recommended suitable range of BP leaves (5%-15%) in animal production (Zhang *et al*., 2020).The 12% BP leaves group displayed the largest number of differential metabolites, abnormal accumulation of certain metabolites (e.g., benzoyl-CoA, 3-chlorocatechol), and intensified fluctuations in TCA cycle intermediates, suggesting that high-level BP leaves may cause excessive metabolic disturbance, overloaded detoxification pathways, or redox imbalance. Similarly, Dong *et al*. (2025) reported that long-term supplementation with 15% BP leaves exerted negative effects on growth performance. In conclusion, based on metabolomic indicators, the optimal dietary supplementation level of BP leaves for white-feathered broilers is recommended to be 8%.

Conclusions: Dietary 4% Broussonetia papyrifera (BP) leaves had no adverse effect on broiler growth performance but significantly enhanced serum antioxidant indices. The 8% BP group showed favorable antioxidant effects and optimal metabolic balance. These findings confirm that 4-8% BP leaves can effectively improve broiler antioxidant capacity, immune function and metabolic homeostasis, providing a theoretical basis for its application as an unconventional feed resource in poultry production.

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Authors contribution: WH Conceptualization, Feeding, Formal analysis, Methodology and Writing original draft; FYM Methodology, Validation, feeding trial; AAB Methodology, Manuscript revision and critical editing; TRZ Disease Prevention and Sampling, Methodology; ZYC Software, Visualization, Formal analysis, Manuscript revision and critical editing; XJY Formal analysis and data processing, Conceptual design and project management, Supervision of the study, Draft preparation, Manuscript revision and critical editing. LAA Formal analysis and biostatistics analysis; MA, RT Manuscript revision and critical editing.

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