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Original Research Article

Dietary supplementation with *Neolamarckia cadamba* leaf extract improves broiler meat quality by enhancing antioxidant capacity and regulating metabolites

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ABSTRACT

This study was to evaluate the effect of supplementing the diet of broilers with *Neolamarckia cadamba* leaf extract (NCLE) on meat quality by evaluating antioxidant parameters and the expression of genes in the p38 mitogen-activated protein kinase/nuclear factor-erythroid 2-related factor 2/antioxidant responsive element (p38 MAPK/Nrf2/ARE) signaling pathway, coupled with LC–MS-based metabolomic analysis. A total of 480 one-day-old male broilers were randomly allocated to four treatment groups—a control (CON) group, which was fed a basal diet, and three NCLE treatment groups, which were fed the basal diet supplemented with 100, 200, or 400 mg/kg NCLE (N1, N2, and N3 groups, respectively) for 42 d. Compared with the CON group, meat quality was improved in the N2 and N3 groups, as evidenced by the higher pH_{45min} ($P < 0.05$) and lower shear force ($P < 0.05$) in breast muscle (BM) and lower drip loss at 48 h ($P < 0.05$) in leg muscle (LM). Moreover, BM antioxidant capacity was significantly enhanced in the N3 group, characterized by an increase in the total antioxidant capacity (T-AOC), the concentrations of glutathione peroxidase (GSH-Px) and catalase (CAT), and the relative mRNA expression of *p38 MAPK*, extracellular-signal regulated kinase (*ERK1/2*), c-Jun N-terminal kinase (*JNK*), *Nrf2*, *CAT*, and *GSH-Px* ($P < 0.05$). Similarly, LM in the N3 group displayed higher T-AOC, increased GSH-Px and CAT concentrations, reduced malonaldehyde contents ($P < 0.05$), and upregulation of the relative mRNA levels of *JNK*, *Nrf2*, heme oxygenase, *CAT*, and superoxide dismutase (*SOD*) ($P < 0.05$). Metabolomics analysis revealed that D-arabinono-1,4-lactone and lyso-PAF C-16-d4 were negatively correlated with shear force and cooking loss ($P < 0.05$) and displayed increased abundance in BM of the N3 group. L-Serine levels were upregulated while D-fructose 1,6-diphosphate contents were downregulated in the three NCLE groups. Finally, the differential metabolites in both BM and LM were involved in amino acid metabolism pathways. Our results indicated that NCLE supplementation improved meat quality by enhancing antioxidant enzyme activities, promoting the expression of genes

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in the p38 MAPK/Nrf2/ARE signaling pathway, and regulating amino acid metabolism. The optimal NCLE concentration was found to be 400 mg/kg.

1. Introduction

Poultry meat is widely consumed worldwide owing to its high nutritional value, affordability, and few religious restrictions (Fan et al., 2018; Petracci et al., 2019). Given the increased demand for poultry meat, high-density cages have become the primary method for broiler breeding. However, these cages can lead to unfavorable production conditions, including restricted movement, high temperatures, and high ammonia concentrations, which can induce stress responses in broilers and negatively impact their body defense systems (Bai et al., 2020; Son et al., 2022). Consequently, the broilers are susceptible to infection by pathogenic bacteria, resulting in gut health disturbance and reduced growth performance (Dai et al., 2022). Over the past few decades, antibiotics have been extensively used to control the pathogenic load and promote the growth of broilers. However, this practice has resulted in numerous issues, including antibiotic resistance and environmental pollution, which pose threats to human health (Zeng et al., 2021). Accordingly, China has implemented a complete ban on the use of growth-promoting antibiotics for livestock and poultry (Tan et al., 2022), and there is now an urgent need for effective alternatives to these antibiotics to avoid pathogen infection in broilers bred in high-density cages.

Meat quality parameters, such as meat color, tenderness, water-holding capacity, flavor, and safety, are key determinants of consumer purchase choices (Mir et al., 2017). However, certain adverse factors during broiler feeding and transportation can lead to the excessive production of reactive oxygen species and, consequently, oxidative stress, which can negatively affect meat quality (Tuell et al., 2020). This can result in a rapid drop in pH, poor meat color, reduced water-holding capacity, and impaired texture (Lan et al., 2020). The activities of antioxidant enzymes in muscle play a direct role in determining its antioxidant capacity, a process that is regulated by the p38 mitogen-activated protein kinase/nuclear factor-erythroid 2-related factor 2/antioxidant responsive element (p38 MAPK/Nrf2/ARE) signaling pathway (Xu et al., 2018; Rocchetti et al., 2020). Plant extracts are a promising alternative to antibiotics owing to their wide availability, eco-friendliness, high efficiency, and good safety profiles. Many studies have reported their effectiveness in replacing antibiotics in the livestock and poultry industries (Cozzi et al., 2021; Gutierrez et al., 2022). Furthermore, plant extracts are rich in bioactive components, such as flavonoids, polyphenols, and saponins, which have a significant capacity for scavenging reactive oxygen species and relieving oxidative stress (More et al., 2021).

Neolamarckia cadamba, a fast-growing tree found in China, India, and Egypt, is traditionally used as herbal medicine. The leaves of this plant represent a vast source of biomass owing to their large size (15 to 50 cm long, 8 to 25 cm wide) and to the fact that they can be harvested 4 to 6 times per year (Zhang et al., 2022a). The leaves *N. cadamba* are a good feed resource for animals given their relatively high nutritional value (13.97% crude protein, 30.43% neutral detergent fiber, 22.59% acid detergent fiber, and 4.19% water soluble carbohydrates) and good digestibility (50.42% dry matter digestibility) (He et al., 2018; Wang et al., 2017). Furthermore, the leaves contain an abundance of bioactive chemicals, including flavonoids (5.62%) and phenolic acids (5.56%) (He et al., 2018), which contribute to their antimicrobial, antioxidant, and anti-inflammatory properties. In our previous studies, we found that both the fresh and powder forms of *N. cadamba* leaves could inhibit undesirable microorganisms in legume silage (Wang et al., 2019). Dietary supplementation with 5% *N. cadamba* leaves altered the composition of the gut microbiota and enhanced the growth performance of partridge chickens (Chen et al., 2021a). Moreover, *N. cadamba* leaf extract (NCLE) improved digestibility and reduced ruminal methane production by influencing the makeup of the ruminal microbial community (Zhang et al., 2022b). The results of these studies highlight the potential of NCLE as a poultry feed additive to alleviate oxidant stress during high-density cage feeding and live broiler transportation. However, the effects of NCLE on the antioxidant capacity and meat quality of broilers, as well as the putative underlying mechanisms, remain unknown. Meat metabolomics, with gas or liquid as the stationary phase, has been widely used to identify potential biomarkers of meat quality under a variety of treatments (Muroya et al., 2020). This approach also allows the monitoring of meat quality traits through the identification of differential metabolites and specific metabolic pathways.

Accordingly, in the present study, we investigated the effect of dietary NCLE supplementation on broiler meat quality through the evaluation of antioxidant parameters and the expression of genes involved in the p38 MAPK/Nrf2/ARE signaling pathway, concomitant with liquid chromatography–mass spectrometry (LC–MS)-based metabolomic analysis. Our findings provide a theoretical reference for understanding how NCLE improves meat quality as well as identifying the underlying mechanisms.

2. Materials and methods

2.1. Animal ethics statement

All procedures involving animals were conducted in accordance with the Chinese guidelines for animal welfare and were approved by the Institutional Animal Care and Use Committee of South China Agricultural University (Permit No: 2021F120).

2.2. Determination of the chemical composition of NCLE

N. cadamba plants were cultivated in and harvested from the experimental field of South China Agricultural University (23°19′ N, 113°34′ E, Guangzhou, China). Fresh materials were wilted to constant weight at room temperature and ground for NCLE production. In brief, NCLE was obtained by first extracting with 60% methanol at a solid-to-liquid ratio of 1:20 for 2.5 h at 60 °C, and then filtrating, condensing, vacuum drying, and storing in a drying vessel. Total flavonoids were detected by aluminum chloride colorimetric assay as previously described (Singh et al., 2013). Total phenols and hydrolysable tannins were measured using Folin–Ciocalteu colorimetry according to Makkar (2010). The hydrochloric acid–acetone–butanol assay was used for the detection of condensed tannins as described by Huang et al. (2011).

Flavonoids and chlorogenic acids in NCLE were detected by high-performance liquid chromatography (HPLC) and high-

resolution mass spectrometry (HRMS). A total of 100 mg of NCLE powder was extracted in an ultrasonic bath with 70% menthol (2 mL) for 30 min and centrifuged for 10 min. Then, 20 μL of the resulting supernatant was filtered through a 0.22-μm membrane filter and homogenized in 980 μL of methanol. The HPLC conditions for the flavonoids were as follows: the column was an ACQUITY UPLC BEH C18 (2.1 × 100 mm, 1.7 μm; Waters Corporation, Milford, MA, USA); the oven temperature was 40 °C; the mobile phase consisted of solvent A (0.1% formic acid) and solvent B (menthol); the gradient elution program was 0 to 1 min, 10% B; 1 to 3 min, 10% to 33% B; 3 to 10 min, 33% B; 10 to 15 min, 33% to 50% B; 15 to 20 min, 50% to 90% B; 20 to 21 min, 90% B; 21 to 22 min, 90% to 10% B; and 22 to 25 min, 10% B. The flow rate was 0.25 mL/min and the injection volume was 5 μL. The HPLC conditions for chlorogenic acids were as follows: the column was an HSS T3 (2.1 × 50 mm, 1.8 μm; Waters Corporation); the oven temperature was 40 °C; the mobile phase consisted of solvent A (0.1% acetic acid) and solvent B (acetonitrile with 0.1% acetic acid); the gradient elution program was 0 to 2 min, 10% B; 2 to 6 min, 10% B; 6 to 8.1 min, 60% B; and 8.1 to 12 min, 10% B. The flow rate was 0.3 mL/min and the injection volume was 2 μL.

HRMS data were recorded on a Q Exactive Hybrid Quadrupole–Orbitrap Mass Spectrometer equipped with a heated electrospray ionization (ESI) source (Thermo Fisher Scientific, USA) employing the selected ion monitoring (SIM) acquisition method. The ESI source parameters were set as follows: spray voltage, −2.8 kV; sheath gas pressure, 40 arbitrary unit; auxiliary gas pressure, 10 arbitrary unit; sweep gas pressure, 0 arbitrary unit; capillary temperature, 320 °C; and auxiliary gas heater temperature, 350 °C. Mass spectra data were acquired using Xcalibur 4.1 (Thermo Fisher Scientific), processed with TraceFinder 4.1 for Clinical Research (Thermo Fisher Scientific), and transferred into Excel format. The chemical composition of NCLE is shown in Table 1.

2.3. Broiler feeding and management

A total of 480 one-day-old male broilers were purchased from Qinglong New Agricultural Technology Co., Ltd (Qingyuan, China). The broilers were randomly assigned to 4 treatment groups of 20 broilers with 6 replicates and reared in cages (125 cm × 100 cm × 50 cm) in a temperature-controlled environment. The birds were maintained on a 24-h constant light schedule and allowed free access to feed and water throughout the experiment. Epidemic prevention was carried out following conventional procedures. The basal diet met or exceeded the requirements for broilers and was formulated based on National Research Council recommendations (NRC, 1994). The composition and nutrient levels of the mini-broiler (d 0 to d 20) and mid-broiler (d 21 to the end of the experiment) diets are presented in Table S1. The nutrient composition (crude protein, total calcium, total phosphorus) of diets was analyzed according to the methods described in Association of Official Analytical Chemists (AOAC, 2006). The methionine, cystine, and methionine + cystine contents were determined according to China National Standards (GB/T 18246-2019 and GB/T 15399-2018). Metabolizable energy was calculated according to Nutrient Requirements of Poultry (NRC, 1994).

The experimental treatment groups were as follows: a CON group, provided with 0 mg/kg NCLE; and 3 NCLE treatment groups, in which the birds were provided with 100 mg/kg NCLE (N1 group), 200 mg/kg NCLE (N2 group), or 400 mg/kg NCLE (N3 group). After a one-week preliminary experiment, the formal trials were conducted as mentioned above.

Table 1
Chemical compositions of *Neolamarckia cadamba* leaves extract.

Item	Content, g/kg dry matter
Spectrophotometric determination	
Total flavonoids	585.0 ± 15.50
Total phenols	73.0 ± 5.35
Hydrolysable tannins	70.8 ± 5.31
Condensed tannin	61.8 ± 5.23
Flavonoids metabolome determination	
Procyanidin B2	0.22 ± 0.01
Rutin	0.09 ± 0.00
Kaempferol 3-neohesperidoside	0.09 ± 0.00
Narcissin	0.09 ± 0.00
Typhaneoside	0.06 ± 0.00
Nicotiflorin	0.06 ± 0.00
Astragalin	0.05 ± 0.00
Other 195 flavonoids	<0.01 ± 0.00
Chlorogenic acid metabolome determination	
Chlorogenic acid	84.7 ± 1.18
Iso-chlorogenic acid A	2.11 ± 0.13
Iso-chlorogenic acid B	0.26 ± 0.07
Iso-chlorogenic acid C	5.05 ± 0.17
Neochlorogenic acid	0.07 ± 0.01
Other chlorogenic acid	<0.01 ± 0.00

2.4. Data and sample collection

Broiler weight and feed consumption were determined weekly throughout the experiment and the average daily feed intake (ADFI), the average daily gain (ADG), and the feed-to-gain ratio (F/G) were calculated. Blood samples were collected according to Liu et al. (2021). Briefly, at the end of the experiment (d 49), approximately 10 mL of blood was collected from the wing vein into tubes containing heparin sodium. Plasma was obtained through sedimentation and centrifugation (1,006 × *g* for 10 min at 4 °C) and stored at −20 °C for further testing. Subsequently, three broilers were randomly selected from each replicate and were euthanized by exsanguination. After defeathering, the abdominal cavity was opened and the breast muscle (BM) and leg muscle (LM) were stripped and weighed. Each was divided into three parts for meat quality assessment, examination of antioxidant-related genes, and metabolomic analysis, respectively.

2.5. Meat quality parameters

The pH of the BM and LM was measured 45 min after euthanasia with a glass electrode pH meter (PHS-3C, INESA Scientific Instrument Co., Ltd, Shanghai, China). The meat color was measured on the exterior surface of intact, skinless BM and LM immediately after muscle sample separation with a high-quality colorimeter (NS800, Shenzhen 3NH Technology Co., Ltd, China). Meat color was assessed based on the CIE L^* a^* b^* color space (L^* = lightness, a^* = redness, b^* = yellowness). Each sample was measured three times at different locations and the average value was calculated. The meat water-holding capacity, including cooking loss and drip loss, was evaluated 24 h after collection as previously described (Zhuang and Savage, 2012). To determine the cooking loss, weighed BM and LM samples were packed into sealed polyethylene plastic bags and cooked in a water bath for 2 min at 75 °C. Subsequently, the muscle samples were wiped dry and weighed, and the difference between before and after cooking was considered the cooking loss. To calculate the drip loss, weighed BM and LM samples were hung in a sealed polyethylene plastic bag at 4 °C and were weighed after 24 and 48 h. Shear force was determined using a digital display muscle tenderness meter (C-LM3B, Nanjing Mingao Instrument Co., Ltd, China). Once the muscle sample was cooked, it was cut at three locations in the direction of the vertical pectoral muscle fibers, and

the average of the readings was considered the shear force value (Cao et al., 2020).

2.6. Antioxidant parameters of breast muscle and leg muscle

The antioxidant parameters of muscles, including total antioxidant capacity (T-AOC) and superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione (GSH), catalase (CAT), and malondialdehyde (MDA) contents, were determined using the respective ELISA kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.7. Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from BM and LM using the HiPure Universal RNA Mini Kit (Megi Biotechnology Co., Ltd, Guangzhou, China) and reverse transcribed into cDNA with HiScript III RT SuperMix for qPCR (+gDNA eraser) (Nanjing Vazyme Biotechnology Co., Ltd, Nanjing, China) following the manufacturer's instructions. Fluorescence qPCR was subsequently performed with ChamQ Universal SYBR qPCR Master Mix (Nanjing Vazyme Biotech Co., Ltd, Nanjing, China) on an Illumina Real-Time System. The reaction mixture consisted of 10 μL of 2× ChamQ 66 Universal SYBR qPCR Master Mix, 0.4 μL of forward and reverse primer, 0.2 μL of cDNA, and 9.4 μL of ddH_2O. The amplification program comprised 1 cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 20 s, and finally 1 cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s for melting curve analysis; qPCR was performed with six biological replicates and two technical replicates. Relative mRNA expression levels were normalized to that of β-actin and calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers targeting genes involved in the p38 MAPK/Nrf2/ARE signaling pathway (*p38 MAPK*, extracellular-signal regulated kinase [*ERK1/2*], c-Jun N-terminal kinase [*JNK*], *Nrf2*, heme oxygenase 1 [*HO-1*], *CAT*, *SOD*, and *GSH-Px*) were shown in Table S2.

2.8. Non-targeted metabolomic analysis

A total of 48 muscle samples (24 breast and 24 leg) were uniformly ground under liquid nitrogen. Then, 20 mg of each sample was extracted using 400 μL (70%, vol/vol) of methanol internal standard liquid, vortexed for 5 min (1500 rpm), and placed in an ice bath for 15 min. The mixture was subsequently centrifuged at 12,000 × *g* for 10 min at 4 °C and 300 μL of supernatant was stored at −20 °C for 30 min. After centrifuging again (12,000 × *g*, 4 °C, 3 min), 200 μL of supernatant was transferred into a vial and submitted to HPLC using an Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA). An ACQUITY UPLC HSS T3 C18 column (1.8 μm, 2.1 × 100 mm; Waters Corporation) was used for hydrophilic interaction liquid chromatography separation. The column temperature was 40 °C, the flow rate was 0.40 mL/min, and the injection volume was 2 μL. Analysis was performed in negative ESI mode. The mobile phase for gradient elution consisted of ultrapure water with 1% formic acid as mobile phase A and acetonitrile with 1% formic acid as mobile phase B. The elution gradient was 95% mobile phase A for 10 min, which was linearly reduced to 10% in 2 min, and then increased to 95% in 0.1 min, with a 1.9 min re-equilibration period. The conditions for MS analysis in negative ESI mode were: voltage, 2500 V; gas flow, 8 L/min; fragmentor voltage, 135 V; gas and sheath temperature, both 325 °C; sheath flow, 11 L/min; and nebulizer pressure, 40 psi.

The data were preprocessed as follows: Proteo Wizard software was used to convert raw LC–MS data to the MzML format, following which peak area extraction, peak alignment, and retention time rectification were performed using XCMS. Peak area correction was undertaken using the support vector regression (SVR) method and peaks with a missing rate of >50% were filtered out. Metabolites were identified by searching an in-house database, a public database, an AI database, and metDNA. Quality control (QC) samples were included after every 15 detection samples to monitor the stability and repeatability of the instrument. The detail information for top 50 metabolites are shown in Table S3. Multivariate data analysis, namely, Pareto-scaled principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA), were performed in R 3.4.1 (base package 3.5.1) and MetaboAnalystR (v. 1.0.1), respectively. A heat map of all the metabolites was created in R (Complex Heatmap, v. 1.2.1). Metabolites were considered differentially abundant using variable importance in projection (VIP) ≥ 1, $P < 0.05$ (Student's *t*-test), and absolute Log_2 FC ≥ 1.0 as selection criteria. Identified metabolites were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) compound database (http://www.kegg.jp/kegg/compound/) and then mapped to KEGG pathways (http://www.kegg.jp/kegg/pathway.html). Significantly enriched pathways were identified with a hypergeometric test *P*-value for a given list of metabolites.

2.9. Statistical analysis

IBM SPSS 20.0 was used to analyze the effect of NCLE on slaughter performance, meat and immune parameters, antioxidant indexes, and gene expression (qRT-PCR). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to identify significant differences among the groups. *P*-values < 0.05 were considered significant. Spearman's correlation analysis was conducted using the OmicShare online platform (https://www.omicshare.com).

3. Results

3.1. The composition of NCLE

The NCLE composition as determined by spectrophotometry and HPLC–HRMS is presented in Table 1. NCLE was found to be rich in bioactive substances. The total flavonoid, total phenol, hydrolyzable tannin, and condensed tannin contents of NCLE were 585.0, 73.0, 70.8, and 61.8 g/kg, respectively. However, the content of procyanidin B2 was only 0.22 g/kg, while those of the other detected flavonoids were all below 0.10 g/kg; this indicated that most flavonoids in NCLE are unknown and may exert positive bioactive effects on broilers. Chlorogenic acids were also found in NCLE, including chlorogenic acid (84.70 g/kg), iso-chlorogenic acid C (5.05 g/kg), and iso-chlorogenic acid A (2.11 g/kg).

3.2. Growth performance, slaughter index, and muscle quality traits

The growth performance and slaughter index of broilers fed with different proportions of NCLE are shown in Table 2. The initial weight of the broilers did not differ significantly across the 4 treatments, indicative of satisfactory uniformity among the animals used in this study. The F/G ratio was significantly decreased in the N2 and N3 treatment groups ($P < 0.05$) compared with that in the CON group. However, no significant effect of NCLE ($P > 0.05$) was detected on the ADG, ADFI, or final weight. Similarly, the slaughter rate, the BM rate, and the LM rate did not differ significantly between the CON group and the NCLE supplementation groups. As shown in Table 3, there was a difference in BM and LM quality traits among the different treatment groups. For BM, diets supplemented with 200 or 400 mg/kg NCLE (N2 and N3 groups) induced a significant increase in the pH_{45min} and a decrease in shear force compared with that seen in the CON group ($P < 0.05$). Breast muscle

Table 2
Effect of *Neolamarckia cadamba* leaves extract on growth and slaughter performance of broilers ($n = 6$).

Item	CON	N1	N2	N3	*P*-value	Linear	Quadratic
Initial weight, g	31.7 ± 0.62	33.4 ± 0.59	32.2 ± 0.60	33.3 ± 0.48	0.12	0.19	0.38
Final weight, g	1391 ± 7.09	1432 ± 29.50	1454 ± 21.60	1458 ± 38.70	0.30	0.07	0.15
Average daily feed intake, g/d	62.7 ± 1.09	62.4 ± 1.32	61.9 ± 1.53	62.1 ± 1.41	0.45	0.48	0.52
Average daily gain, g/d	32.4 ± 0.17	33.3 ± 0.71	33.9 ± 0.52	33.9 ± 0.92	0.32	0.07	0.17
F/G ratio, g/g	1.94 ± 0.03[a]	1.88 ± 0.04[ab]	1.83 ± 0.03[b]	1.83 ± 0.04[b]	0.03	0.03	0.03
Slaughter rate, %	91.6 ± 0.33	91.3 ± 0.13	92.5 ± 1.07	92.2 ± 0.47	0.46	0.26	0.53
Breast muscle rate, %	12.7 ± 0.31	12.9 ± 0.35	13.7 ± 0.44	13.2 ± 0.82	0.56	0.32	0.50
Leg muscle rate, %	13.6 ± 0.55	13.6 ± 0.68	13.6 ± 0.60	13.4 ± 0.46	0.99	0.79	0.96

CON = feed for basal diet; N1, N2 and N3 = feed for basal diet with 100, 200 and 400 mg/kg *Neolamarckia cadamba* leaves extracts; F/G ratio = feed to gain ratio.
[a,b]Within a same row, means with different superscripts means a significant effect ($P < 0.05$).

Table 3
Effect of *Neolamarckia cadamba* leaves extract on breast and leg muscle quality traits of broilers ($n = 6$).

Item	CON	N1	N2	N3	*P*-value	Linear	Quadratic
BM							
pH_{45min}	6.25 ± 0.02[c]	6.29 ± 0.01[cb]	6.32 ± 0.01[ab]	6.33 ± 0.01[a]	<0.01	<0.01	<0.01
*L**	60.20 ± 0.82	61.60 ± 1.38	59.10 ± 1.02	60.90 ± 1.19	0.47	0.75	0.89
*a**	3.88 ± 0.68	4.40 ± 0.50	4.66 ± 0.87	4.31 ± 0.54	0.87	0.60	0.70
*b**	10.30 ± 0.59	7.99 ± 0.69	9.01 ± 0.47	10.10 ± 0.58	0.06	0.67	0.04
Cook loss, %	26.33 ± 0.90	27.50 ± 0.81	24.90 ± 0.54	24.90 ± 1.64	0.27	0.15	0.32
Drip loss, %							
24 h	3.40 ± 0.93	3.04 ± 0.81	3.15 ± 0.78	2.52 ± 0.61	0.88	0.46	0.76
48 h	4.56 ± 1.08	4.47 ± 0.99	4.65 ± 0.95	4.16 ± 0.67	0.98	0.80	0.95
Shear force, N	29.90 ± 3.08[a]	30.50 ± 2.93[a]	21.90 ± 2.64[b]	20.80 ± 1.92[b]	0.03	0.01	0.03
LM							
pH_{45min}	6.41 ± 0.01	6.42 ± 0.01	6.41 ± 003	6.42 ± 0.02	0.99	0.85	0.98
*L**	52.70 ± 1.40	53.30 ± 0.56	51.70 ± 5.40	54.90 ± 1.32	0.32	0.39	0.39
*a**	4.17 ± 0.27	4.22 ± 0.49	4.95 ± 0.62	5.20 ± 0.55	0.38	0.09	0.24
*b**	7.78 ± 0.81	6.22 ± 0.35	6.33 ± 0.38	6.92 ± 0.54	0.20	0.35	0.11
Cook loss, %	31.80 ± 1.23	32.20 ± 0.61	30.10 ± 1.19	29.00 ± 1.43	0.22	0.05	0.13
Drip loss, %							
24 h	4.58 ± 0.57	3.72 ± 0.63	3.30 ± 0.65	2.71 ± 0.91	0.32	0.06	0.17
48 h	5.98 ± 0.52[a]	6.54 ± 0.62[a]	5.02 ± 0.41[b]	4.30 ± 0.90[b]	0.01	0.03	0.03
Shear force, N	25.6 ± 2.68	19.6 ± 1.77	19.9 ± 2.76	19.6 ± 2.27	0.25	0.11	0.15

CON = feed for basal diet; N1, N2 and N3 = feed for basal diet with 100, 200 and 400 mg/kg *Neolamarckia cadamba* leaves extracts; BM = breast muscle; LM = leg muscle; *L** = lightness; *a** = redness; *b** = yellowness.
[a–c]Within a same row, means with different superscripts means a significant effect ($P < 0.05$).

in the N3 group also showed better meat color performance, with higher *L** and *a** and lower *b** values, as well as better water-holding capacity (less cooking loss and drip loss, $P > 0.05$). For LM, compared with the CON group, a significant decrease ($P < 0.05$) in drip loss was detected in the N2 and N3 groups at the 48-h sampling point. The addition of NCLE to the diet also improved the leg muscle quality, as evidenced by the increase in the *L** value and the decrease in the *b** value, cooking loss, and shear force; however, the differences in these parameters were not significant ($P > 0.05$).

3.3. The effect of NCLE on the antioxidant capacity of broiler muscle

Dietary NCLE supplementation enhanced the antioxidant capacity of both BM and LM. For BM (Fig. 1A), compared with the CON group, the T-AOC and the concentrations of GSH-Px and CAT were significantly increased in the N3 group ($P < 0.05$), while the T-AOC was significantly increased in the N2 group ($P < 0.05$). Additionally, SOD and GSH activity showed an increasing tendency, while MDA contents showed a decreasing tendency in the NCLE group relative to that in the CON group ($P > 0.05$). Similar differences in the antioxidant capacity of the LM were observed between the CON and the NCLE treatment groups (Fig. 1B). The T-AOC and the activities of GSH-Px and CAT were all higher ($P < 0.05$), whereas the MDA content was lower ($P < 0.05$), in the N3 group than in the CON group. Additionally, T-AOC was higher and the MDA concentration was lower ($P < 0.05$) in the N2 group than in the CON group. No significant difference in SOD or GSH activity in the LM was observed among the treatments.

3.4. The relative mRNA expression of genes in the p38 MAPK/Nrf2/ARE signaling pathway

The relative mRNA expression levels of factors involved in the p38 MAPK/Nrf2/ARE signaling pathway are shown in Fig. 2A and B. In both BM and LM, the relative mRNA expression levels of most genes in the p38 MAPK/Nrf2/ARE signaling pathway evaluated were significantly upregulated in the NCLE supplementation groups, especially the N3 group. For BM, the relative mRNA expression levels of *p38 MAPK*, *ERK1/2*, *JNK*, *Nrf2*, *CAT*, and *GSH-Px* in the N3 group were significantly upregulated ($P < 0.05$) when compared with those in the CON group. Furthermore, compared with the controls, the relative mRNA expression levels of *p38 MAPK*, *ERK1/2*, *JNK*, *Nrf2*, and *SOD* were significantly upregulated ($P < 0.05$) in the N2 treatment group, while those of *ERK1/2*, *JNK*, *Nrf2*, and *SOD* was significantly upregulated in the N1 group ($P < 0.05$). For LM, meanwhile, compared with the CON group, the relative mRNA expression levels of *JNK*, *Nrf2*, *HO-1*, *CAT*, and *SOD* were significantly upregulated ($P < 0.05$) in the N3 group, those of *p38 MAPK* and *JNK* were significantly upregulated in the N2 group ($P < 0.05$), and those of *ERK1/2*, *JNK*, and *Nrf2* were significantly downregulated in the N1 group ($P < 0.05$).

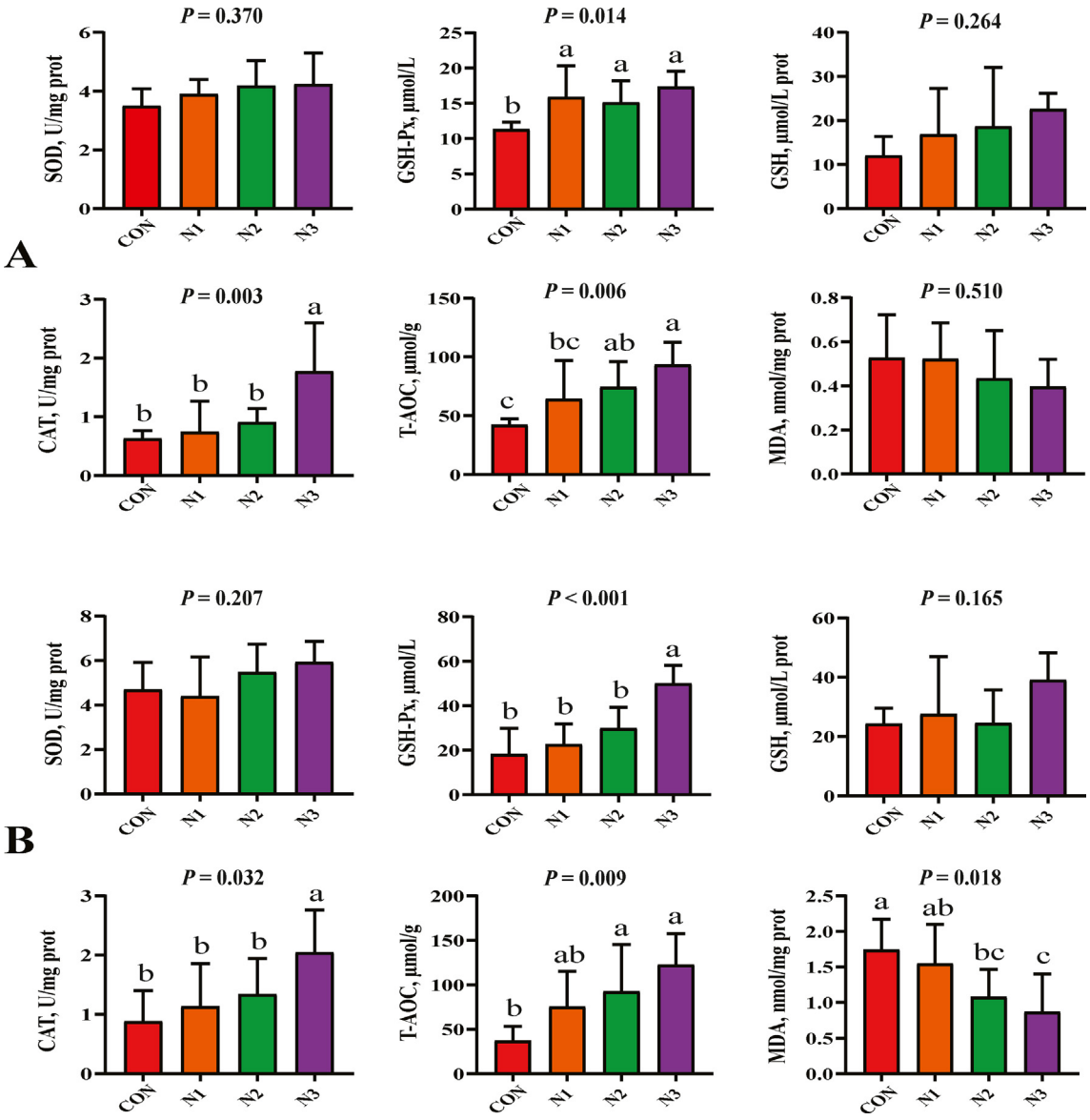

Fig. 1. The antioxidant capacity of breast muscle (A) and leg muscle (B). CON = feed basal diet; N1, N2 and N3 = feed basal diet with 100, 200 and 400 mg/kg *Neolamarckia cadamba* leaves extract; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; GSH = glutathione; CAT = catalase; T-AOC = total antioxidant capacity; MDA = malondialdehyde.

3.5. Spearman's correlation analysis between meat quality and antioxidant parameters

Spearman's correlation analysis was conducted to examine the relationship between meat quality and antioxidant parameters. Correlation coefficients (r) greater than 0.40 or lower than −0.40 were considered statistically significant if the corresponding P-values were lower than 0.05. For BM (Fig. 3A), the pH value showed a positive correlation with GSH-Px activity ($r = 0.45$, $P < 0.01$) and the mRNA levels of *HO-1* ($r = 0.45$, $P < 0.05$), cooking loss showed a positive correlation with the MDA content ($r = 0.41$, $P < 0.05$), and shear force showed a negative correlation with GSH-Px activity ($r = -0.48$, $P < 0.05$) and the mRNA expression levels of *Nrf2* ($r = -0.42$, $P < 0.05$). For LM (Fig. 3B), the pH value showed a positive correlation with *CAT* mRNA levels ($r = 0.41$, $P < 0.05$); cooking loss and shear force both showed a negative correlation with SOD activity ($r = -0.46$, $P < 0.05$ and $r = -0.47$, $P < 0.05$, respectively); yellowness (b^*) showed a negative correlation with SOD activity ($r = -0.50$, $P < 0.05$) and *CAT* mRNA expression levels ($r = -0.41$, $P < 0.05$); drip loss at 24 h showed a highly negative correlation with the mRNA expression levels of *HO-1* ($r = -0.63$, $P < 0.001$) and *GSH-Px* ($r = -0.71$, $P < 0.001$); and drip loss at 48 h showed a negative correlation with the mRNA levels of *CAT* ($r = -0.46$, $P < 0.05$), *JNK* ($r = -0.49$, $P < 0.05$), *Nrf2* ($r = -0.55$, $P < 0.01$), *HO-1* ($r = -0.50$, $P < 0.05$), *GSH-Px* ($r = -0.54$, $P < 0.01$), and *SOD* ($r = -0.49$, $P < 0.05$).

3.6. Metabolomic analysis of breast muscle and leg muscle

The composition and proportion of metabolites in BM and LM are depicted in the stacked column charts in Fig. 4 (the raw data are shown in Table S4). The identified metabolites were dominated by benzene and its substituted derivatives (23.46%), followed by heterocyclic compounds (14.41%); organic acids and their derivatives (14.41%); amino acids and their metabolites (8.98%); and aldehydes, ketones, and esters (8.74%). As shown in Fig. 5A and C, the PCA indicated that there was an obvious separation between the CON and NCLE groups, especially for LM. Additionally, PC1 and PC2 of BM/LM

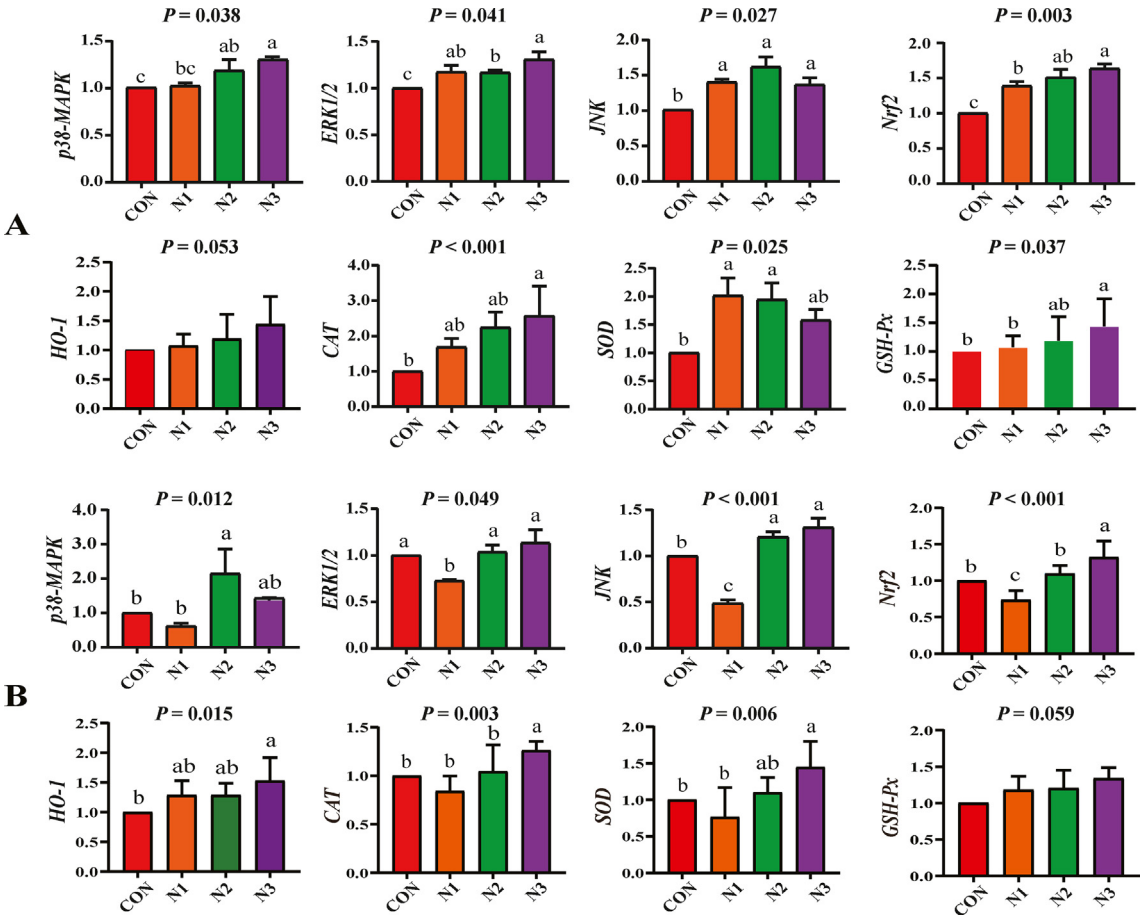

Fig. 2. The relative mRNA expression of MAPK-Nrf2-ARE signaling pathway of breast muscle (A) and leg muscle (B). CON = feed basal diet; N1, N2 and N3 = feed basal diet with 100, 200 and 400 mg/kg *Neolamarckia cadamba* leaves extract; *p38 MAPK* = p38 mitogen-activated protein kinase; *ERK1/2* = extracellular-signal regulated kinase; *JNK* = c-Jun N-terminal kinase; *Nrf2* = nuclear factor-erythroid 2-related factor 2; *HO-1* = heme oxygenase 1; *CAT* = catalase; *SOD* = superoxide dismutase; *GSH-Px* = glutathione peroxidase.

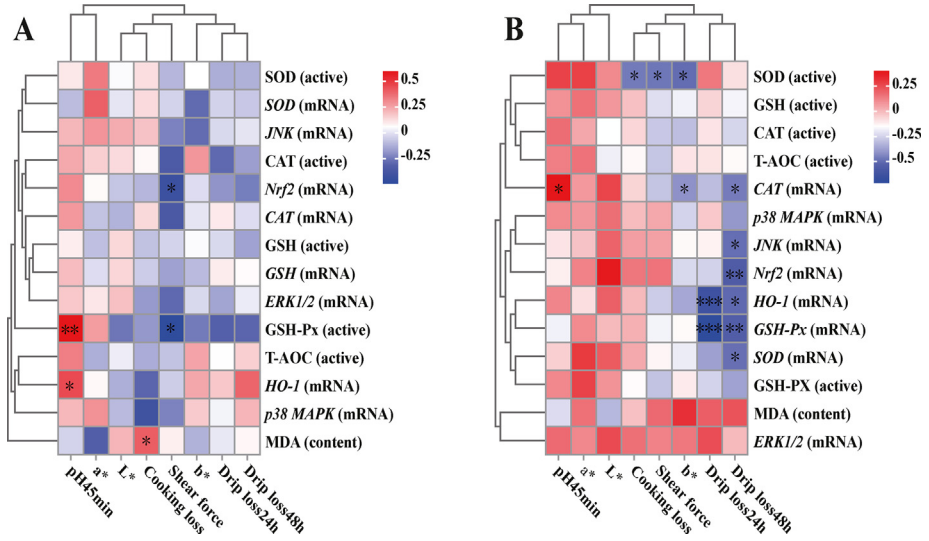

Fig. 3. Spearman's correlation analysis among meat quality and antioxidant parameters of breast muscle (A) and leg muscle (B). SOD = superoxide dismutase; JNK = c-Jun N-terminal kinase; CAT = catalase; Nrf2 = nuclear factor-erythroid 2-related factor 2; GSH = glutathione; ERK1/2 = extracellular-signal regulated kinase; GSH-Px = glutathione peroxidase; T-AOC = total antioxidant capacity; HO-1 = heme oxygenase 1; p38 MAPK = p38 mitogen-activated protein kinase; MDA = malondialdehyde. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

explained 17.88%/14.95% and 16.34%/11.94% of the total variation, respectively. Moreover, the OPLS-DA plot for BM and LM (Fig. 5B and D) showed strong clustering and clear separation between the CON and the NCLE groups, indicating that the addition of NCLE to the diet

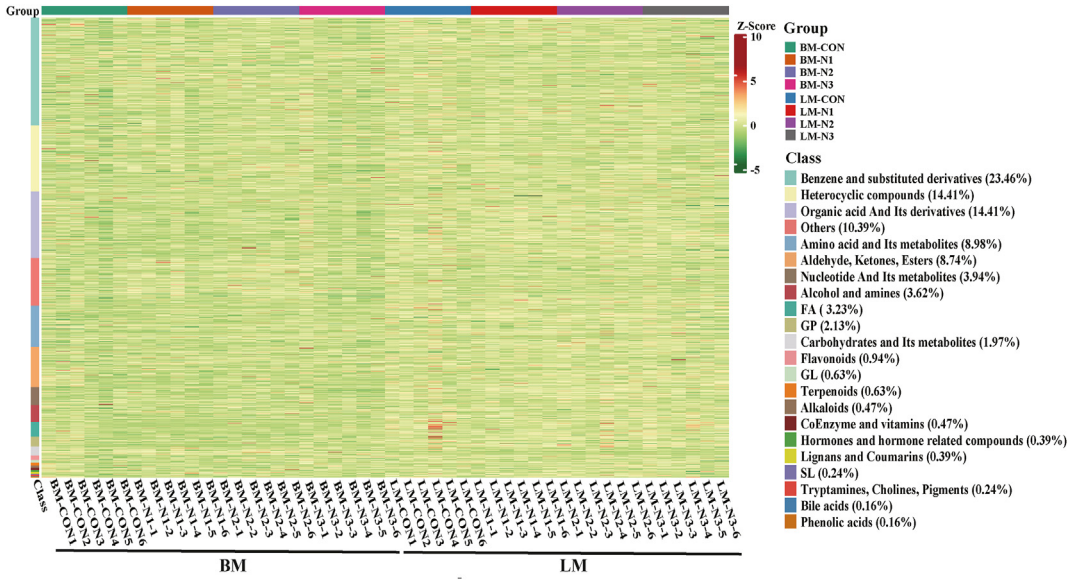

Fig. 4. The biochemical categories of the metabolites identified in breast muscle and leg muscle. BM = breast muscle; LM = leg muscle; CON = feed basal diet; N1, N2 and N3 = feed basal diet with 100, 200 and 400 mg/kg *Neolamarckia cadamba* leaves extract; FA = fatty acid; GP = glycerophospholipid; GL = glycerolipid; SL = sphingolipid.

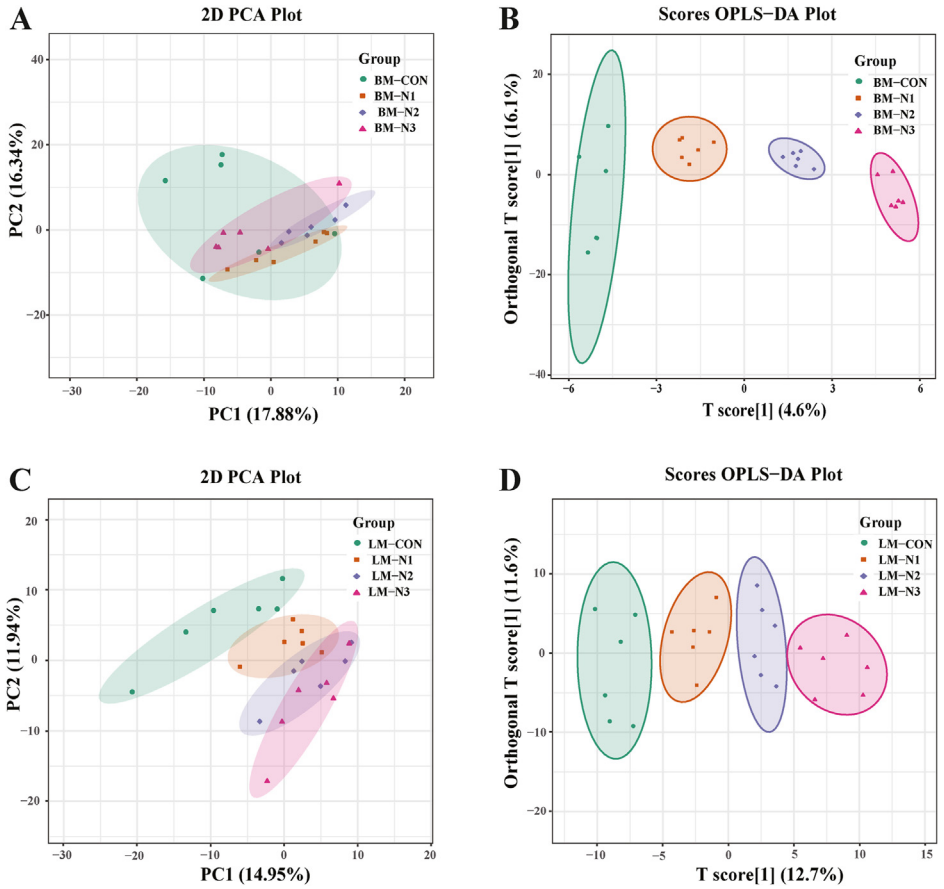

Fig. 5. The principal components analysis score plots (A and C) and orthogonal partial least-squares discriminant analysis scores plots (B and D) based on metabolite profiles of breast muscle and leg muscle. BM = breast muscle; LM = leg muscle; CON = feed basal diet; N1, N2 and N3 = feed basal diet with 100, 200 and 400 mg/kg *Neolamarckia cadamba* leaves extract.

influenced the metabolite profiles of BM and LM. Heat maps of the differential metabolites in the two muscle types are shown in Fig. 6A and B (the raw data are shown in Table S5). The top 24 differential metabolites in BM were classified as amino acids and their metabolites, heterocyclic compounds, organic acids and their derivatives, fatty acids, and nucleotides and their metabolites. In LM, meanwhile,

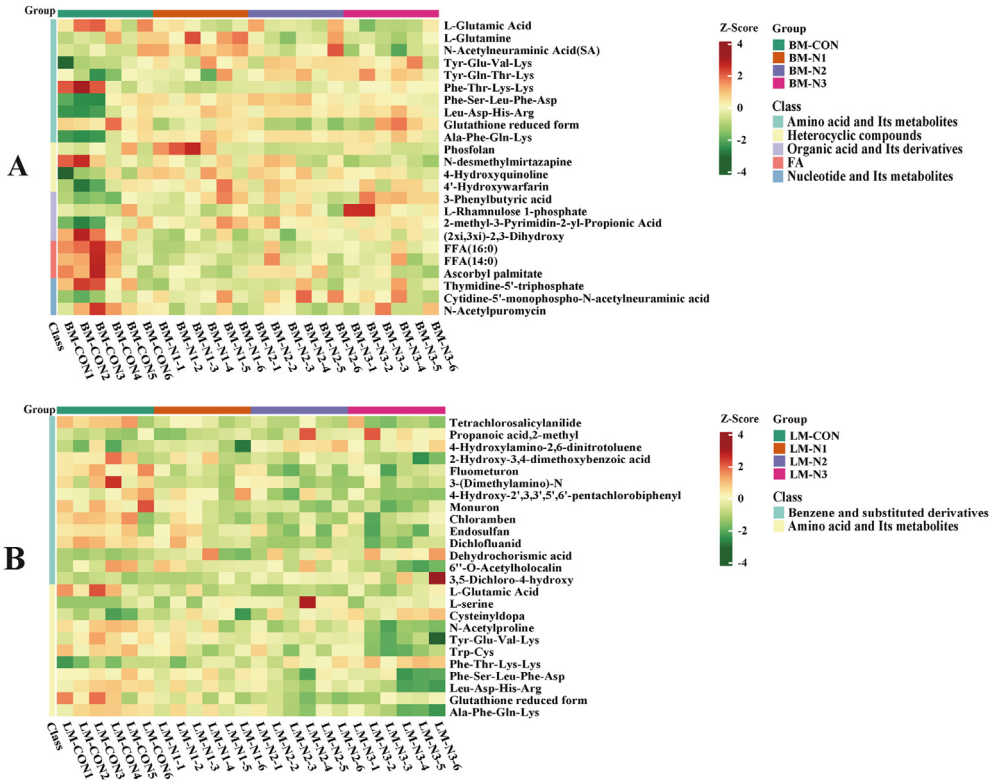

Fig. 6. The differential metabolites heatmap of breast muscle (A) and leg muscle (B). BM = breast muscle; LM = leg muscle; CON = feed basal diet; N1, N2 and N3 = feed basal diet with 100, 200 and 400 mg/kg *Neolamarckia cadamba* leaves extract.

the top 25 differential metabolites were categorized as benzene and its substituted derivatives and amino acids and their metabolites. The differences in the effects of NCLE on BM and LM quality might be due to the variation in metabolite abundance. To visualize the metabolites exhibiting differential abundance between the CON and NCLE treatment groups, Venn diagrams were drawn and are shown in Fig. 7A and B (the raw data are shown in Table S6). For BM, the results showed that lyso-PAF C-16-d4 abundance was higher in both the N2 and N3 groups than in the CON group, suggesting that these may be key metabolites associated with the improvements in BM quality. For LM, L-serine contents were increased, whereas D-fructose 1,6-diphosphate levels were decreased in the 3 NCLE treatment groups relative to those in the CON group. Meanwhile, we also conducted Spearman's correlation analysis between meat quality parameters and differential metabolites. Regarding BM (Fig. 7C), the pH value showed a negative correlation with artomunoxanthentrione epoxide ($r = -0.42$, $P < 0.05$), cooking loss showed a negative correlation with lyso-PAF C-16-d4 ($r = -0.47$, $P < 0.05$), shear force showed a negative correlation with D-arabinono-1,4-lactone ($r = -0.45$, $P < 0.05$), and yellowness (b^*) showed a positive correlation with psilocybin ($r = 0.54$, $P < 0.01$). For LM (Fig. 7D), the pH value showed a negative correlation with p-hydroxyphenylethanolamine ($r = -0.41$, $P < 0.05$); yellowness (b^*) showed a negative correlation with citric acid ($r = -0.43$, $P < 0.05$), 3,5,7-trihydroxy-6-methoxy-4H-chromen-4-one ($r = -0.41$, $P < 0.05$), and oxidized photinus luciferin ($r = -0.53$, $P < 0.01$); drip loss at 24 h showed a negative correlation with citric acid ($r = -0.52$, $P < 0.01$) and a positive correlation with D-fructose 1,6-diphosphate ($r = 0.49$, $P < 0.05$); cooking loss showed a negative correlation with FAA (18:1) ($r = -0.41$, $P < 0.05$) and a positive correlation with isoimide ($r = 0.42$, $P < 0.05$) and oxidized photinus luciferin ($r = 0.50$, $P < 0.05$); and shear force showed a negative correlation with 2-methoxy-6-methylbenzoic acid ($r = -0.41$, $P < 0.05$). Therefore, the changes in metabolite abundance in the NCLE groups may explain the observed improvements in meat quality.

The 20 metabolic pathways in BM (sorted according to *P*-value, from lowest to highest) identified through KEGG enrichment analysis are shown in Fig. 8. The pathways that were significantly influenced by NCLE supplementation included 10 pathways associated with "metabolism" (metabolic pathways; pyrimidine metabolism; nitrogen metabolism; glyoxylate and dicarboxylate metabolism; glutathione metabolism; fatty acid biosynthesis; D-amino acid metabolism; arginine biosynthesis; alanine, aspartate and glutamate metabolism; and C5-branched dibasic acid metabolism), four pathways related to "organismal systems" (proximal tubule bicarbonate reclamation, protein digestion and absorption, glutamatergic synapse, and GABAergic synapse), three pathways related to "human diseases" (amyotrophic lateral sclerosis, amphetamine addiction, and alcoholism), one associated with "environmental information processing" (ABC transporters), and one with "cellular processes" (ferroptosis) (Fig. 8A). The top 20 pathways affected by NCLE supplementation in LM included eight linked with "human diseases" (aminoacyl-tRNA biosynthesis, alcoholism, amphetamine addiction, amyotrophic lateral sclerosis, antifolate resistance, central carbon metabolism in cancer, chemical carcinogenesis-reactive oxygen species, and cocaine addiction), six associated with "metabolism" (biosynthesis of cofactors, porphyrin metabolism, glutathione metabolism, cysteine and methionine metabolism, C5-branched dibasic acid metabolism, and cholesterol metabolism), four with "organismal systems" (bile secretion, proximal tubule bicarbonate reclamation, protein digestion and absorption, and circadian entrainment), one with "environmental information processing" (ABC transporters), one with "genetic information processing" (aminoacyl-tRNA biosynthesis), and one

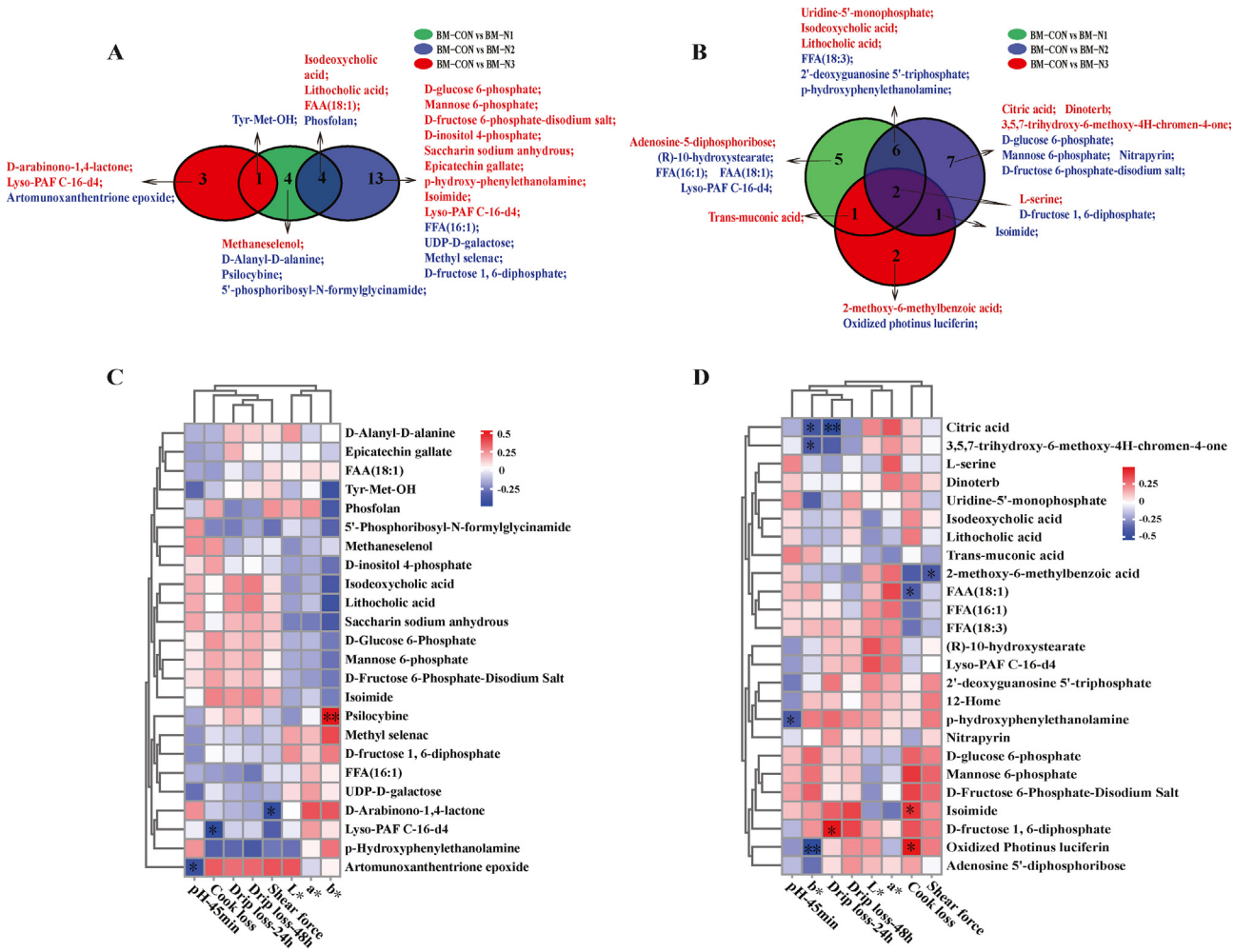

Fig. 7. The Venn diagram of pairwise comparative about differential metabolites of breast muscle (A) and leg muscle (B) and the Spearman's correlation analysis among meat quality and differential metabolites of breast muscle (C) and leg muscle (D). BM = breast muscle; LM = leg muscle; CON = feed basal diet; N1, N2 and N3 = feed basal diet with 100, 200 and 400 mg/kg *Neolamarckia cadamba* leaves extract; differential metabolites were determined by VIP $\geq$ 1, *P*-value < 0.05 with Student's *t*-test and absolute Log_2 FC $\geq$ 1.0; metabolites in red were up-regulated and metabolites in blue were down-regulated; *, $P < 0.05$; **, $P < 0.01$.

with "cellular processes" (ferroptosis) (Fig. 8C). Meanwhile, the numbers of differential metabolites in BM and LM corresponding to the KEGG pathways are shown in Fig. 8B and D, respectively. The enriched differential metabolites in KEGG pathways in BM were dominated by L-glutamic acid, L-glutamine, and reduced glutathione while in LM they were dominated by L-glutamic acid, L-serine, and reduced glutathione.

4. Discussion

Plant extracts comprise secondary metabolites produced during plant growth processes. They contain a variety of bioactive ingredients, such as polysaccharides, alkaloids, phenols, and flavonoids. These extracts are widely used in the livestock industry to improve growth and productive performance (Corino et al., 2021; Liu and Kim, 2023; Song et al., 2023). In this study, NCLE was found to be particularly rich in flavonoids. These polyphenolic compounds can scavenge free radicals and have been reported to exert positive effects on the health of chickens (Ding et al., 2023), weaned piglets (You et al., 2023), and ruminants (Ochoa-García et al., 2021). Additionally, NCLE was also found to contain phenols, including tannins and chlorogenic acid. Lee et al. (2021) and Buyse et al. (2022) both reported that chestnut tannins influenced the composition of the intestinal microbiome of broilers and enhanced their intestinal growth; in contrast, excess tannins were shown to exert a negative effect on nutrient digestion and absorption, especially for protein (Buyse et al., 2021), and the suitable administered dose was below 20 g/kg (Iji et al., 2003). In our study, dietary NCLE supplementation enhanced the growth performance of broilers, namely, it increased the final weight of the birds and significantly decreased the F/G ratio. This improvement may be attributable to the positive impact of NCLE on gut health, including gut microbial balance and intestinal integrity, which led to enhanced nutrient absorption. Additionally, there are notable interactions between the gut microbiota and amino acid metabolism and utilization (Abdallah et al., 2020). Combined, these observations indicate that NCLE has the potential for use as a feed additive, and further research is needed to explore its effects on gut health.

As living standards rise, consumers increasingly focus on meat quality. However, several factors, such as high-density feeding, excess environmental nitrogen content, and heat stress, can result in increased reactive oxygen species production as well as accelerate meat spoilage after broiler slaughter (He et al., 2019). Effective measures such as regulating feeding conditions and supplying antioxidant additives have been widely used in intensive feeding to mitigate these deleterious effects. Plant extracts are known for their

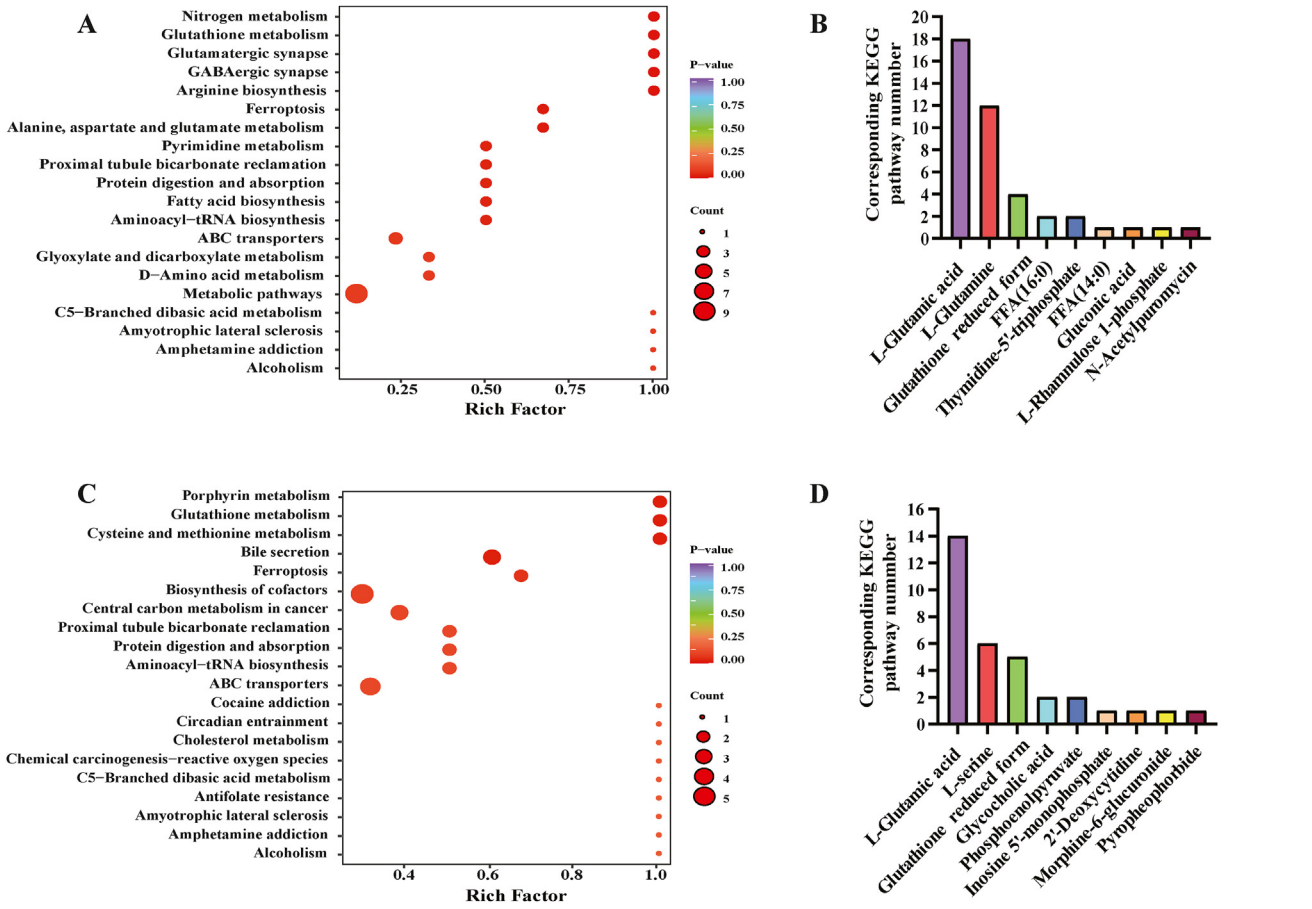

Fig. 8. The KEGG enrichment analysis of metabolic pathways (top 20) in breast muscle (A) and leg muscle (C) and the differential metabolites enriched number of KEGG pathway for breast muscle (B) and leg muscle (D). ABC transporters = ATP-binding cassette transporters.

eco-friendliness, health-promoting effects, low cost, and effective free radical scavenging ability and have a significant capacity for improving meat quality (Shah et al., 2014). The evaluation of the quality of fresh meat commonly includes the determination of parameters such as pH, meat color, water-holding capacity, and shear force (Liu et al., 2021). In our study, the pH of BM was significantly higher in the N2 and N3 groups than in the CON group, indicating that 200 to 400 mg/kg NCLE inhibited glycolysis and delayed the decline in pH after slaughter. Zhang et al. (2012) noted that the post-slaughter pH value is a crucial index that directly reflects the glycolysis process of meat and is closely related to meat quality. In a study investigating the effects of supplementing the diet of broilers with chitosan oligosaccharides, it was observed that the pH of BM increased at both the 45-min and 24-h sampling times (Chang et al., 2022). Additionally, there was a noticeable change in meat color after slaughter, characterized by higher L^* and lower a^* values, which can be attributed to enhanced light scattering resulting from the denaturation of sarcoplasmic proteins (Barbut et al., 2008). The groups supplied with NCLE exhibited higher a^* and lower b^* values in both BM and LM. The water-holding capacity of fresh meat, a crucial quality characteristic, is closely related to tenderness and can be assessed through the determination of the cooking and drip loss rates (Mir et al., 2017). In this study, there was an increase in water-holding capacity and tenderness with NCLE supplementation, with the N2 and N3 groups showing a significant decrease in shear force in BM and drip loss (48 h) in LM. The above results suggested that the addition of 200 to 400 mg/kg NCLE to the diet of broilers notably improved the juiciness, tenderness, and shelf life of BM and LM.

The overproduction of free radicals, which occurs when the levels of oxidants exceed those of antioxidants, leads to oxidative stress (Chen et al., 2021b). Generally, the antioxidant capacity is evaluated through the determination of antioxidant parameters such as T-AOC and SOD, GSH-Px, GSH, and CAT activities. Meanwhile, oxidative stress is an unavoidable consequence of high-density cage breeding for broilers, which results in the excessive production of reactive oxygen species and, consequently, a decline in meat quality (Zhang et al., 2022c). Antioxidants in poultry can convert superoxide radicals to H_2O_2 and then to H_2O. In the current study, antioxidant activity (T-AOC and the contents of GSH-Px and CAT) increased in the N3 group, indicating that the dietary supplementation of 400 mg/kg NCLE improved the antioxidant capacity of both BM and LM. Moreover, the content of MDA, regarded as a marker of oxidative stress, was decreased in the N3 group, thus further explaining the antioxidant capacity-enhancing effect of NCLE. This may be due to the abundance of bioactive compounds in the extract, which is supported by the fact that the addition of 1,000 mg/kg chlorogenic acid-enriched extract from *Eucommia ulmoides* leaf to the diet of broilers was reported to increase SOD activity and decrease the MDA content (Zhao et al., 2019). Shi et al. (2022) also reported that the dietary supplementation of total flavonoids derived from *Artemisia ordosica* mitigated the decrease in antioxidant activity and the overproduction of MDA in serum, liver, and spleen in lipopolysaccharide-challenged broilers. The MAPK/

Nrf2/ARE signaling pathway plays an important regulatory role during the antioxidant process. Nrf2 is a redox-sensitive nuclear transcription factor that binds to ARE in the nucleus and activates the expression of genes encoding key antioxidant enzymes such as *HO-1*, *CAT*, *SOD*, and *GSH-Px*, thus playing a protective role in cells (Zhang et al., 2019). MAPK is a critical ubiquitous intracellular kinase that phosphorylates Nrf2, while p38 MAPK, ERK1/2, and JNK are the most widely characterized MAPKs (Zhang et al., 2018). Studies have reported that the MAPK signaling pathway can activate and modulate Nrf2/ARE (Hu et al., 2020). In this study, dietary NCLE supplementation, especially at the 400 mg/kg concentration, upregulated the relative expression of genes in the MAPK/Nrf2/ARE signaling pathway both in BM and LM. Similar results were reported by Shi et al. (2022), who demonstrated that the supplementation of broiler diets with total flavonoids extracted from *A. ordosica* improved antioxidant capacity by alleviating a decrease in the mRNA expression of *Nrf2*, *CAT*, *SOD*, and *GSH-Px* in broilers challenged with lipopolysaccharide. The tenderness of muscle tissue is closely related to its protein content and metabolic levels. Oxidative stress resulting from lipid peroxidation can degrade muscle tissue protein, resulting in reduced tenderness and poor meat quality (Zhang et al., 2013). Additionally, reactive oxygen species can oxidize lipids and proteins on cell membranes, leading to their disruption, leakage of cellular fluid, and reduced water-holding capacity in muscle tissue, which also negatively affects meat quality (Bejaoui et al., 2023). These results further support that the improvement in meat quality observed following NCLE supplementation was due to the enhancement of antioxidant capacity. Furthermore, the results of Spearman's correlation analysis between meat quality and antioxidant parameters showed that pH was positively correlated with some antioxidant activities and the mRNA expression of genes in the MAPK/Nrf2/ARE signaling pathway, whereas water-holding capacity and shear force were negatively correlated with certain antioxidant parameters. These observations further support that NCLE can modulate the MAPK/Nrf2/ARE signaling pathway and enhance antioxidant capacity, ultimately improving the meat quality of both BM and LM.

Numerous metabolites can influence meat quality and flavor. In our study, HPLC—MS-based metabolomic analysis was used to identify differential metabolites in BM and LM between the CON and NCLE treatment groups. Interestingly, the composition and proportions of the identified metabolites in BM and LM were wholly consistent. In both groups, the top five differentially abundant metabolites were benzene and its substituted derivatives, heterocyclic compounds, organic acids and their derivatives, amino acids and their metabolites, and aldehyde/ketones/esters. Li et al. (2022) and Liu et al. (2021) also reported that these metabolites were dominant in chicken meat. The PCA results revealed that dietary NCLE supplementation significantly altered the abundance of the identified metabolites in BM and LM. The OPLS-DA results further indicated that the metabolic profiles of BM and LM differed significantly from each other according to the dietary supplementation level. A heat map of the differential metabolites among the different NCLE supplementation groups indicated that the differential metabolites in BM were mainly classified as amino acids and their metabolites, heterocyclic compounds, organic acids and their derivatives, fatty acids, and nucleotides and their metabolites. Meanwhile, the differential metabolites in LM were primarily categorized as benzene and its substituted derivatives and amino acids and their metabolites. Amino acids and their metabolites are decisive for broiler meat quality, which is mainly influenced by breed, age, growth conditions, feed, and additives (Deng et al., 2022; Li et al., 2022; Jayasena et al., 2014). The results of this study clearly showed that the BM peptides (Phe-Ser-Leu-Phe-Asp, Leu-Asp-His-Arg, and Ala-Phe-Gln-Lys) were more abundant in the NCLE groups than in the CON group. Peptides are not only necessary for the biosynthesis of proteins and other factors, but also have important physiological functions, including in immunoregulation (antibacterial, anti-inflammatory, and antioxidant activities), hypertension, diabetes, obesity, and cancer (Jia et al., 2021). Thus, the greater peptide abundance in the NCLE groups could explain their better meat quality and antioxidant capacity performance relative to the CON group. Moreover, the abundance of fatty acid metabolites [FAA (16:0), FFA (14:0), and ascorbyl palmitate] decreased with NCLE supplementation, possibly due to the inhibition of the fatty acid oxidation in the NCLE groups, leading to reduced FFA generation. The above results indicated that dietary NCLE supplementation can improve broiler meat quality by influencing the composition and abundance of muscle metabolites. Notably, some differential metabolites in LM were classified as pesticides (fluometuron, monuron, chloramben, endosulfan, and dichlofluanid) and their abundance decreased with the addition of NCLE to the diet. This showed that dietary NCLE supplementation can improve meat safety by reducing the levels of pesticide residues in livestock products; however, the associated mechanisms of action need further clarification.

In this study, the Venn diagram comparing metabolites of BM and LM revealed specific differential metabolites between each group. Lyso-PAF C-16-d4 was found to be upregulated in LM in both the N2 and N3 groups. This metabolite is considered a biologically inactive product resulting from the conversion of phospholipid platelet-activating factor (PAF) by phospholipase A2 during the inflammatory response (Gao et al., 2022). Gavriil et al. (2019) reported that the consumption of a plant extract supplement increased PAF catabolism through lyso-PAF acetyltransferase, which suggests that the increased levels of lyso-PAF C-16-d4 in the N2 and N3 groups may reflect the potential anti-inflammatory effect of NCLE. Oxidative stress is commonly associated with inflammation (Abu et al., 2023), and the increased abundance of lyso-PAF C-16-d4 could be related to this. Additionally, the results of Spearman's correlation analysis showed the existence of a negative correlation between lyso-PAF C-16-d4 and cooking loss, which could explain the decreased cooking loss in BM of the N2 and N3 treatment groups. D-Arabinono-1,4-lactone oxidase is a FAD-dependent oxidoreductase that functions synergistically with glycosyltransferase (Avalon et al., 2021). In the N3 group, D-arabinono-1,4-lactone was upregulated and showed a negative correlation with shear force, suggesting that it may contribute to improving antioxidant capacity and meat quality. Psilocybin, a psychoactive alkaloid extracted from mushrooms (Calder and Hasler, 2023), has shown potential in treating depression, anxiety, and addiction; however, little is known about its impact on meat quality. D-Fructose 1,6-diphosphate is an important intermediate metabolite in glycolysis and gluconeogenesis (Shen et al., 2022; Zhang et al., 2021b), while L-serine is a precursor for several amino acids and metabolites, and its levels significantly influence the biosynthesis of purines and pyrimidines (Weigand et al., 2020). In this study, the observed increase in the abundance of L-serine and the decline in D-fructose-1,6-diphosphate contents in the LM indicated that dietary NCLE supplementation improved amino acid metabolism and inhibited glyco-metabolism. Furthermore, the positive correlation detected between D-fructose 1,6-diphosphate and drip loss could explain the improvement in LM quality.

To further explore the most relevant pathways in BM and LM, we submitted all the metabolites to KEGG pathway analysis. The most enriched KEGG pathways in BM (metabolic pathways, ABC transporters, and D-amino acid metabolism) and LM (biosynthesis of cofactors, ABC transporters, and protein digestion and absorption) were frequently reported in previous studies (Liu et al., 2021; Zhang et al., 2021a; Ge et al., 2022). Moreover, L-glutamic acid,

L-glutamine, and reduced glutathione, differentially abundant metabolites in BM, were involved in 18, 14, and 4 KEGG pathways, respectively. Meanwhile, L-glutamic acid, L-serine, and reduced glutathione, which showed differential abundance in LM, were found to be involved in 14, 6, and 5 KEGG pathways, respectively. Glutamic acid is the key component of muscle protein and is regarded as a crucial amino acid for meat umami taste enhancement (Hu et al., 2021), while serine is directly related to the sweet taste of meat (Zhang et al., 2022a). Glutamic acid is also a constituent amino acid of glutathione, a well-characterized antioxidant (Shimamoto et al., 2020). Glutamine is not only a substrate for protein biosynthesis and de novo nucleotides biosynthesis (Han et al., 2019) but also contributes to enhancing immunoglobulin levels (Zhou et al., 2019) as well as muscle repair and building (Durainayagam et al., 2019). Notably, the above-mentioned differential metabolites were classified as amino acids and their metabolites, indicating that NCLE supplementation to broiler diets influenced meat quality through its regulatory effects on amino acid metabolism pathways. These effects may involve the gut microbiome and gut morphology and lead to improved feed protein utilization and amino acid transport for muscle protein synthesis. Several studies have reported that the addition of plant extracts to the diet improved feed efficiency, cecal amino acid metabolism capacity, and muscle amino acid contents (Fortuoso et al., 2019; Liu et al., 2020). This suggests that the improvement in meat quality observed with NCLE supplementation may be due to the regulatory effect of NCLE on muscle amino acid metabolism.

5. Conclusion

In the current study, we demonstrated that dietary NCLE supplementation greatly improved the meat quality of BM (higher pH_{45min} and lower shear force) and LM (lower drip loss at 48 h). The antioxidant capacity of BM was significantly enhanced in the N3 group, characterized by increases in the T-AOC, the concentrations of GSH-Px and CAT, and the relative mRNA expression levels of *p38 MAPK*, *ERK1/2*, *JNK*, *Nrf2*, *CAT*, and *GSH-Px*. Similarly, LM in the N3 group displayed higher T-AOC, increased GSH-Px and CAT activities, lower MDA contents, and upregulation of the relative mRNA expression levels of *JNK*, *Nrf2*, *HO-1*, *CAT*, and *SOD*. Metabolomics analysis revealed that D-arabinono-1,4-lactone and lyso-PAF C-16-d4 were negatively correlated with shear force and cooking loss and displayed increased abundance in BM of the N3 group. The contents of L-serine were upregulated and those of D-fructose-1,6-diphosphate were downregulated in the 3 NCLE treatment groups. Finally, the differential metabolites in BM and LM were found to be involved in amino acid metabolism pathways. Combined, our results indicated that dietary NCLE supplementation improved meat quality by enhancing antioxidant enzyme activities, promoting the expression of genes involved in the p38 MAPK/Nrf2/ARE signaling pathway, and regulating amino acid metabolism. The optimal level of NCLE supplementation to the diet of broilers was found to be 400 mg/kg.

Author contributions

Cheng Wang: Data curation, Writing-Original draft preparation. **Dandan Chen:** Methodology, Data curation. **Shuo Wu:** Software, Validation. **Wei Zhou:** Visualization, Investigation. **Xiaoyang Chen:** Supervision, Funding acquisition. **Qing Zhang:** Formal analysis, Supervision. **Li Wang:** Supervision, Funding acquisition.

Declaration of competing interest

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

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Appendix supplementary data

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