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Research article

Walnut meal improves meat quality by modulating intestinal microbes in white feather broilers

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ABSTRACT

Improving the number of amino acids and unsaturated fatty acids in the diet is a good way to raise the quality of the meat. Currently, most research on the quality of broiler meat focuses on genetic traits; nevertheless, it is unclear how meat quality is regulated. This experiment was conducted to investigate the effects of different supplemental levels of walnut meal (WM) on growth performance, amino acid and fatty acid composition, microbial composition, and meat quality of white feather broilers. 1 week old white feather broilers (n = 120; Body weight 83.76 ± 2.32 g), were randomly divided into 3 treatments and 4 replicates. Walnut meal of basic diet (CK), 5 %(WM-L) and 10 %(WM-H) were added to the diets of white feather broilers, respectively. The results showed that walnut meal could increase L* 24 h (24 h brightness) of breast muscle of white feathered broilers ($p < 0.05$). The amount of essential amino acids (e.g., isoleucine, methionine, leucine, tryptophan, and phenylalanine), umami amino taste acids (glutamic acid), and PUFA/SFA (polyunsaturated fatty acid) (n-3PUFA and n-6 PUFA) in breast muscle increased as the dose was increased. Furthermore, walnut meal regulated amino acid flavour metabolism by increasing the relative abundance of *Bacteroides*, *bifidobacterium*, and *enterococcus faecalis*, according to 16S rRNA sequencing and functional prediction analysis. The correlation showed that amino acid and fatty acid composition was one of the key factors affecting pH value, meat color and tenderness of chicken. In conclusion, dietary addition of walnut meal can increase the content of essential amino acids and unsaturated fatty acids and the relative abundance of beneficial bacteria of broilers, which is of great significance for improving meat quality of white feather broilers.

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1. Introduction

Since the nutritional value of feed and market price will affect the production and sale of chicken meat, adjusting the nutritional composition is one of the effective means to improve the meat quality of white feather broilers [1]. The use of feed additives and the replacement of some conventional feed are conducive to reducing feed cost and improving meat quality [2,3]. intestinal digestion and absorption function and barrier function are very important for white feather broilers. Especially in the context of "banning feed resistance", the risk of economic losses due to intestinal diseases in the broiler industry is increasing [4]. General feed additives play an increasingly important role in promoting animal growth and development, reducing feed cost, and improving meat quality. Research conducted domestically on general feed additives for white feather broilers has mainly focused on plants and their extracts, as well as probiotics and their metabolites. Studies on plants and their extracts have focused on Chinese herbs, polysaccharides, and essential oils. Many of these studies aim to regulate the body's immune and antioxidant functions. Meanwhile, studies on essential oils and probiotics for broilers have focused on their combined use with other feed additives to alleviate the effects of challenge models for pathogenic bacteria such as *Clostridium perfringens*, pathogenic *Escherichia coli*, and *Salmonella*, whereas feed rich in natural plant protein is not developed and applied [5].

Walnut, also known as *Juglans regia*, is a plant from the walnut family. It has been highly respected since ancient times due to its rich nutritional and medicinal value. Walnuts are not only nutritious but also play a vital role in maintaining the normal physiological function of the human body, promoting growth and development, and enhancing immunity [6]. The walnut industry emerged due to the excellent ecological benefits of walnuts, their rich nutrition and health functions, and the safety of grain and oil. In recent years, the industry has become a pillar in many places, with China ranking first in the world for walnut planting area and output. Yunnan province has the highest production, with a planting area of over 43 million mu [7]. Therefore, the in-depth exploitation of walnut and its by-products is crucial for the healthy and sustainable development of the walnut industry, both socially and economically [8]. Currently, the use of walnut resources in China is primarily limited to the extraction of walnut oil. The remaining walnut meal is often treated as waste and used as feed, fertilizer, or discarded, resulting in a significant waste of resources and environmental pollution. However, recent studies have shown that walnut meal contains up to 40 % protein and is easily digestible. Seventy percent of the protein content is gluten, while 18 % is globulin, 7 % is albumin, and the remaining percentage is composed of other alcohol-soluble proteins [9]. These proportions meet the requirements for a protein-rich diet, where animal proteins should account for half of the total protein intake. The 18 different amino acids in walnut meal are complete, among which the content of arginine, glutamic acid, and aspartic acid is higher [10]. Walnut meal can be used as a high-quality source of feed, effectively solving the protein-feed shortage in China, and improving feed safety and utilization rates. Additionally, it can serve as a theoretical basis for studying the regulation mechanism of walnut meal on meat quality and breeding white feathered broilers in line with dietary health and nutrition requirements.

As people's living standards improve, the demand for high-quality chicken has increased. Currently, there is a significant gap in

Table 1
Nutrient level composition of basic diet.

Ingredients (%)	Iitial period (1–21 days of age)	Fttening period (2–42 days of age)
Corn	58.50	60.10
Limestone	1.60	1.35
Soybean oil	3.04	4.50
Soyabean meal	28.00	30.00
Fermented soybean meal	5.00	–
Salt	0.22	0.26
Threonine	0.10	0.09
Lysine	–	0.10
Methionine	0.14	0.15
Calcium monohydrogen phosphate	1.40	1.45
Premix[a]	2.00	2.00
Total	100	100
Nutrients		
Metabolizable energy[b](/MJ·kg 1)	12.33	12.76
Crude protein	21.04	18.97
Crude fat	5.60	7.06
Calcium	1.00	0.90
Methionine	0.43	0.40
Lysine	1.08	1.01
Threonine	0.83	0.77
Total phosphate	0.65	0.60

Note.

[a] Premix provides vitamin A $_9$ 500 IU, vitamin D_3 500 IU, vitamin E 20 IU, vitamin K 1.2 mg, vitamin B_1 2.2 mg, vitamin B_2 5.0 mg, vitamin B_6 2.0 mg, niacin 30 mg, pantothenic acid 12.0 mg, folic acid 0.8 mg, biotin 0.18 mg, iodine 0.35 mg, selenium 0.30 mg, manganese 100 mg, iron 80 mg, copper 8 mg, and zinc 75 mg. The premix contained no antibiotics or chemically synthesized antibacterial agents. The values presented are measured quantities in percentages.

[b] The values for metabolizable energy were computed, while the levels of other nutrients were determined through measurement.

chicken quality in China. Feed costs make up 60 %–80 % of production costs. However, the rising demand for feed raw materials in China's livestock industry and insufficient domestic supply have led to an increase in the price of feed raw materials such as corn and soybeans, resulting in higher breeding costs. Reducing feeding costs is a prerequisite for maintaining the development of animal husbandry. This study investigates the growth performance and meat quality of white feathered broilers in relation to different doses of walnut meal feed. The aim is to determine whether walnut meal feed can enhance the meat quality of broilers without negatively affecting their health status. Additionally, the study seeks to determine the optimal dose of walnut meal to provide a reference for its development and utilization in the broiler breeding industry.

2. Materials and methods

2.1. Mixed feed preparation

Walnut meal comes from Yunnan Zijiang Food Co., LTD. It is the residue left after walnut oil extraction, which is ground and screened (60 mesh). White feather broilers from Shandong Dacheng Collective Hunan Shuncheng Industrial Co., Ltd. were fed walnut meal powder at a rate of 5 % or 10 % of their basic diet shown in Table 1.

2.2. Design of experiments and collection of samples

Sixty white feather broilers with initial body weight (IBW) = 83.76 ± 2.32 g were randomly divided into 3 groups with 3 replicates per group and 10 broilers per replicate. The experiment was randomly divided into three treatment groups: blank control group (CK: basal diet), WM-L(5 % walnut meal) and WM-H (10 % walnut meal). Broilers were placed in stainless steel cages, naturally ventilated, free to eat and drink, indoor relative humidity was maintained at 50 %, the temperature was maintained at 32–34 °C (1–7 days), and gradually decreased to 23 °C at a rate of 3 °C per week until the end of the experiment.

After 42 days of the experiment, five white feather broilers were randomly selected from each group, and their final weight was recorded after a 12-h fast. The jugular vein was used for bleeding after the shock. The left pectoral muscle of the broilers was used to measure food quality (pH value, color difference, shear force, etc.) and nutrient composition (crude fat and crude protein), while the right pectoral muscle was used to measure fatty acid and amino acid composition. The contents of the cecum were collected, frozen, and stored at −80 °C for further analysis.

2.3. Growth index

The body weight, test days and feed intake of white feather broilers on 42 days were recorded. Average body weight (BW), average daily gain (ADG= (final weight - initial weight)/test days), average daily feed intake (ADFI = total feed intake/test days), feed to gain ratio (F/G = total substance consumption/total weight gain) were calculated [11].

2.4. Meat quality analyses

Take a sample of the left pectoral muscle for color, Indicators such as pH value, drip loss, cooking loss, shear force, fat and protein are processed according to the procedure of Li et al. [12].

2.4.1. pH and meat color

The pH values of the pectoral muscles were measured using a carcass pH-star (MATTHAUS, Germany), and the pectoral muscle's brightness (L*), redness (a*), and yellowness (b*) values were measured using a chromometer (CR-410, Minolta, Japan) at 45 min and 24 h after slaughter. Each meat sample was measured three times.

2.4.2. Drip loss

Measure the weight of the chest muscles of equal size (W1). Suspend the meat in the centre of a plastic cup and leave it to hang for 24 h at 4 °C. After 24 h, wipe the surface of the meat and weigh it (W2), and the calculation formula is as follows:

$$\text{Drip loss}(\%) = (W_1 - W_2) / W_2 \times 100\%$$

2.4.3. Cooking loss

Weigh the meat sample W3 of the same size, put it in a cooking bag, cook it in a water bath for 15 min, cool it to room temperature, remove the water on the surface of the meat, weigh W4, the calculation formula is as follows: and calculate the formula as follows:

$$\text{Cooking loss}(\%) = (W_3 - W_4) / W_3 \times 100\%$$

2.4.4. Shear force

After the cooking loss was measured, the chest muscle was vertically placed on the C-LM3 digital muscle cutter and cut into 3 meat samples of the same size and thickness, and the values were recorded.

2.4.5. Crude protein and fat

(1) The determination of crude protein was carried out using the Kjelhardt nitrogen determination method as outlined in GB5009.5–2016, which is a national standard for food safety. (2) The determination of crude fat was carried out using the Soxhlet extraction method as outlined in GB5009.6–2016, which is also a national standard for food safety [13].

2.5. Determination of the composition of amino acids and fatty acids

Amino acid analysis: A sample of appropriate quantity is taken from the sample container and transferred to a 2 mL centrifuge tube. The tube is then filled with 600 μL of a 10 % acetic acid methanol solution in water (1:1, v/v). A glass bead is added, and the tube is vortexed for 2 min. The sample is then centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant is transferred to a new tube and diluted with 980 μL of the same solution. The sample is then diluted with water (1:1, v/v) solution and 100 μL of the diluted sample is added to 100 μL of the 10 ppb Trp-d3 internal standard. The mixture is then filtered through a 0.22 μm membrane filter and the filtrate is transferred to a test tube for analysis. The test is conducted using 21 amino acid standards (Shanghai Huagong Chemical Reagent Co., Ltd., China) as the reference material [14,15].

The determination of fatty acids was conducted by the following procedure: A quantity of the sample was taken and placed in a 2 mL centrifuge tube, to which 1 mL of chloroform: methanol (2:1) solution was added. This was then mixed with 100 mg of glass beads, and the mixture was subjected to two rounds of grinding. The resulting suspension was then subjected to ultrasonication at room temperature for 30 min at 12,000 rpm. After this, the mixture was centrifuged at 4 °C for 5 min at 1500 rpm. The supernatant was then transferred to a 15 mL centrifuge tube, and 2 mL of 1 % sulfuric acid in methanol solution was added. The samples were mixed and incubated at 80 °C in a water bath for 30 min. Following this, the samples were cooled, and 1 mL of hexane was added for extraction. The samples were then mixed and allowed to settle for 5 min. This was followed by the addition of 5 mL of water (4 °C) for further purification. The samples were then centrifuged at 3500 rpm for 10 min at 4 °C. A 700 μL aliquot of the supernatant was transferred to a 2 mL centrifuge tube, and 100 mg of sodium sulfate was added. The sodium sulfate powder was removed from the solution by evaporation, and the resulting solution was mixed and centrifuged at 12,000 rpm for 5 min. A 200 μL aliquot of the supernatant was transferred to a 2 mL centrifuge tube and mixed with 200 μL of hexane. The mixture was then centrifuged at 12,000 rpm for 5 min. A 300 μL aliquot of the supernatant was transferred to a 2 mL centrifuge tube and mixed with 15 μL of 500 ppm hydroxysuccinic acid. The internal standard, methyl acetate, was mixed and 200 μL of the supernatant was added to the test tube for analysis. Gas chromatography (GC) was performed using a Trace 1300 gas chromatograph (Thermo Fisher Scientific, USA) with a mass spectrometer (MS) detector (TSQ 9000, Thermo Fisher Scientific, USA). The analytical conditions were based on the recommendations of L.R. Hoving and colleagues [16,17].

2.6. Composition of microorganisms in the cecum

The test sample of −80 °C was sent to BioDeep BioInformation Technology (Suzhou, China) for further analysis. The FastDNA®Spin Kit for Soil was used to extract total DNA (Omega, USA) and to detect the purity and concentration of NanoDrop2000 DNA (Thermo Scientific, NC2000). Specific primers (338F and 806R) were used to amplify the V3–V4 region of 16S rRNA gen and using QIIME2 version 2019.4 to Alpha diversity analysis of data, including Chao 1, Shannon, Simpson and Good 's coverage analysis, etc., The raw data was uploaded to the NCBI website with the join number SRA: SRR26909438 to SRR2690945.

2.7. Statistical analyses

The experimental data were preliminarily sorted by Excel and statistically analyzed by SPSS23.0 software and GraphPad Prism 9 software. The data were represented by mean ± standard error (X±SEM). Correlation analysis was performed using the cloud platform of BioDeep Metabolism Detection Company for analysis and mapping. $P > 0.05$ meant no significant difference, and $P < 0.05$ meant significant difference.

Table 2
Effects of different doses of walnut meal on performance of broilers.

Item[a]	Treatments[b]			*p*-value
	CK	WM-L	WM-H	
Initial BW, g	82.79 ± 3.61	82.01 ± 3.21	85.50 ± 3.52	0.761
Final BW, g	1386.41 ± 63.72[a]	1116.68 ± 44.97[b]	1329.66 ± 23.68[a]	0.011
ADFI, g	853.45	673.19	780.87	–
ADG, g	36.21 ± 1.73[a]	28.74 ± 1.26 [b]	34.56 ± 1.58[a]	0.012
F/G ratio	23.78 ± 1.10	23.59 ± 0.95	22.78 ± 1.01	0.768

The values in the table are expressed as a mean ± standard error. [a-b]Means with different letters within a row differ significantly ($p < 0.05$).
[a] BW: body weight; ADFI: average daily feed intake; ADG: average daily gain; F/G: feed/gain ratio.
[b] CK = base diet (SBM); WM-L = SBM+5 % walnut meal; WM-H=SBM+10 % walnut meal.

3. Results

3.1. Effects of different doses of walnut meal on performance of broilers

Growth performance is one of the most important performance indexes. As shown in Table 2, The level of walnut meal supplementation increased, the final weight ($p = 0.011$) and daily gain ($p = 0.012$) of white feathered broilers tended to increase. These results indicate that the addition of walnut meal did not have a negative effect on the growth and development of white feather broilers.

3.2. Effects of different doses of walnut meal on meat quality of broilers

As can be seen from Table 3, compared with the control group, walnut meal increased L* 24 h of breast muscle of broilers at 42 days of age ($p < 0.05$), but there were no significant differences in shear force, dripping loss, cooking loss, pH24 h, breast muscle fat and protein ($p > 0.05$).

3.3. Effects of different doses of walnut meal on amino acid composition of broilers

Compared with the control group, the contents of essential amino acids (isoleucine, methionine, leucine, tryptophan, phenylalanine, arginine, methionine) were increased by addition of walnut meal ($p < 0.05$), while the contents of arginine, homocysteine, glutamine, EAA, FAA and TAA were decreased (Table 4).

3.4. Effects of different doses of walnut meal on fatty acid composition of broilers

In the breast muscle of the white feather broilers, a total of 47 fatty acids were detected. Out of these, 31 fatty acids (such as C18:0, C18:1n-9c, and C20:4n-6) did not show any significant difference in their contents when compared to the control group. However, the contents of the remaining 16 fatty acids showed significant differences ($p < 0.05$). The broiler treatment groups had C16:0, C18:0, C18:1n-9c, and C18:2n-6 as the main fatty acids, which accounted for 80.09 %, 79.32 %, and 78.1 % of the total fatty acids, respectively. Compared to the control group, the breast muscle of the broilers showed a significant increase ($p < 0.05$) in the contents of PUFA, n-3 PUFA, n-6 PUFA, and PUFA/SFA, including essential fatty acids α-linolenic acid (C18:3n-3) and linoleic acid (C18:2n-6) in humans ($p < 0.05$) (Table 5).

3.5. Metabolomics analysis of cecum in broilers

3.5.1. Analysis of intestinal microbial diversity

The study aimed to analyze the effects of varying proportions of walnut meal on the intestinal microbial composition of broilers. To achieve this, the cecal microflora was analyzed using 16SrRNA sequencing. A total of 16,648 OTUs were generated across all samples, with 894 OTUs present in all three groups. The control group had 6607 unique OTUs, while the WM-L and WM-H groups had 3318 and 4311 unique OTUs, respectively (Fig. 1a). To comprehensively evaluate the alpha diversity of microbial communities, Chao and Observed species indices were used to characterize the richness, Shanno and Simpso indices to characterize the diversity, and Pielou's evennes index to characterize the evenness. Good's coverag index representation coverage (The alpha Diversity index can be calculated at: http://scikitbio.org/docs/latest/generated/skbio.diversity.alpha.html#module-skbio.diversity.alpha), compared with the

Table 3
Effects of different doses of walnut meal on meat quality of broilers.

Item[a]	Treatments[b]			*p*-value
	CK	WM-L	WM-H	
Drip loss, %	8.22 ± 0.11	7.03 ± 0.4	6.49 ± 0.13	0.886
Cooking loss, %	24.05 ± 0.72	26.41 ± 1.9	25.4 ± 2.25	0.644
Shear force, N	2.95 ± 0.45	3.99 ± 0.73	3.25 ± 0.4	0.412
pH 45 min	6.31 ± 0.04	6.33 ± 0.15	6.07 ± 0.13	0.220
pH 24 h	6.44 ± 0.10	6.16 ± 0.09	6.28 ± 0.06	0.077
L* 45 min	43.36 ± 0.87	46.62 ± 1.24	46.56 ± 1.29	0.072
a* 45 min	5.33 ± 0.72	5.42 ± 0.68	4.14 ± 0.61	0.362
b* 45 min	12.38 ± 0.84	13.58 ± 0.57	11.75 ± 0.84	0.248
L*24 h	45.61 ± 0.79[b]	50.01 ± 0.63[a]	51.15 ± 1.05[a]	<0.001
a*24 h	7.66 ± 0.95	5.89 ± 0.69	7.45 ± 0.74	0.253
b*24 h	13.46 ± 0.56	15.62 ± 0.52	15.00 ± 0.82	0.053
Protein g/100 g	21.5 ± 0.774	22.88 ± 0.3308	22.84 ± 0.8553	0.312
Fat g/100 g	1.98 ± 0.14	1.66 ± 0.24	1.9 ± 0.2	0.511

The values in the table are expressed as a mean ± standard error. [a-b]Means with different letters within a row differ significantly ($p < 0.05$).
[a] L*: luminance; a*: redness; b*: yellowness.
[b] CK = base diet (SBM); WM-L = SBM+5 % walnut meal; WM-H=SBM+10 % walnut meal.

Table 4
Effects of different doses of walnut meal on amino acid composition of broilers (%).

Item[a]	Treatments[b]			*p*-value
	CK	WM-L	WM-H	
Gly	6.73 ± 0.18[a]	4.62 ± 0.12[b]	4.44 ± 0.11[b]	<0.001
Ala	8.06 ± 0.07[b]	9.08 ± 0.09[a]	7.91 ± 0.1[b]	<0.001
GABA	0.03 ± 0[b]	0.04 ± 0[b]	0.05 ± 0[a]	<0.001
Ser	8.47 ± 0.05[a]	7.93 ± 0.07[b]	8.48 ± 0.08[a]	<0.001
Pro	2.08 ± 0.02[a]	1.94 ± 0.03[b]	1.79 ± 0.02[c]	<0.001
Val	3.4 ± 0.03[a]	3.38 ± 0.02[a]	2.99 ± 0.07[b]	<0.001
Thr	3.82 ± 0.08[a]	2.08 ± 0.12[b]	2.44 ± 0.17[b]	<0.001
Iso	2.92 ± 0.03[a]	2.98 ± 0.05[a]	2.79 ± 0.04[b]	0.017
Leuc	5.19 ± 0.08[b]	5.56 ± 0.05[a]	5.27 ± 0.13[b]	0.037
Asn	2.95 ± 0.03[c]	3.58 ± 0.07[b]	3.8 ± 0.07[a]	<0.001
Asp	3.5 ± 0.08	3.34 ± 0.13	3.37 ± 0.12	0.576
Hcy	0.23 ± 0.01[a]	0.1 ± 0[b]	0.1 ± 0[b]	<0.001
Gln	7.86 ± 0.06[a]	6.5 ± 0.14[b]	6.4 ± 0.04[b]	<0.001
Lys	6.24 ± 0.1[a]	4.93 ± 0.03[c]	5.58 ± 0.05[b]	<0.001
Glu	7.79 ± 0.09	8.17 ± 0.12	7.92 ± 0.1	0.068
Met	2.07 ± 0.04[b]	2.44 ± 0.02[a]	2.34 ± 0.05[a]	<0.001
His	17.73 ± 0.19[c]	20.18 ± 0.3[b]	21.47 ± 0.48[a]	<0.001
Phe	2.57 ± 0.04[c]	2.99 ± 0.04[a]	2.84 ± 0.05[b]	<0.001
Arg	2.86 ± 0.03[c]	4.15 ± 0.07[b]	4.4 ± 0.11[a]	<0.001
Tyr	4.48 ± 0.04[b]	4.94 ± 0.07[a]	4.56 ± 0.09[b]	0.001
Try	0.92 ± 0.01[c]	1.08 ± 0.01[a]	1.01 ± 0[b]	<0.001
EAA	27.13 ± 0.13[a]	25.42 ± 0.16[b]	25.27 ± 0.39[b]	<0.001
FAA	39.96 ± 0.22[a]	38.97 ± 0.34[b]	37.67 ± 0.16[c]	<0.001
TAA	2261.27 ± 34.45[a]	1689.28 ± 57.19[b]	1518.32 ± 33.2[c]	<0.001

EAA = Sum of (Lys, Thr, Phe, Met, Val, Trp, Leu, Ile).
FAA=Sum of (Leu, Ile, Arg, Gln, Val, Hcy, Gly, Glu, Trp, Met).
The values in the table are expressed as a mean ± standard error. [a-c]Means with different letters within a row differ significantly ($p < 0.05$).
[a] TAA: total amino acids; FAA: flavor amino acids; EAA: essential amino acids.
[b] CK = base diet (SBM); WM-L = SBM+5 % walnut meal; WM-H =SBM+10 % walnut meal.

control group, the indexes of Chao, observed species, Shannon, Simpson, and Pielou's evenness were down-regulated in the experimental groups, whereas those of good's coverage were up-regulated ($p < 0.05$) (Fig. 1b). The results showed that walnut meal treatment decreased the species richness and diversity of the cecal microflora of the broilers and increased the species coverage of microflora. Principal coordinate and NMDS analysis of cecal microflora diversity data of broilers in each group showed that the samples of the control group and the experimental group could be distinguished, indicating that the microbial community composition was different between the groups (Fig. 1d–e).

3.5.2. Microbial composition analysis of cecum

At the phylum classification level, compared with the control group, the contents of Actinomyces and Bacteroidetes were significantly increased, that of Firmicutes significantly decreased ($p < 0.05$) (Fig. 2a), and there was no significant difference in the relative abundance of proteobacteria ($p > 0.05$) (Fig. 2c–f). At the genus classification level(Fig. 2b), compared with the control group, the abundances of *Bacteroides*, *Coecalis*, *Bifidobacteria*, and *Koala* were significantly increased in all treatment groups ($p < 0.05$) (Fig. 2g–j).

3.5.3. Screening of marker species

Combining effect size measurement (LEfSe) with linear discrimination analysis (LDA), we analyzed the cecal microbiota of broilers, thirteen orders and 29 families were identified by LDA score, a total of 28 genera were selected as dominant bacteria, most of the specific taxa were derived from WM-H, suggesting that walnut meal treatment had a strong effect on the gut microbiome of the broilers (Fig. 3a). The main bacteria in the CK group were *Firmicutes* and *Clostridia* bacteria (LDA>4), those of the WM-L group were *Proteobacteria* and *Bacillus* (LDA>4), and those of the MM-H group were *Coriobacteria* and *Phascolarctobacteria* (LDA>4) (Fig. 3b).

3.5.4. Correlation network analysis of dominant species

In the control group, Firmicutes and Bacteroides were positively correlated with each other and Proteus in the microbiome. However, the regulatory trend of this network was more obvious in the walnut meal treatment group (Fig. 4a). In addition, more complex microbial interaction patterns were found among Firmicutes and Bacteroides, proving the existence of more core nodes (e.g., unidentified_*Lachnospiraceae*, *Phascolarctobacterium*, and *Butyricicoccus)*. The unidentified_*Christensenellaceae* and *Lactobacillus* in the control group were mainly negatively correlated, and after hydrolysis processing mainly negative correlation flora is unclassified_*Rikenellaceae* and unclassified_*Coriobacteriaceae*. Gate-level network analysis also revealed a more complex symbiosis within the cecal microbiome in the walnut meal treated group compared to the control group, as demonstrated by a larger number of edges in the WM network than in the control group (Fig. 4b).

Table 5
Effects of different doses of walnut meal on fatty acid composition of broilers (%).

Item[a]	Treatments[b]			*p*-value
	CK	WM-L	WM-H	
C10:0	0.02 ± 0.00[a]	0.01 ± 0.00[b]	0.01 ± 0.00[c]	<0.01
C14:1	0.54 ± 0.16[c]	1.73 ± 0.71[b]	3.03 ± 0.57[a]	<0.01
C16:0	30.13 ± 0.72[a]	29.44 ± 1.05[a]	28.17 ± 0.73[b]	0.01
C16:1t	0.25 ± 0.05[a]	0.23 ± 0.04[a]	0.17 ± 0.01[b]	<0.01
C16:1	2.90 ± 0.38[a]	2.25 ± 0.49[b]	1.92 ± 0.16[b]	<0.01
C18:0	18.06 ± 1.39	18.25 ± 1.68	17.23 ± 1.03	0.492
C18:1n9t	0.18 ± 0.00[b]	0.27 ± 0.06[a]	0.30 ± 0.02[a]	<0.01
C18:1n12	0.40 ± 0.07[a]	0.31 ± 0.04[b]	0.28 ± 0.02[b]	<0.01
C18:1n9c	20.90 ± 1.78	18.87 ± 3.07	18.53 ± 1.28	0.218
C18:1n7	2.53 ± 0.06[a]	1.85 ± 0.05[b]	1.86 ± 0.09[b]	<0.01
C18:2n6	11.00 ± 0.70[c]	12.76 ± 0.70[b]	14.17 ± 0.67[a]	<0.01
C18:3n3	0.31 ± 0.03[c]	0.38 ± 0.07[b]	0.49 ± 0.04[a]	<0.01
C20:2	0.38 ± 0.03[ab]	0.34 ± 0.04[a]	0.40 ± 0.03[b]	0.025
C20:3n6	0.87 ± 0.07[a]	0.73 ± 0.12[b]	0.72 ± 0.05[b]	<0.01
C20:4n6	3.67 ± 0.28	4.10 ± 0.72	4.16 ± 0.27	0.236
C20:5n3	0.24 ± 0.02[a]	0.17 ± 0.03[b]	0.17 ± 0.01[b]	<0.01
C22:0	0.04 ± 0.01[a]	0.03 ± 0.00[ab]	0.03 ± 0.00[b]	0.042
C22:5n6	0.25 ± 0.02	0.27 ± 0.05	0.26 ± 0.01	0.656
C24:0	0.02 ± 0.01[a]	0.01 ± 0.00[b]	0.01 ± 0.00[b]	<0.01
C24:1	0.22 ± 0.03[b]	0.39 ± 0.14[a]	0.49 ± 0.03[a]	<0.01
Total Fas	4192.74 ± 312.30	4009.15 ± 640.45	4240.88 ± 182.25	0.670
SFA	49.82 ± 2.07	49.25 ± 2.67	46.90 ± 1.76	0.127
MUFA	31.36 ± 1.84	29.83 ± 3.14	30.50 ± 1.37	0.576
PUFA	18.83 ± 0.42[c]	20.92 ± 0.54[b]	22.60 ± 0.66[a]	<0.01
PUFA/SFA	0.37 ± 0.10[c]	0.42 ± 0.06[b]	0.48 ± 0.14[a]	<0.01
n-3 PUFA	1.57 ± 0.13[b]	1.60 ± 0.14[b]	1.79 ± 0.07[a]	0.027
n-6 PUFA	15.96 ± 0.51[c]	18.04 ± 0.23[b]	19.47 ± 0.60[a]	<0.01
n-6/n-3	10.20 ± 0.99	11.31 ± 0.92	10.88 ± 0.39	0.138

The values in the table are expressed as a mean ± standard error. [a-c]Means with different letters within a row differ significantly ($p < 0.05$).
[a] PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids; SFA: saturated fatty acids.
[b] CK = base diet (SBM); WM-L = SBM+5 % walnut meal; WM-H=SBM+10 % walnut meal.

3.5.5. Effect of walnut meal on prediction of intestinal microbial function in broilers

The metagenomic sequences were analyzed by PICRUSt to obtain the predictive KEGG pathway. The results show that the metabolic regulation of host gene enrichment is mainly involved in carbohydrate metabolism, amino acid metabolism, cofactor, and vitamin metabolism, terpenoid and polyketide acid metabolism, lipid metabolism, energy metabolism, glycan biosynthesis, and nucleotide metabolism (Fig. 5a). In addition, compared to control, 22 pathways (e.g., transport and catabolism, isogenic biodegradation and metabolism, cofactor and vitamin metabolism, lipid generation, glycan biosynthesis and metabolism, energy metabolism, and carbohydrate metabolism) were up-regulated in the WM-L group, and 13 pathways (e.g., cell movement, signal transduction, membrane transport, and polyketoic acid metabolism) were down-regulated. WM-H up-regulates 6 pathways (e.g., cell motility, transcription translation and replication, biodegradation, and metabolism of allobiotin) and down-regulated 20 pathways (e.g., glycan biosynthesis and metabolism, energy metabolism, carbohydrate metabolism), there is less regulation of human neurodegenerative diseases, infectious diseases, cardiovascular diseases and immune diseases (Fig. 5b).

3.6. Correlation analysis of meat quality

Mantel and chord-graph correlation were employed to analyze the correlation between the intestinal microbiome level and the amino acid and fatty acid composition of the breast muscle in broilers. The aim was to explore the mechanism of improving meat quality with walnut meal. The results indicate that there is a correlation between the changes in pH 45 min, L* 45 min, a* 45 min, and Drip loss (Fig. 6a) and the amino acid composition, while the changes in pH 45 min, L* 45 min, a* 45 min, and a*24 h (Fig. 6b) are correlated with the fatty acid composition. In the analysis of amino acid correlation, 12 genera showed significant negative correlation with the amino acid composition of pectoral muscle. These included *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Faecalis* and *Dorea*. On the other hand, 18 genera showed significant positive correlation with the amino acid composition of pectoral muscle (Fig. 6c–d).

4. Discussion

Corn, wheat and soybean meal (SBM) are used as nutrients in the main animal feeds, however, there is a need to explore and evaluate alternative feeds to cope with variable costs and the availability of these conventional feeds [18]. Studies have shown that 150 g/kg Macadamia nut cake does not affect its growth performance and can be used as a potential alternative feed to partially replace corn and SBM [19]. In this study, the addition of walnut meal to the diet improved the growth performance (final BW and ADG) of

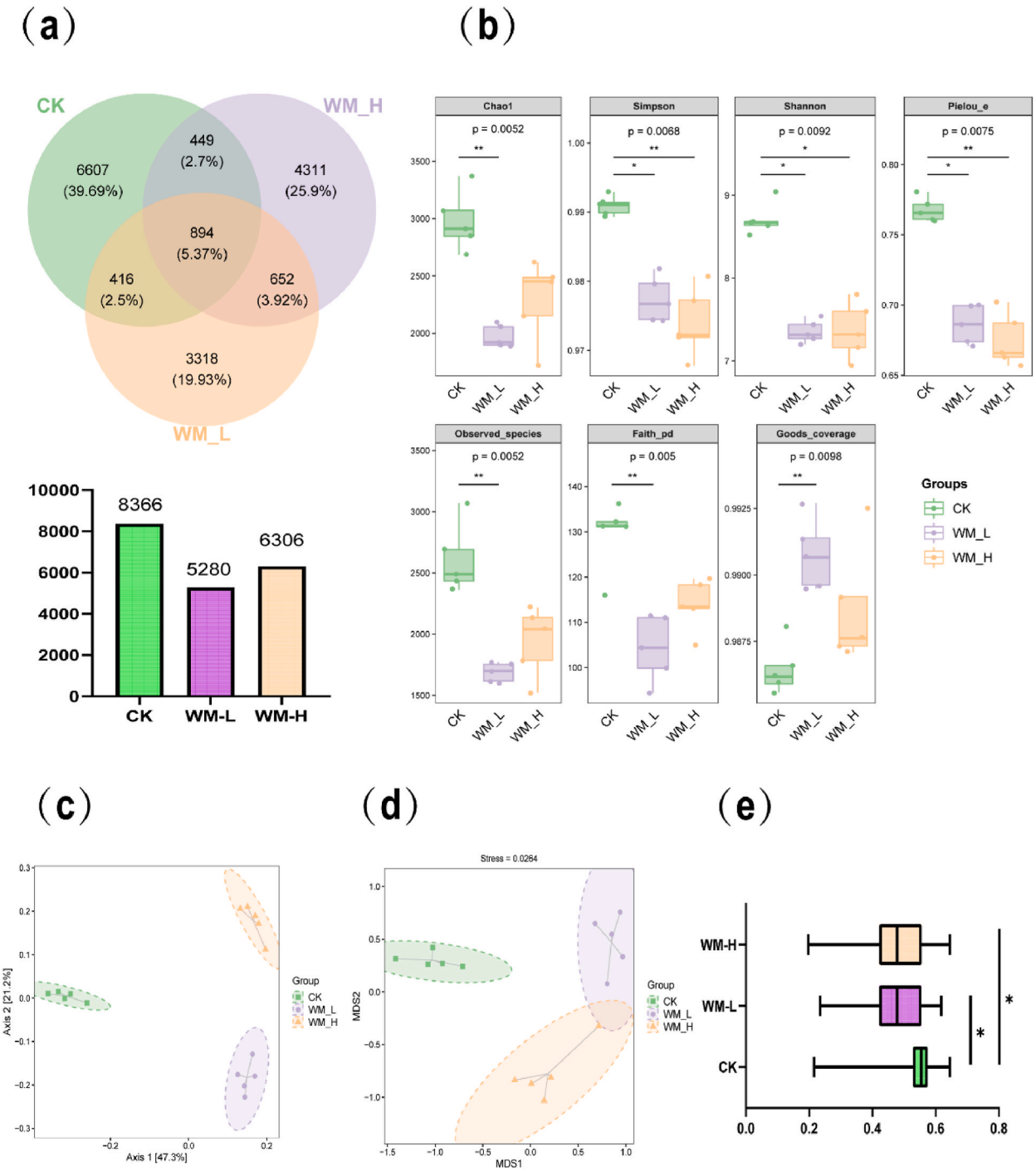

Fig. 1. Analysis of intestinal microbial diversity (n = 5).
(a) Venn diagram; (b) α-diversity: Chao, Observed species, Shannon, Simpson, and Pielou's evenness and good's coverage indices; (c-e) beta diversity. $*p < 0.05$,$**p < 0.01$,$***p < 0.001$ compared with the CK group.

white feathered broilers compared with the control group. This may be because walnut meal is rich in protein or amino acids, which improves the lipid metabolism or intestinal flora of broilers, thereby improving the growth performance of broilers.

Meat quality is an important economic factor that directly influences consumers' preference for chicken meat. Various characteristics of meat quality are associated with different meat processing methods [20]. For instance, meat that is tender is suitable for grilling, while chewy meat is better for braising. Studies have shown that the addition of 10 % walnut meal affects the quality of meat,

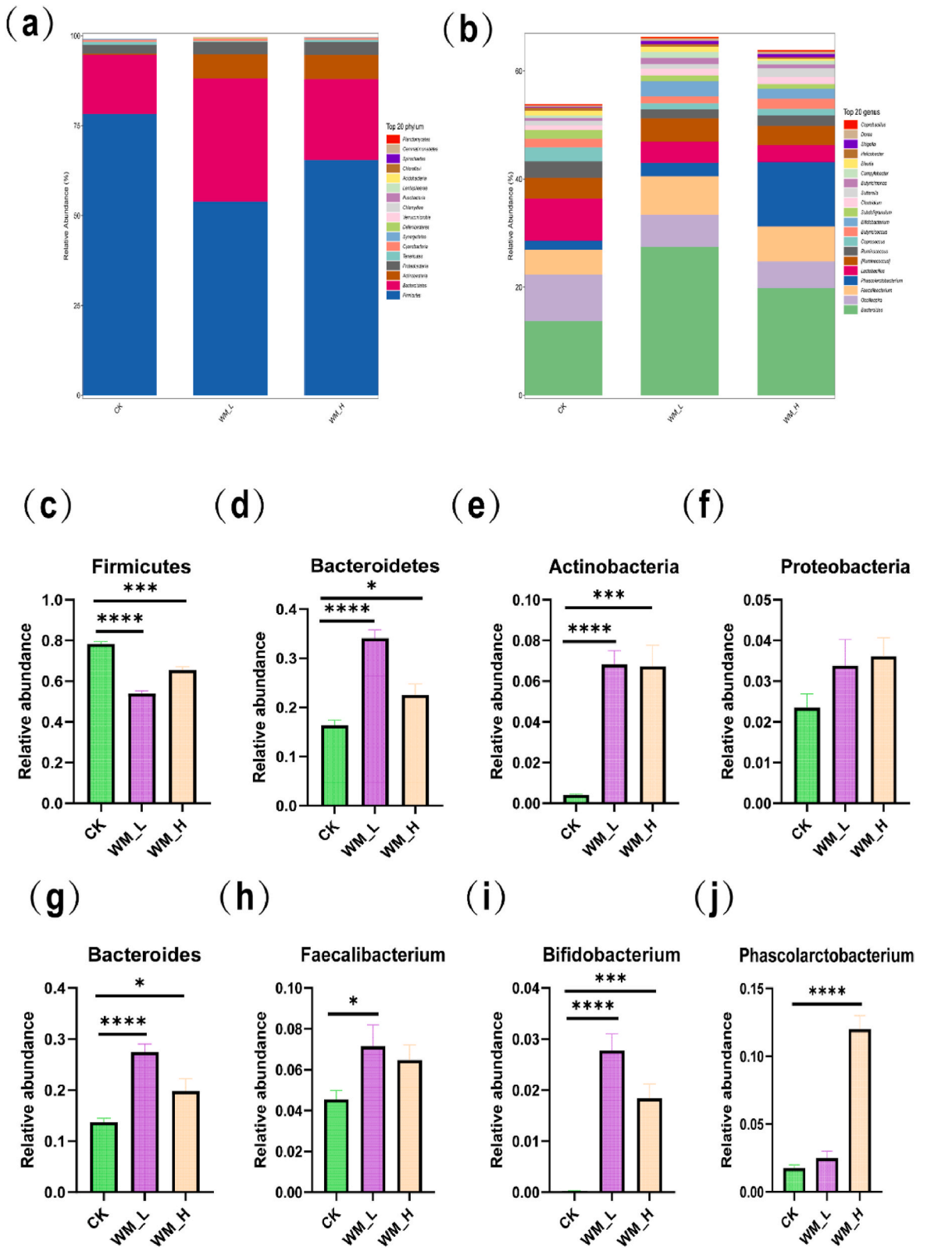

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Fig. 2. Microbial composition analysis of cecum (n = 5).
(a) phylum level analysis. (b) Generic microbial composition; each bar represents the average relative abundance of each bacterial taxon within a group. (c-f) Relative abundances of Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria under different doses of walnut meal. (g-h) Relative abundances of *Bacteroides*, *Coecalis*, *Bifidobacteria*, and *Koala* under different doses of walnut meal in the genus class. The data were analyzed using one-way analysis of variance. Statistical significance was determined at the following levels: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

including tenderness, pH, meat color and medium and long chain fatty acid content [21].It was observed that the addition of walnut meal had no significant effects on the breast muscle redness (a* at 45 min), yellowness (b* at 45 min), dripping loss, cooking loss, shear force, pH at 24 h, or fat content of the broilers. With the increase of walnut meal dosage, the L* at 1 h and the protein contents of the breast muscle of the broilers were increased. The color of meat depends on the amount of myoglobin in the muscle, and the reason meat turns dark red with increased exposure under any feeding pattern is because oxygenated myoglobin (bright red) is oxidized to methemoglobin (brown) [22].

Amino acid content in muscle is an important index to evaluate the nutritional value of protein and one of the main chemical indexes affecting the flavor quality of chicken. There are many kinds of amino acids in chicken, which correspond to different tastes for humans and have important effects on meat quality. Amino acids have been found to directly activate the taste pathway ion channels that control umami and sweetness in human type II receptor cells [23]. Compared with the control group, the content of amami amino acid (one of the main components affecting meat taste) in WM-H group was significantly increased. From sensory evaluations of synthetic solutions based on meat components, it has been suggested that Glu contributes to umami and broth flavor [24]. Compared with the control group, the contents of essential amino acids (isoleucine, methionine, leucine, tryptophan, phenylalanine, arginine, methionine) were increased by addition of walnut meal, while the contents of arginine, homocysteine, glutamine, EAA, FAA and TAA were decreased.Essential amino acids such as isoleucine, methionine, leucine, tryptophan, and phenylalanine were increased in a dose-dependent manner in comparison with the control group. Compared with the control group, the addition of walnut meal increased the contents of essential amino acids (isoleucine, methionine, leucine, tryptophan, phenylalanine, arginine, methionine) and decreased the contents of arginine, homocysteine, glutamine, EAA, FAA and TAA, possibly because walnut meal has the potential to regulate amino acid transformation and synthesis. Studies have reported that 150 mg/kg GML can significantly increase the contents of lysine, aspartic acid, glutamic acid, tyrosine, umami amino acid and total amino acid in pectoral muscle [25]. In addition, amino acids are not only the carriers of protein construction, but also participate in the intracellular signaling pathways of protein anabolism and promote protein synthesis. From the perspective of functional amino acids, it was found that leucine, tryptophan, and methionine was dose dependent. The selected combination of functional amino acids polyphenols can completely restore the performance of chickens affected by coccidiosis and improve the digestibility of AA [26]. These results suggest that walnut meal may have similar effects.

The content and proportion of fatty acids are important criteria related to the health-promoting properties of meat [27]. Poultry meat is a good source of polyunsaturated fatty acids, especially n-3 polyunsaturated fatty acids, including eicosapentaenoic acid C20:5n-3 and docosahexaenoic acid C22:6n-3, which have positive effects on brain and cardiovascular-system function [28]. Other important criteria include, for example, the ratio of the n-6/n-3 ratio of fatty acids to AI and TI, with lower AI and TI values positively associated with a lower risk of severe coronary artery abnormalities [29]. In this study, walnut meal treatment increased the content of PUFAs, n-3 PUFAs, and n-6 PUFAs in the breast muscle of the broilers. It may be because walnut meal contains some components that make the conversion between saturated and unsaturated fatty acids occur, thus increasing the content of unsaturated fatty acids. Further identification of active components is needed. n-3 PUFA has been shown to be effective in treating bronchial asthma, neuropsychiatric disorders, and cognitive brain function in children; it can also prevent future cardiovascular disease in adults [30], Fatty acid composition, especially n-3 PUFA, is regulated by changes in dietary lipid intake and absorption levels [31]. In this study, the fatty acid composition of white feather broilers fed with walnut meal is more beneficial to human health. In addition to n-3 polyunsaturated fatty acids, other fatty acids, including essential fatty acids alpha-linolenic acid (C18:3n3) and linoleic acid (C18:2n6), also have health benefits in preventing brain, retinal, and cardiovascular diseases. The four double bonds of arachidonic acid (ARA) tend to lead to oxygenation, which leads to many metabolites that are important for the normal function of the immune system, promoting allergy and inflammation; resolving inflammation; mood; and appetite [32]. Consistent with the results of this study.

Complex microecosystems exist in the digestive tract of animals, which participate in the basic process of digestion and absorption, and have the functions of preventing pathogen colonization, improving the intestinal environment, and protecting the intestinal health of the host [33]. Chicken intestinal microorganisms are rich in species and play an important role in improving growth performance and maintaining physical health [34]. More and more studies have shown that the development of intestinal microbes is a dynamic process, with some differences in microbial diversity and community composition among different parts [35]. The chicken intestine can be divided into different intestinal segments, such as duodenum, jejunum, ileum, cecum, and colorectal [36]. The cecum is the most diverse part of the chicken gastrointestinal tract. Studies have shown that Bacteroides, Firmicutes, Proteus, and Actinomyces are the main bacteria in chicken intestinal microbiota [37], which is consistent with the results of our experiment. However, there are some differences in the relative abundance of the main dominant bacterial phyla, which may be caused by differences in chicken breed and feeding diet [38]. It was found that Firmicutes, Bacteroides, and Actinomyces accounted for 97.42 %, 98.25 %, and 98.29 % of the cecum microbe composition of the white feather broilers, respectively. Firmicutes produce short-chain fatty acids, which are absorbed directly into the host intestinal wall as an energy source and are positively associated with weight gain and immune function in birds and mammals [39]. The probiotic effect of *Bacteroides* has attracted the attention of many scholars. *Bacteroides* can improve the utilization of polysaccharides by the host [40], improve the immunity of the host [41], and maintain the balance of intestinal flora

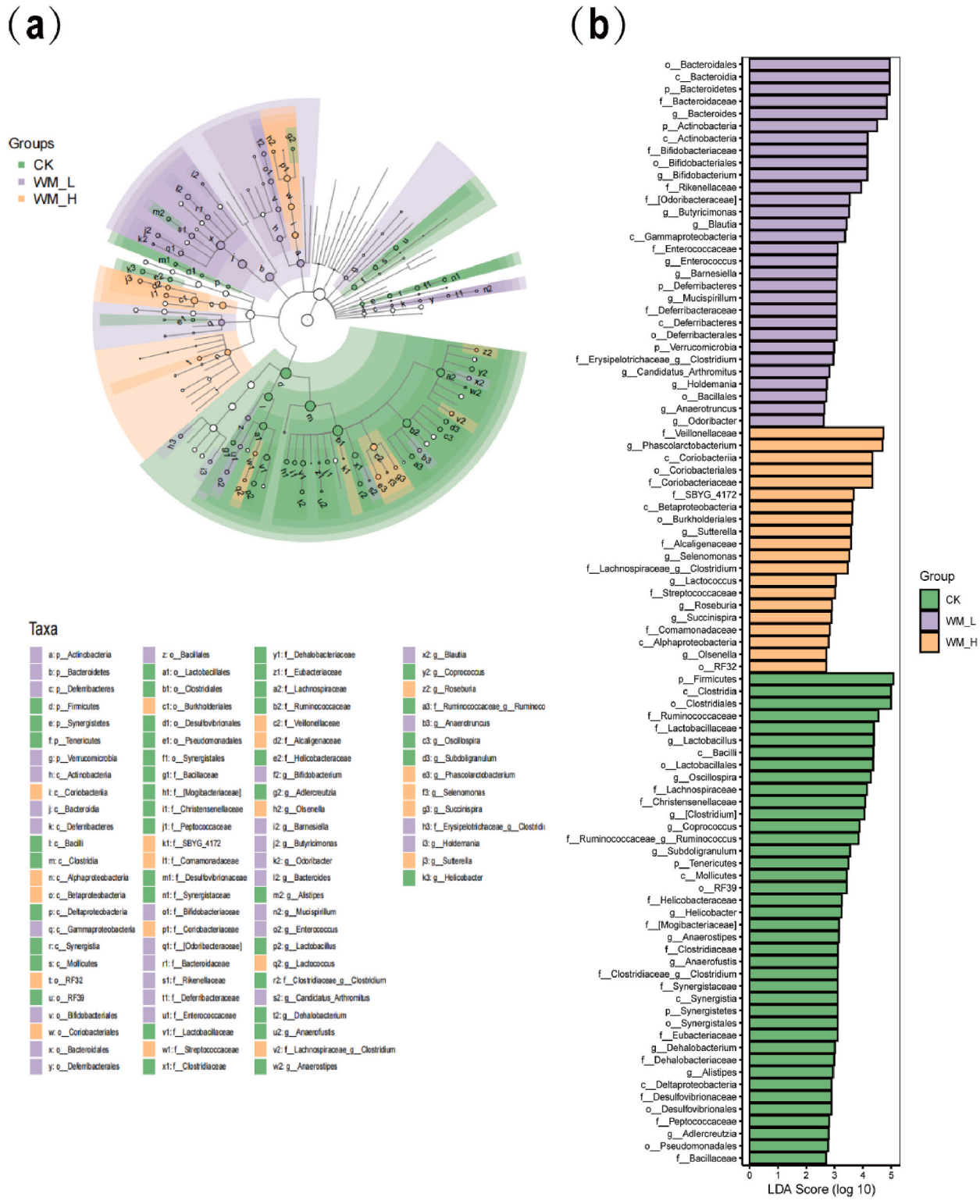

Fig. 3. LEfSe analysis and LDA score screening markers (n = 5).
(a) The branching diagram shows the significantly different microbial species between the two groups (p, c, o, f, g from the inside out), with green and orange nodes on the phylogenetic tree representing microbial species that played important roles in the control and treatment groups, respectively. (b) Species with significant differences (default is 2.0) where the LDA score is greater than the estimate, and the length of the histogram represents the LDA score.

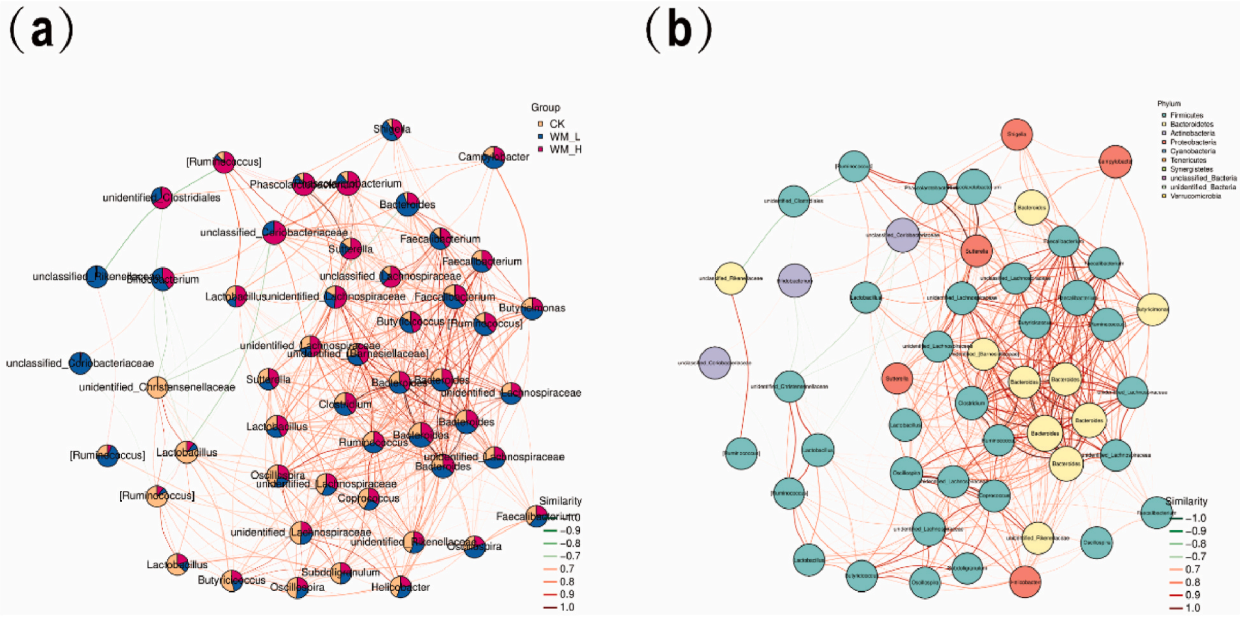

Fig. 4. Dominant species network analysis.
(a) Modular map of dominant species network (average abundance top 50 ASV/OTU), with a correlation of R≥|0.7| or higher, the diagram uses red lines to indicate a positive correlation and green lines to indicate a negative correlation. (b) Seed network diagram of dominant species annotated horizontally, with red lines indicating a positive correlation, and green lines indicating a negative correlation. The colors are represented by modules. Gray is Module 1, orange is module 2, yellow is module 3, and green is module 4.

[42]. *Bifidobacteria* can regulate intestinal microbiota and is an important intestinal probiotic [43]. *Koala Bacillus*, an obligate anaerobic and Gram-negative bacterium that produces short-chain fatty acids, including acetate and propanate, and may be related to the metabolic state and mood of the host, colonizes extensively in the human gastrointestinal tract [44]. The results of this study showed that the consumption of walnut meal rich in vegetable protein could increase the content of beneficial bacteria in the cecum of broilers. In addition, symbiotic network analysis ($R \geq 0.7$ and $p \leq 0.05$) showed that the number of nodes and correlation between bacteria in each group increased with the increase of walnut meal dose. The higher the network complexity, the richer the microbial diversity, which may represent the better the dynamic balance of intestinal microbiota [45]. Intestinal homeostasis inhibits the colonization of pathogens and is beneficial to nutrient absorption and physiological health [46].

Functional prediction results showed that the dietary contents of the WM groups were rich in Retinol_metabolism, Proteasome, Lipopolysaccharide_biosynthesis, PPAR_signaling_pathway, African_trypanosomiasis, and D−Arginine_and_D−ornithine_metabolism. The critical contribution of the proteasome to healthy cellular proteostasis has been increasingly recognized in recent years [47]. Peroxisome proliferator-activated receptors (PPARs) belong to the ligand-activated nuclear receptor family. They play a crucial role in regulating metabolism, and certain PPAR ligands have been suggested as potential treatments for various diseases, including metabolic syndrome, neurodegenerative diseases, diabetes, and cardiovascular disease [48]. The results show that different doses of walnut meal affect the synthesis of amino acids and fatty acids by regulating the changes of microbe composition and diversity in the cecum.

Recent studies have convincingly shown that large numbers of amino-acid-fermenting bacteria reside in the gut [49]. It has been shown that the essential amino acid "lysine", produced by bacteria, is absorbed in the body, and integrated into whole host proteins [50]. In a study of a nitrogen-adequate diet in humans, gut-microbiome-derived lysine and threonine were shown to be essential for free lysine and threonine contents [51]. Various strains of *Lactobacillus* and *Bifidobacteria* have been found to produce gamma-aminobutyric acid, a neuroactive substance [52]. It is well known that imidazole propionate can be converted to histidine-derived metabolites by gut microbiota [53]. In addition, the gut microbiota can produce tryptophan due to the presence of tryptophan decarboxylase in the gut [54].

The study found that arginine, leucine, tryptophan, and methionine increased in a dose-dependent mannerl, suggesting the involvement of cecal microorganisms in amino acid synthesis and catabolism [55]. Based on the correlation analysis of amino acids and fatty acids between microorganisms and pectoral muscle, it was found that the amino acid synthesis and catabolic pathways of Ruminococcus, Coprococcus, Oscillospira, unclassified Clostridiales, and unidentified Christensenellaceae flora, such as the Arginine and D-ornithine metabolism pathways, play a regulatory role. The correlation between meat quality and amino acid composition was found to be linked to changes in pH 45 min, L* 45 min, a* 45 min, and drip loss. Similarly, the fatty acid composition was found to be correlated with changes in pH 45 min, L* 45 min, a* 45 min, and a*24 h. It is important to note that the composition of amino acids and fatty acids is a crucial factor that affects the quality of chicken food, particularly the pH, meat color, and tenderness of fresh meat. The pH of meat directly indicates the quality of its meat, affecting its color, water retention, tenderness, and taste, which can reflect the rate of muscle glycolysis after death [56]. In this study, walnut meal treatment tended to lower pH at 45 min and 24 h. Lactic acid secreted

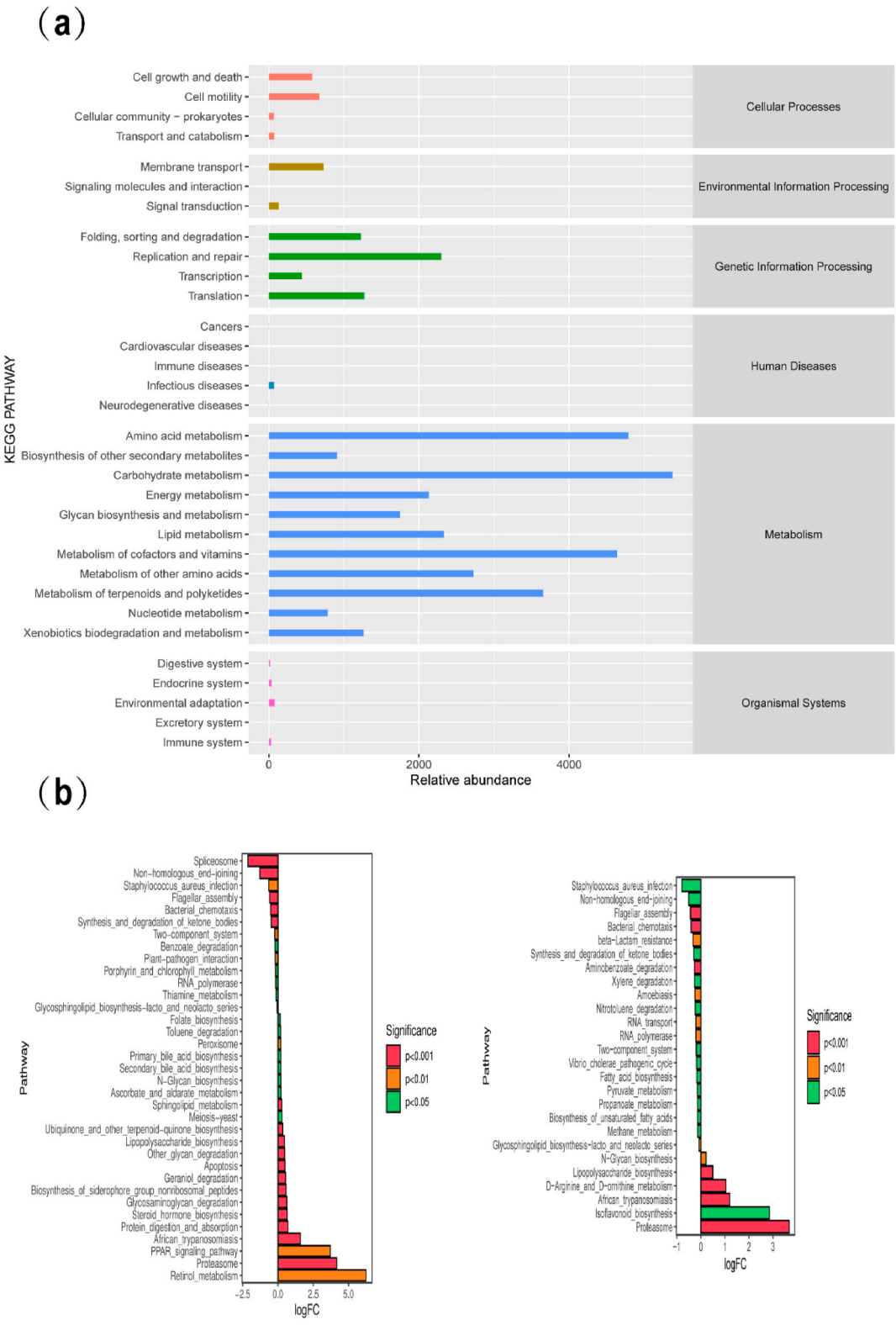

Fig. 5. KEGG functional pathway analysis (n = 5).
(a) Analysis of the functional metabolic pathway in KEGG. (b) Differential analysis of KEGG metabolic pathway.

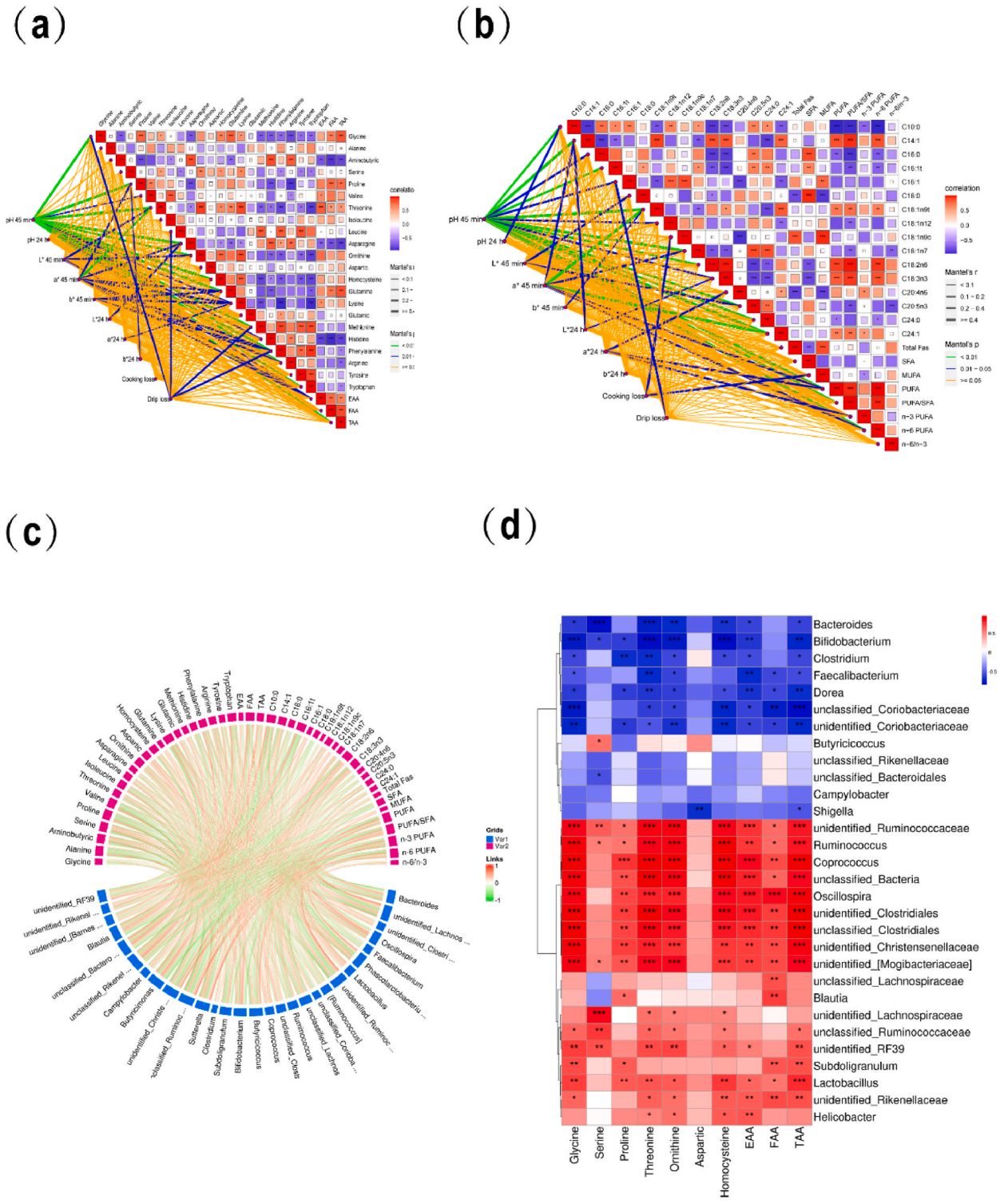

Fig. 6. Correlation analysis of meat quality (n = 5).
(a) Correlation analysis of amino acid and meat quality. (b) Correlation between fatty acids and meat quality. (c) Correlation analysis of amino acids and fatty acids with intestinal flora of the top 30 genera ($p < 0.05$). (d) Analysis of the correlation between amino acids and the top 30 intestinal flora. In correlation analysis, red represents a positive correlation and blue or green represents a negative correlation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

by muscle glycogen after death causes pH levels to drop. In addition, the decrease of pH value is conducive to the degradation of myofibrillar protein [57] Meat color is an important metric in the evaluation of sensory characteristics that affect consumer preferences. Changes in meat color depend on the amount and chemical composition of myoglobin. Both deoxymyoglobin and oxygenated myoglobin can be oxidized to high ferrimyoglobin, resulting in brown meat [58]。Lipid oxidation is closely related to meat color and produces free radicals that promote the accumulation of ferrimyoglobin, resulting in reduced color stability and accelerated meat discoloration [59]. This study showed that the composition and content of amino acids and fatty acids in pectoral muscle of broilers treated with walnut meal changed, which may be the cause of affecting meat color and pH, but the specific mechanism of the study needs further research.

5. Conclusion

This study demonstrates that the inclusion of walnut meal in the diet of broilers can enhance their nutritional characteristics. It can increase the protein content in the breast muscle of white feather broilers, promote the deposition of flavouring substances such as glutamic acid, leucine, isoleucine, tryptophan, and methionine in the muscle, and increase the body weight and average daily gain of white feather broilers. The study found that the contents of PUFA, n-3PUFA and n-6 PUFA in the breast muscle of white feathered broilers increased, and the PUFA/SFA composition ratio improved. This study also proved for the first time that walnut meal can effectively increase the content of *Bacteroides*, *Bifidobacterium*, *Coriobacteriia* and *Phascolarctobacterium* in cecum microorganisms. Involving Retinol_metabolism, Lipopolysaccharide_biosynthesis, PPAR_signaling_pathway, African_trypanosomiasis and Regulation of D−Arginine_and_D−ornithine_metabolism and other metabolic signaling pathways. The correlation between meat quality and walnut meal was investigated, and it was found that adding 10 % walnut meal to the diet of white feather broilers can improve the quality of their breast muscle. This improvement is achieved by altering the microbe composition of the cecum and regulating the metabolism of amino acids and fatty acids in the muscle. These findings provide valuable insights for the industrialization of white feather broilers. In conclusion, walnut meal has the potential to enhance the flavor and nutritional value of white feather broilers when used as a feed formula additive.

Ethical statement

All procedures are approved by the Life Science Ethics Committee of Yunnan Agricultural University, Ethics number:202209012.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Xingjiao Jiang: Writing – original draft, Formal analysis, Data curation. **Jiangrui Yang:** Validation, Methodology, Data curation. **Lihui Yu:** Writing – review & editing. **Zhengjiang Zhou:** Writing – review & editing. **Lijun Yu:** Writing – review & editing. **Yankai Luo:** Supervision. **Linxian Shan:** Supervision. **Ruijuan Yang:** Conceptualization. **Haizhen Wang:** Validation, Software. **Xiaocui Du:** Validation, Software. **Qichao Huang:** Validation, Software. **Cunchao Zhao:** Writing – review & editing. **Yan Liu:** Validation, Software. **Jun Sheng:** Funding acquisition, Conceptualization. **Chongye Fang:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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