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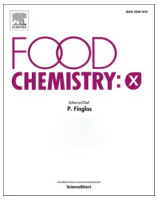

Alfalfa leaf meal as a new protein feedstuff improves meat quality by modulating lipid metabolism and antioxidant capacity of finishing pigs

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ABSTRACT

The effects of alfalfa leaf meal (ALM) on the meat quality of finishing pigs are largely unknown. Here, we investigated the effects of ALM diet on meat quality by replacing 0%, 25%, 50%, and 75% of soybean meal in the diet of finishing pigs, respectively. The findings showed that 25% ALM diet increased the IMF, cooked meat rate, a* and antioxidant capacity of *longissimus dorsi* (LD), improved amino acid composition, increased MUFA content, and increased LD lipid synthesis and mRNA expression of antioxidation-related genes. At the same time, ALM diet altered serum lipid metabolism (TG, FFA). Correlation analysis showed that antioxidant capacity was positively correlated with meat quality. In addition, metabolomic analysis of LD showed that the main metabolites of 25% ALM diet altered stachydrine and L-carnitine were associated with meat quality and antioxidant capacity. In conclusion, ALM replacing 25% soybean meal diet can improve the meat quality of pigs.

1. Introduction

China is the world's largest producer and consumer of pork, with a per-person yearly consumption of >30 kg. Therefore, healthy development of the finishing pig industry is important to China's national economy and people's livelihood. At present, there is a serious shortage of high-quality protein feedstuffs in China. The soybeans used in animal husbandry production are heavily dependent on imports. Therefore, solutions to the development and utilization of new domestic high-quality protein feedstuff resources are urgently needed. Cottonseed meal and rapeseed meal are two types of abundant plant protein feedstuffs. However, due to an imbalance in the ratio of amino acids present, a low rate of absorption, and anti-nutritional elements like free gossypol and isothiocyanate, their usage is restricted (Konkol et al., 2019). Therefore, alternative new protein feedstuff resources are being sought. Alfalfa is a perennial leguminous plant, that is rich in protein, and has a good balance of amino acids, minerals, and vitamins. Moreover, the protein concentrate extracted from alfalfa is a good bioactive substance affecting the metabolism and digestion of pigs. The addition of 2%–3% alfalfa protein concentrate in pigs' diet leads to higher body weight gain and feed conversion (Pietrzak & Grela, 2015). Previous study has shown that low fiber alfalfa meal partially replaces traditional soybean meal (SBM) as a protein feedstuff in laying hens diet, which is beneficial to egg quality without affecting production performance (Laudadio, Ceci, Lastella, Introna & Tufarelli, 2014). Alfalfa leaf meal (ALM) has a higher crude protein content and lower fiber content than alfalfa meal, which has utility in replacing SBM to alleviate the dependence on imports. However, whether ALM can be used as a new protein feedstuff in finishing pigs has not been reported, and more research is required to determine how ALM affects the growth parameters and meat quality of finishing pigs.

With improvements in living standards, people's requirements for quality meat products are increasing. Meat quality reflects appearance, palatability, nutritional value, flavor, and physical/chemical properties. Evaluation indexes of meat quality include meat color, marbling pattern, pH, flavor, and odor. These traits are closely related to each other (Lebret & Čandek-Potokar, 2022). Lipid oxidation can affect meat color, water holding capacity, shear force, flavor, and odor (Rossi et al., 2013).

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There are many factors affecting lipid oxidation, including slaughter and other causes of oxidative stress in animals (Liu et al., 2009; Xing, Gao, Tume, Zhou & Xu, 2019). Oxidation occurs frequently when meat and meat products are stored, changing the lipids' and proteins' physical and molecular characteristics (Jin et al., 2020). Therefore, with the increase of meat production and consumption, it has become increasingly important to control lipid oxidation in meat and its products (Wu, Xiao, Yin, Zhang & Richards, 2021). Studies have shown that reducing lipid oxidation and increasing antioxidant capacity can successfully prevent reactive oxygen species (ROS) generation and improve meat quality (Rossi et al., 2013). In addition, intramuscular fat (IMF) affects pork quality. IMF can improve meat color, tenderness and flavor, while IMF content below 2% can make meat dry and give-off a bad odor (Gong et al., 2019). Therefore, promoting intramuscular fat deposition is an important strategy for improving meat quality.

It has been reported that alfalfa contains abundant beneficial components such as flavonoids and saponins, which are important active ingredients with antioxidant and free radical scavenging effects. Many studies have demonstrated that alfalfa meal promotes growth and improves meat quality in livestock. For example, adding a proper amount of alfalfa meal to the diet can significantly increase the meat feed ratio of finishing pigs (Wang et al., 2018). Additionally, adding alfalfa to the diet enhances Beijing-you chicken growth parameters and meat quality (Zheng, Mao, Tian, Guo & Meng, 2019). Alfalfa meal supplementation improves the fatty acid composition of sheep muscle and increases backfat thickness, marbling score, and antioxidant capacity (Su et al., 2022).

Therefore, the purpose of this study was to assess the effects of ALM on growth parameters and meat quality of finishing pigs by replacing different proportions of SBM, and to explore the appropriate proportion of ALM to replace SBM in the production of high-quality pork as well as the mechanism influencing pork quality. These results offered a rationale for feeding ALM to pigs as well as a useful guide for alleviating the shortage of high-quality protein feedstuff and promoting the production of high-quality pork.

2. Material and methods

2.1. Animal ethics

All animal experiments and procedures were approved by the Institutional Animal Welfare and Research Ethics Committee of Henan Agricultural University (Zhengzhou, China) (Permit No: 21–1127).

2.2. Animals and experimental design

The same batch of 64 healthy castrated boars, with an initial body weight (Duroc × Landrace × Yorkshire, 67.35 ± 0.70 kg), was assigned into 4 groups at random, with 4 finishing pigs in each replicate. Different amounts of alfalfa leaf meal (ALM) were substituted for soybean meal (SBM), including the control group (CON, a diet consisting of common corn and soybean meal), the 25% ALM diet group (ALM replacing 25% of SBM in the control diet), the 50% ALM diet group (ALM substituting 50% of SBM in the control diet), and the 75% ALM diet group (ALM replacing 75% of SBM in the control diet). The diet was designed according to the American NRC (2012) pig nutrition standard, with similar levels of energy and protein. Diet composition and nutrition levels are presented in Supplementary Materials (Table S1). During the trial period, the finishing pigs were fed twice a day with free access to diet and water; feed intake was recorded every day. The experiment lasted for 67 d including a 7d pre-trial period and 60 d trial period.

2.3. Growth parameters

On day 1 and day 60 of the experiment, the finishing pigs were weighed after fasting for 12 h. The initial and final body weights (LWBS, live weight before slaughtering) were recorded on Day 1 and Day 60, respectively. All these results are based on 60 days. The initial and final weights are obtained by weighing each pig with a weighbridge. By keeping track of the body weight and feed intake of pigs, the average daily feed intake (ADFI), average daily gain (ADG), and feed/gain weight ratio (F/G) were computed. Calculation formula: ADFI = average daily supplement (kg) - average daily surplus (kg); ADG = (average final weight - average initial weight)/60; F/G = ADFI/ADG.

2.4. Carcass traits

Four finishing pigs in each group were slaughtered at day 60 to determine the dressing percentage. The pigs were taken to the slaughter house and given free access to water before being slaughtered. Then, in accordance with Chinese guidelines, one pig was slaughtered every 15 min by electrical stunning, exsanguination, dehairing, evisceration, and splitting down the middle (Science and Technology Ministry of China, 2006). The body weight after bleeding, depilation, and removal of head, hoof, tail, and viscera (except suet and kidney) was recorded as the carcass weight. Dressing percentage (%) = (carcass weight/LWBS) × 100. The backfat thickness of the first rib, the last rib, and the last lumbar vertebra of the left carcass were measured using vernier calipers. The distance from the depression of the first cervical vertebra to the front line of the pubic symphysis was recorded as the straight length of the carcass, and the distance from the junction of the first rib and sternum to the middle line of the pubic symphysis was recorded as the oblique length of the carcass. The cross-sectional area of the same part of the LD between the 12th and 13th ribs of the finishing pig was the eye muscle area. The above indexes were measured according to the previous research techniques (Li et al., 2018).

2.5. Blood sampling and analysis

Blood samples were obtained from the jugular vein before feeding on Day 60 of the trial period and the finishing pigs were fasted for 12 h through the night before blood collection. Serum samples were centrifuged at 3000 r/min for 10 min and stored at −20 ℃ before use. Serum contents of total protein (TP), albumin (ALB), total cholesterol (TC), triglyceride (TG), blood urea nitrogen (BUN), alanine aminotransferase (ALT), and free fatty acid (FFA) were detected using colorimetry. The determination methods were carried out in strict accordance with the manual of Nanjing Jiancheng Biological kit (Nanjing, China). TP, ALB, TC, TG, BUN, ALT were tested by automatic biochemical analyzer (Hitachi-7020, Hitachi, Japan). FFA was tested by enzyme marker (Tecan Spark 10 M, Switzerland).

2.6. Meat color and pH measurement

The pH values of LD were measured at a position of 6 cm from the last rib to the dorsal midline using a pH meter (Testo 205, Testo Instrument Co., Ltd., Germany). The values of 45 min and 24 h after slaughter were recorded as pH_{45min} and pH_{24h}, respectively. LD meat color was recorded after slaughter for 24 h by MinoltaCR-400 colorimeter (Konica Minolta, Tokyo, Japan), including Lightness (L*), redness (a*), yellowness (b*). Each indicator was measured three times.

2.7. Meat water holding capacity assessment

LD were cut into a specific shape (about 2 × 2 × 2 cm), the initial weight was recorded, then the sample was placed into a drip loss pipe and kept closed, to keep the meat sample away from the pipe wall, and stored at 4 ℃ for 24 h. Then, the sample was weighed again after wiping the surface water with filter paper, which was recorded as the final weight. Drip loss was calculated by initial and final weights (Su et al., 2022). About 50 g of three LD cuboid samples (muscle membrane and attached fat were removed) were weighed, packed in a sealed bag and

immersed in a water bath to the internal temperature of 75 ℃. After cooling to room temperature, the meat sample was dried with filter paper and weighed again. Cooking loss (%) = (meat weight before cooked/meat weight after cooked) × 100 (Li et al., 2018).

2.8. Meat shear force detection

An electronic muscle tenderness meter was used to measure the shear force (#C-LM36, Engineering College of Northeast Agricultural University, China). Three points were cut along the direction perpendicular to the pectoral muscle fiber after cooking of the LD samples (Li et al., 2018).

2.9. Determination of antioxidant capacity of muscle, serum and liver

The LD and liver (about 3–5 g in the middle of the large leaf) samples of each finishing pig were taken and stored at −80 °C until use. Glutathione peroxidase activity (GSH-PX) and serum total antioxidant capacity (*T*-AOC) were determined using colorimetric method, superoxide dismutase activity (SOD) was determined using hydroxylamine method, catalase activity (CAT) was determined using UV method, thiobarbituric acid (TBA) was used to determine malondialdehyde (MDA). The Nanjing Jiancheng biological kit's instructions were followed while measuring all the indicators (Nanjing, China). By using a full-band enzyme labeling device (SynergyH4, BioTek Company, USA), the antioxidant capacity of the muscle, serum, and liver was discovered.

2.10. Determination of mRNA expression levels by RT-qPCR

Following the directions on the TRIzol reagent package, total mRNA was extracted from the LD of finishing pigs (China Bioengineering Co., Ltd., China). The reverse transcription reaction was performed using Novozan HiScipt II 1st strand cDNA synthesis (+gDNA wiper) kit. The *GAPDH* mRNA (loading control) and target sequence specific primers, including lipid metabolism genes and antioxidant genes, were designed by Primerbank and synthesized by Henan Shangya Biotechnology Co., LTD, China. All primer sequences in this study are listed in Table S2. The dissolution curve was collected for primer specificity verification, and the relative expression of the gene was analyzed by $2^{-\triangle\triangle Ct}$ method.

2.11. Measurement of chemical composition of LD

The moisture content of each sample was determined by drying in an electric oven (105 ℃) to a constant weight. The crude protein content was determined by Kjeldahl nitrogen determination method and the IMF was calculated by the Soxhlet extraction method. The detection method is based on previous research methods (Li et al., 2018).

2.12. Determination of amino acid content of muscle

To identify the amino acids present in LD samples, an automated amino acid analyzer was utilized (LC5090, Fuli, Zhejiang, China). The pretreatment procedure was carried out according to previous research methods (Liu et al., 2020). The procedure of sample pretreatment was as follows: a 25 mg sample was ground and crushed, put into a 5 mL ampoule to which was then added 3 mL of 6 mol/L hydrochloric acid; the ampoule was then sealed with high-temperature drawing seal of alcohol lamp, and subsequently put into the oven for hydrolysis at 110 ℃ for 24 h. The hydrolyzed sample was then transferred from the ampoule bottle to the evaporating dish, and the washing solution from the ampoule bottle (multiple washes with water) was also transferred to the evaporating dish, and then steamed dried in a water bath at 80 ℃. The evaporating dish was then washed with derivative buffer solution several times; the washing solution was transferred into a 25 mL volumetric flask; And the volume was fixed with derivative buffer solution. Finally, the solution was filtered using a 0.45 μm microporous membrane. The UltinateAQ-C18 chromatographic column (5 μm, 4.6 × 250 mm) was used to detect a wavelength of 360 nm and the column temperature was 27 ℃. According to the standard sample provided in the kit of amino acid analysis reagent, the standard curve was delimited and quantified by area external standard method.

2.13. Determination of fatty acid content of muscle

Fatty acid determination was carried out according to the gas chromatograph protocol (GC-2010PLUS, SHIMADZU, Germany). The pretreatment procedure was carried out according to previous research methods (Liu et al., 2020). Sample pretreatment was as follows: 0.5 g dried meat samples were crushed and placed into a 15 mL test tube with a stopper; 4 mL isooctane was added and the tube was placed in a vortex mixer for 30 s, and then placed in a 37 ℃ constant temperature shaker overnight. Aliquots of 4 mL of 2 mol/L potassium hydroxide methanol solution were added, vortexed and mixed for 30 s for rapid methylation. The solutions were left to stand for 30 min for layering, then approximately 1 g of sodium hydrogen sulfate was added into the test tube, shaken violently to neutralize the remaining potassium hydroxide, and left to stand until the solution was clear. The methylated supernatant was filtered using a 0.45 μm microporous membrane. The chromatographic column was a polyethylene glycol strong polar stationary phase with a length of 30 m, an inner diameter of 0.25 mm, and a film thickness of 0.25 μm. The detector was a flame ionization detector (FID). Sampler temperature was 220 ℃, while detector temperature was 280 ℃, and the programmed temperature rose to 50 ℃ for 1 min. The temperature in stage 1 was increased to 220 ℃ at a rate of 25 ℃/min for 0 min, and the temperature in stage 2 was raised to 230 ℃ at the rate of 3 ℃/min for 18 min. The carrier gas was nitrogen. The standard curve was delimited and quantified by area external standard method.

2.14. Nontargeted metabolomics analysis of LD

Samples of 50 mg were accurately weighed for metabonomic analysis. Each LD sample was added with 400 μL methanol:water (4:1, v/v) and 0.02 mg/mL L-2-chlorophenylalanine. The sample solution grinded for 6 min (−10 ℃, 50 Hz), and low temperature ultrasonic extraction 30 min (5 ℃, 40 kHz). The samples were placed at −20 ℃ for 30 min and centrifugation for 15 min (13,000*g*, 4 ℃), then the supernatant was transferred to sample vials for LC-MS/MS analysis. Thermo Fisher Scientific's UHPLC-Q Exactive system with an ACQUITY HSS T3 column (100 mm 2.1 mm i.d., 1.8 m; Waters, USA) serves as the instrument platform for LC-MS analysis at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) (Li, Al-Dalali, Zhou & Xu, 2022).

The Progenesis QI (Waters Corporation, Milford, USA) software pretreated the LC/MS raw data, deredundant, extracted from the data matrix, and peak-pooled. The HMDB (https://www.hmdb.ca/), Metlin (https://metlin.scripps.edu/), and Majorbio Database were the primary databases used to identify the metabolites.

The data was examined using the Majorbio cloud platform's web platform (cloud.majorbio.com). The metabolic features detected have been preserved in at least 80% of the samples, and carry on the normalization processing. Meanwhile, variables eliminated from QC samples had a relative standard deviation (RSD) > 30%.

For interactive analysis, the R package "ropls" (Version 1.6.2) was utilized. Based on the Variable importance in the project (VIP) acquired by the OPLS-DA model and the *P*-value produced by the Student's *t*-test, the metabolites with VIP > 1, $P < 0.05$ were considered to be significantly different metabolites. Finally, on the basis of the KEGG database (https://www.genome.jp/kegg/), metabolic pathway analysis was carried out. The above research analysis is based on previous studies (Li, Al-Dalali, Zhou & Xu, 2022).

2.15. Statistical analysis

Statistical analysis and mapping were performed using GraphPad Prism (v.8, GraphPad Software, La Jolla, CA, USA). All data are expressed as mean ± Standard Error of Mean (SEM). Between-groups comparisons were made using one-way ANOVA or Student's *t*-test (CON vs 25% ALM). Data were analyzed through the free online platform of Majorbio Cloud Platform (cloud.majorbio.com). At the same time, the metabolites were searched and identified, and the main databases were the HMDB (http://www.hmdb.ca/), Metlin (https://metlin.scripps.edu/), and Majorbio Database. Based on Pearson's analysis, the relationship between meat quality parameters and antioxidant level, the relationship between lipid metabolism and antioxidant genes expression, and the relationship between different metabolites and meat

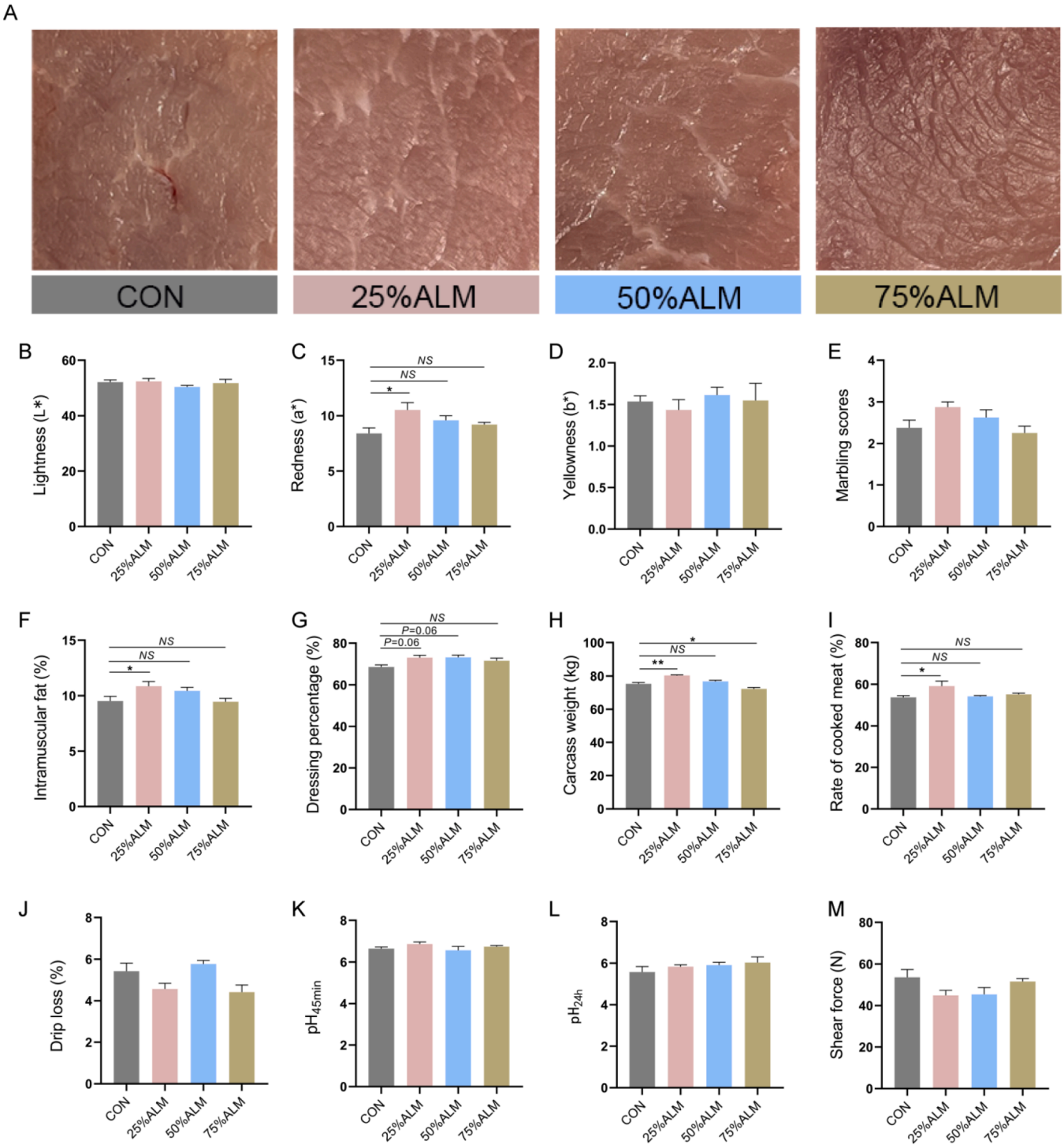

Fig. 1. Effects of ALM on the carcass traits and meat quality of LD of finishing pigs by replacing different proportions of SBM in the diet. (A) Morphological observation. (B) Lightness, L*. (C) Redness, a*. (D) Yellowness, b*. (E) Marbling scores. (F) Intramuscular fat content. (G) Dressing percentage. (H) Carcass weight. (I) Rate of cooked meat. (J) Drip loss. (K) pH_{45min}. (L) pH_{24h}. (M) Shear force. The analytical method was LSD. Value was presented as mean ± SEM; *NS*, not significant, $P \geq 0.1$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

quality were analyzed.

3. Results

3.1. Growth parameters and carcass traits

The results showed that the F/G ratio in the 25% ALM diet group was significantly lower ($P < 0.05$) compared to the CON group, while there was no significant difference ($P > 0.05$) in 50% ALM diet and 75% diet ALM compared to CON (Fig. S1A). Compared with CON, there was no significant difference ($P > 0.05$) in the average daily gain in 25% ALM diet, while those in 50% ALM diet and 75% ALM diet were significantly lower ($P < 0.05$) (Fig. S1B). Moreover, the average daily feed gain in all experimental groups were significantly decreased ($P < 0.01$) (Fig. S1C). These results implied that the diet supplemented with ALM replacing 25% SBM diet was most beneficial for finishing pigs.

The 25% ALM diet and the 50% ALM diet groups had increasing trends ($P = 0.06$) in the dressing percentage compared with CON (Fig. 1G). The carcass weight of finishing pigs in 25% ALM diet significantly increased ($P < 0.01$), while that in 75% ALM diet significantly

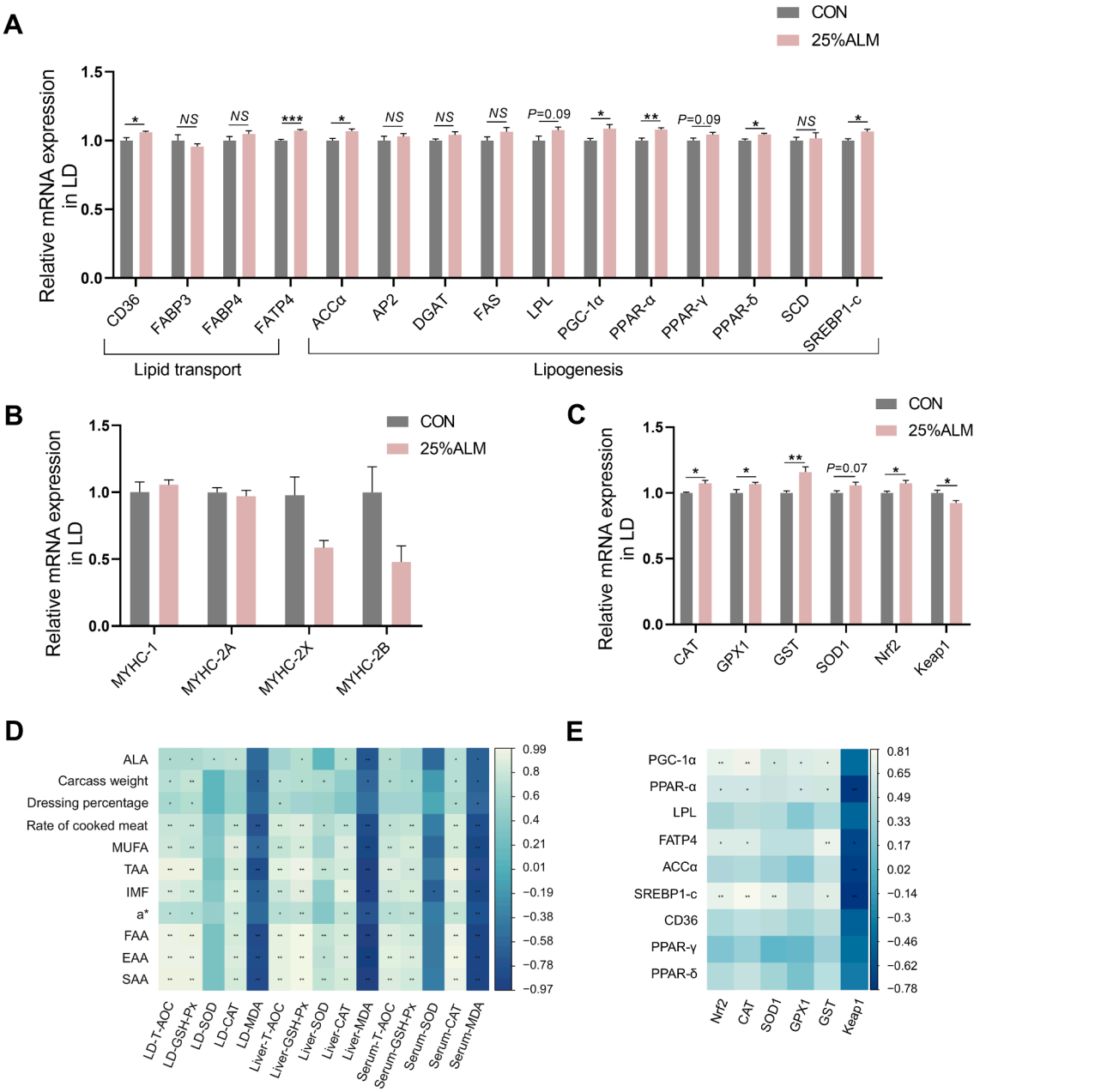

Fig. 2. Effect of ALM replacing 25% SBM on expression of genes in LD of finishing pigs. (A) The mRNA expression of genes related to lipid metabolism. (B) mRNA expression of genes related to muscle fiber types. (C) The mRNA expression of genes related to antioxidant capacity. (D) Correlation analysis of antioxidant capacity and meat quality related parameters. ALA, α-linolenic acid; MUFA, Saturated fatty acids; IMF, Intramuscular fat; a*, redness; TAA, Total amino acid; FAA, Flavor amino acid; EAA, Essential amino acid; SAA, Sweet amino acid. (E) Correlation analysis of antioxidant genes and lipid metabolism genes. The analysis method was Student's *t*-test. Value was presented as mean ± SEM. *NS*, not significant, $P \geq 0.1$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

decreased ($P < 0.05$) compared with CON (Fig. 1H). In addition, heart weight and skin thickness in treatment groups were increased compared with CON (Fig. S2F and J), while no apparent changes in liver weight (Fig. S2G), kidney weight (Fig. S2H), back fat thickness (Fig. S2I), and carcass length (Fig. S2K-L) were found.

3.2. Effects of ALM diet on antioxidative activities of finishing pigs

As shown in Table 1, compared with CON, the contents of *T*-AOC, GSH-Px, and CAT in LD, liver and serum in 25% ALM diet were significantly increased, and the MDA content was significantly decreased ($P < 0.05$), respectively. The 25% ALM diet had significantly increased SOD contents in liver ($P < 0.01$). These results indicated that ALM replacing 25% SBM diet could improve the antioxidant capacity of finishing pigs.

The expression levels of antioxidant genes (*CAT*, *GPX1*, *GST*, *SOD1*, and *Nrf2*) in LD of 25% ALM diet were also increased, while the expression of *Keap1* was inhibited (Fig. 2C). These results indicated that ALM diet may be involved in the activation of the *Nrf2* pathway, in which the expression of *Keap1* was inhibited, which then upregulated the expression of antioxidant enzyme genes (*SOD*, *CAT,* and *GSH-Px*).

3.3. Serum metabolites

As presented in Table S3, compared to CON, the contents of triglyceride ($P < 0.05$) and insulin ($P < 0.01$) were significantly increased, and the free fatty acid content was significantly decreased ($P < 0.001$) in 25% ALM diet; Meanwhile no significant changes ($P > 0.05$) were found in the levels of TP, ALB, TC, BUN, and ALT in 25% ALM diet.

3.4. Meat quality

3.4.1. Meat quality related parameters

The a* of pork meat color in 25% ALM diet were significantly increased ($P < 0.05$) compared with CON (Fig. 1B-D). Compared with CON, a significant increase ($P < 0.05$) in the rate of cooked meat was noted in 25% ALM diet (Fig. 1I), with no significant difference ($P > 0.05$) in the marbling scores (Fig. 1E), dripping loss (Fig. 1J), pH (Fig. 1K-L), shear force (Fig. 1M), and eye muscle area (Fig. S2M) of pork in the experimental groups. The IMF content of LD in 25% ALM diet was significantly improved ($P < 0.05$) over CON (Fig. 1F). The above results indicated that ALM replacing 25% SBM diet was optimum for carcass traits and meat quality of finishing pigs.

Table 1
Effects of ALM replacing 25% SBM on antioxidant capacity of finishing pigs.

Items	CON	25% ALM	SEM	*P*-value
LD				
T-AOC (μmol/g)	0.014^{b}	0.017^{a}	0.000	0.000
GSH-Px (U/g)	529.01^{b}	633.17^{a}	35.342	0.007
SOD (U/g)	1058.24	1072.58	71.000	0.843
CAT (U/g)	54.40^{b}	72.29^{a}	4.815	0.001
MDA (nmol/g)	66.21^{a}	50.15^{b}	4.351	0.001
Liver				
T-AOC (μmol/g)	0.016^{b}	0.019^{a}	0.001	0.014
GSH-Px (U/g)	435.63^{b}	761.49^{a}	64.081	0.000
SOD (U/g)	897.76^{b}	1152.80^{a}	80.338	0.004
CAT (U/g)	65.69^{b}	78.50^{a}	5.686	0.039
MDA (nmol/g)	74.06^{a}	41.96^{b}	3.907	0.000
Serum				
T-AOC (μmol/mL)	0.018^{b}	0.021^{a}	0.001	0.001
GSH-Px (U/mL)	730.82^{b}	868.42^{a}	42.885	0.004
SOD (U/mL)	1250.45	1256.99	18.359	0.725
CAT (U/mL)	75.39^{b}	107.70^{a}	5.703	0.000
MDA (nmol/mL)	89.85^{a}	75.52^{b}	5.157	0.011

T-AOC, Total antioxidant capacity; GSH-Px, Glutathione peroxidase; SOD, Superoxide dismutase; CAT, Catalase; MDA, Malondialdehyde.
The analysis method was Student's *t*-test. Values with different superscript letters in each row are significantly different ($P < 0.05$).

The characteristics of muscle fibers (Fig. S2A-C), moisture content (Fig. S2D), and crude protein content (Fig. S2E) of LD showed no significant changes ($P > 0.05$) in treatment groups compared with CON. Moreover, the mRNA expression of genes related to muscle fiber types in each treatment group showed no apparent changes ($P > 0.05$) compared to CON, based on the results of qPCR verification (Fig. 2B).

3.4.2. Fatty acid content of LD

As presented in Table 2, compared with CON, the levels of decanoic acid (C10:0) ($P < 0.05$), myristic acid (C14:0) ($P < 0.05$), myristoleic acid (C14:1) ($P < 0.05$), palmitoleic acid (C16:1) ($P < 0.01$), and α-linolenic acid (ALA) ($P < 0.05$) in LD were increased significantly in 25% ALM diet, and the content of monounsaturated fatty acid (MUFA) showed an increasing trend ($P = 0.06$). Meanwhile the contents of heptadecanoic acid (C17:0) ($P < 0.05$), stearic acid (C18:0) ($P < 0.05$), and arachidic acid (C20:0) ($P < 0.05$) in LD were significantly decreased in 25% ALM diet, and the level of arachidonic acid (C20:1) had a decreasing trend ($P = 0.09$). These results indicated that ALM replacing 25% SBM diet could improve the composition of fatty acids in LD of finishing pigs.

The mRNA expression of lipid transport genes (*CD36*, *FATP4*) and lipid synthesis genes (*ACCα*, *LPL*, *PGC-1α*, *PPARα/δ*, *SREBP1-c*) were increased in LD in 25% ALM diet compared with CON (Fig. 2A). Meanwhile, the contents of IMF, ALA, carcass traits, meat quality, MUFA, and various amino acid contents were significantly positively correlated with antioxidant capacity of LD, liver, and serum, and negatively correlated with MDA ($P < 0.05$) (Fig. 2D). Lipid synthesis and

Table 2
Effects of replacing 25% SBM with ALM on fatty acid content of LD of finishing pigs (%).

Items	CON	25% ALM	SEM	*P*-value
C8:0	0.005	0.006	0.000	0.46
C10:0	0.072^{b}	0.095^{a}	0.009	0.04
C11:0	0.002	0.002	0.000	0.78
C12:0	0.073	0.082	0.008	0.30
C13:0	0.011	0.017	0.008	0.48
C14:0	1.108^{b}	1.291^{a}	0.059	0.01
C14:1	0.024^{b}	0.038^{a}	0.006	0.04
C15:0	0.039	0.033	0.005	0.23
C16:0	25.680	25.670	0.525	0.99
C16:1	2.929^{b}	3.437^{a}	0.119	0.00
C17:0	0.187^{a}	0.157^{b}	0.013	0.04
C17:1	0.163	0.158	0.011	0.65
C18:0	13.440^{a}	11.960^{b}	0.469	0.01
C18:1	42.050	43.450	0.843	0.13
C18:2n6	8.240	8.317	0.970	0.90
C18:3n3	0.366^{b}	0.522^{a}	0.065	0.04
C18:3n6	0.080	0.074	0.009	0.53
C20:0	0.184^{a}	0.135^{b}	0.015	0.01
C20:1	0.653^{a}	0.584^{b}	0.037	0.09
C20:2	0.336	0.324	0.035	0.73
C20:3n6	1.863	1.580	0.348	0.43
C20:3n3	0.245	0.208	0.036	0.33
C20:4	0.106	0.113	0.016	0.71
C20:5n3	0.045	0.046	0.009	0.92
C22:0	0.009	0.009	0.000	>0.9
C22:1	0.013	0.012	0.001	0.19
C22:2	0.006	0.007	0.000	0.18
C22:6n3	0.107	0.091	0.009	0.22
C24:1	0.044	0.049	0.006	0.41
SFA	40.800	39.460	0.968	0.20
MUFA	45.890^{b}	47.720^{a}	0.890	0.07
PUFA	13.300	12.810	1.480	0.75
n-6 PUFA	10.184	9.971	0.656	0.73
n-3 PUFA	0.757	0.824	0.078	0.41
n-6 /n-3	13.453	12.101	0.360	0.15

SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.
The analysis method was Student's *t*-test. Values with different superscript letters in each row are significantly different ($P < 0.05$).

transport genes (*PGC-1α*, *PPAR-α*, *FATP4*, and *SREBP1-c*) were significantly positively correlated with antioxidant genes (*Nrf2*, *CAT*, *SOD1*, *GPX1*, and *GST*), and negatively correlated with *Keap1* ($P < 0.05$) (Fig. 2E).

3.4.3. Amino acid content of LD

As shown in Table 3, the contents of phenylalanine (Phe) ($P < 0.01$), aspartic acid (Asp) ($P < 0.01$), glutamic acid (Glu) ($P < 0.01$), glycine (Gly) ($P < 0.05$), alanine (Ala) ($P < 0.05$), threonine (Thr) ($P < 0.01$), lysine (Lys) ($P < 0.01$), sperm amino acid (Arg) ($P < 0.01$), valine (Val) ($P < 0.01$), isoleucine (Ile) ($P < 0.01$), and leucine (Leu) ($P < 0.05$) in LD in 25% ALM diet were significantly increased compared with CON. In addition, the contents of essential amino acids ($P < 0.01$), flavor amino acids ($P < 0.05$), sweet amino acids ($P < 0.05$), and total amino acids ($P < 0.05$) in LD were also significantly increased in 25% ALM diet over CON. These results indicated that ALM replacing 25% SBM diet could improve the composition of amino acids in LD of finishing pigs.

3.5. Nontarget metabolomics analysis of LD

Metabolite differences in LD between different groups were studied using non-targeted metabolomics analysis to investigate whether the changes in metabolites were related to meat quality. Based on OPLS-DA analysis, it was found that the score maps between 25% ALM diet and CON were significantly separated (Fig. 3A-B), indicating that there were significant differences in metabolic profiles between the two groups. Through volcano map analysis for the screening of differentially expressed metabolites (VIP > 1.5), several potential biomarkers related to muscle lipid metabolism and antioxidant capacity such as proline betaine (stachydrine) were identified (Fig. S3). From the results of KEGG enrichment analysis for differentially expressed metabolites, we found that ALM replacing 25% SBM diet affected some pathways including retrograde endocannabinoid signaling, Phe metabolism, glycerophospholipid metabolism, and glutathione metabolism (Fig. 3C).

To further study the potential relationships between differentially expressed metabolites and meat quality related parameters, we conducted Spearman correlation analysis as shown in Fig. 3D. The results showed that stachydrine was significantly positively correlated with carcass traits, meat quality related parameters, various amino acid contents, ALA, and MUFA, and significantly negatively correlated with MDA. L-carnitine was positively correlated with meat color, various amino acid contents, and MUFA, and negatively correlated with MDA. There was a significant positive correlation among 2-methylbutyroylcarnitine, ALA, antioxidant capacity related indicators, and various amino acid contents. (R)-3-hydroxybutyrylcarnitine was negatively correlated with ALA, carcass weight, meat quality related parameters, various amino acid contents, and MUFA, and positively correlated with MDA. There was a significant positive correlation among oleyl alcohol, carcass weight, meat quality related parameters, various amino acid contents, and MUFA. Moreover, inosine 2′-phosphate and *cis*-5-tetradecenoylcarnitine were significantly positively correlated with meat quality related parameters, various amino acid contents, and MUFA, and significantly negatively correlated with MDA.

Table 3
Effects of replacing 25% SBM with ALM on amino acid content of LD of finishing pigs (%).

Items	CON	25% ALM	SEM	*P*-value
Phe[1,2]	0.720[b]	0.922[a]	0.020	0.002
Asp[2]	1.625[b]	2.061[a]	0.053	0.004
Glu[2]	2.631[b]	3.296[a]	0.059	0.001
Tyr[2]	0.603	0.734	0.039	0.079
Gly[2,3]	0.902[b]	1.274[a]	0.087	0.024
Ala[2,3]	0.924[b]	1.218[a]	0.078	0.033
Pro[3]	0.790	0.810	0.042	0.682
Ser[3]	0.701	0.960	0.088	0.060
Thr[1,3]	0.783[b]	1.007[a]	0.036	0.008
Lys[1,3]	1.882[b]	2.459[a]	0.058	0.002
Arg	1.124[b]	1.423[a]	0.037	0.004
Met	0.151	0.118	0.079	0.702
Cys	0.093	0.108	0.012	0.307
His	0.983	1.442	0.442	0.375
Val[1]	0.922[b]	1.202[a]	0.029	0.002
Ile[1]	0.825[b]	1.110[a]	0.031	0.003
Leu[1]	1.269[b]	1.743[a]	0.083	0.011
EAA	6.400[b]	8.445[a]	0.206	0.002
FAA	7.403[b]	9.640[a]	0.397	0.011
SAA	5.828[b]	7.726[a]	0.353	0.013
TAA	16.772[b]	22.021[a]	1.212	0.023

The analysis method was Student's *t*-test. Values with different superscript letters in each row are significantly different ($P < 0.05$).
[1] EAA, Essential amino acid.
[2] FAA, Flavor amino acid.
[3] SAA, Sweet amino acid; TAA, Total amino acid.

4. Discussion

4.1. Effect of dietary addition of ALM diet on the performance of finishing pigs

Owing to the unstable global economy, politics, and health related matters in recent years, the production of SBM feed ingredients, which is dependent on imports, as well as pork production, have been severely constrained. Making full use of local feed resources, research and development of multi-dimensional feed formulations are needed for food safety, both in China and throughout the world. Among the feed ingredients that can replace SBM, alfalfa has a higher protein yield and relatively high concentrations of Lys and methionine than other legumes, making it a good protein source for pigs and poultry (Blume, Hoischen-Taubner & Sundrum, 2021). To maximize growth parameters and nutrient digestion, alfalfa meal should only make up 5% of fattening pig feeds due to the amount and kind of fiber it contains. (Liang, Gao & Zhang, 2014). Alfalfa leaves are a high value component of animal feed crops, with a higher protein content (260–300 g/kg) compared to alfalfa stems (100–120 g/kg). Alfalfa leaves have higher protein and lower crude fiber content and thus higher nutritional value than whole alfalfa. In the current study, the highest feed conversion rate, without affecting average daily gain of finishing pigs, was in diets where ALM replaced 25% of the SBM in the diet, indicating that ALM has more feeding value replcaed of SBM. Meanwhile, ALM diet increased the dressing percentage and carcass weight of finishing pigs; however, with increased replacement of SBM, the carcass weight showed a downward trend, and was the lowest when ALM replaced 75% of the SBM in the diet. Alfalfa contains a certain amount of saponins, which added in too high a quantity could reduce the palatability of the feed, thus decreasing the intake of pigs, and may eventually lead to a reduction in body weight gain (Clouard, Meunier-Salaün & Val-Laillet, 2012). The results indicated that 25% was the optimum proportion of ALM for replacing SBM to promote growth and carcass traits of finishing pigs. Higher levels of ALM were unfavorable.

4.2. Effect of ALM diet on antioxidant in the organism

Following intensive research on alfalfa, it has been shown that alfalfa leaf protein is of high quality and excellent for the production of nutritious foods. After protease hydrolysis, alfalfa leaf protein is converted into alfalfa leaf peptides, which have the antioxidant capacity to scavenge free radicals. Flavonoids, especially tricin and apigenin glycosides, have been found to have a higher antioxidant activity in alfalfa than traditional antioxidants such as butylated hydroxytoluene (Goławska, Łukasik, Kapusta & Janda, 2010). Alfalfa also contains coumestrol and apigenin, which show good antioxidant properties in various LDL oxidation systems (Hwang, Hodis & Sevanian, 2001). Alfalfa flavonoids can promote livestock growth parameters, improve carcass traits, eliminate free radicals, and enhance antioxidant

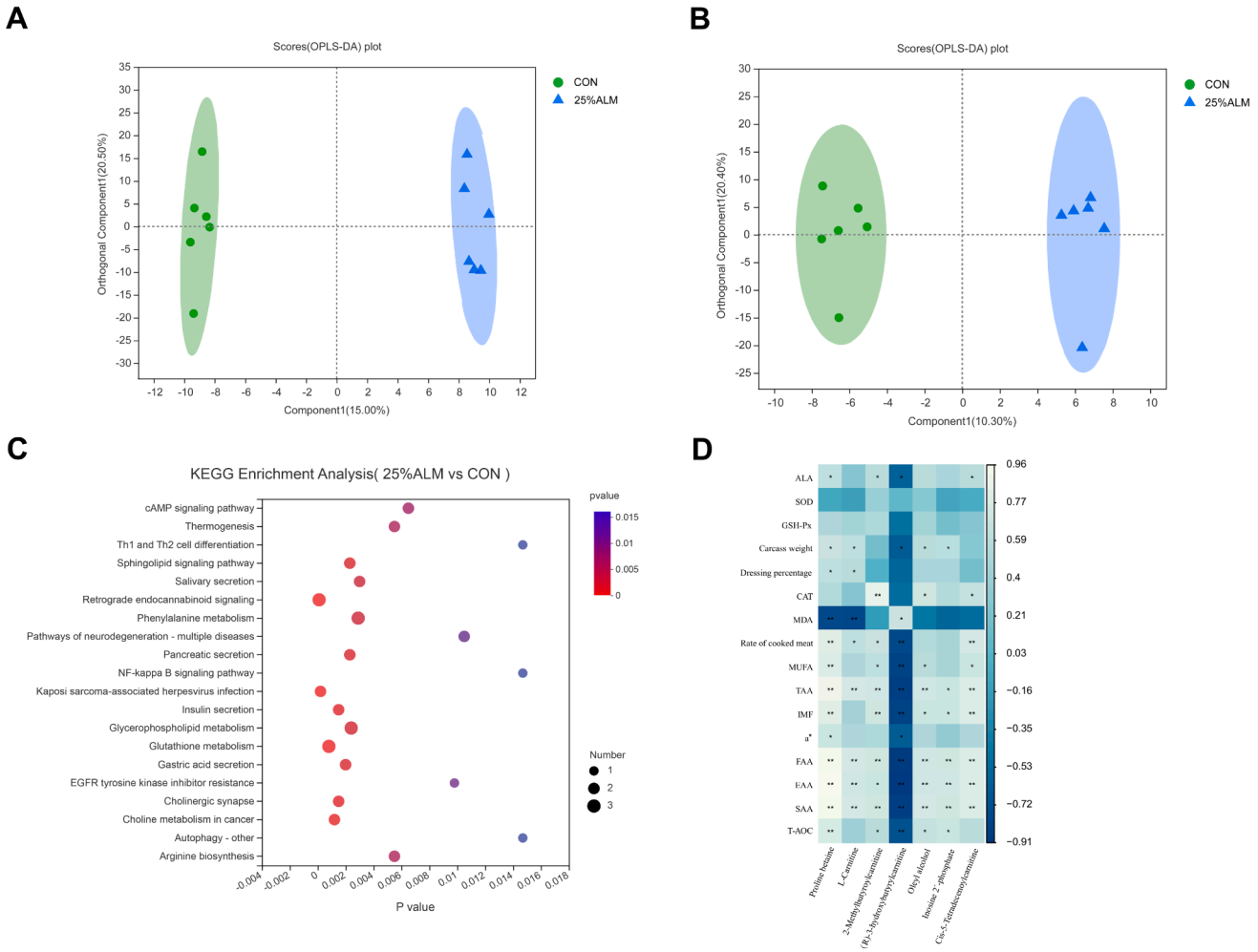

Fig. 3. Effects of ALM on the non-targeted metabolome in LD of finishing pigs by replacing 25% SBM in the diet. (A) OPLS-DA analysis for cation of non-targeted metabolome in LD. (B) OPLS-DA analysis for anion of non-targeted metabolome in LD. (C) KEGG enrichment analysis of differential expressed metabolites. (D) Spearman correlation analysis between differential expressed metabolites and parameters related to meat quality. ALA, α-linolenic acid; MUFA, Saturated fatty acids; IMF, Intramuscular fat; a*, redness; TAA, Total amino acid; FAA, Flavor amino acid; EAA, Essential amino acid; SAA, Sweet amino acid.

properties and host immunity within a certain range (Sun et al., 2023). We therefore hypothesized that the use of appropriate amounts of ALM instead of SBM in finishing pig diets might improve their antioxidant properties. Consistent with our expectation, our study showed that 25% ALM diet significantly increased the content of *T*-AOC, GSH-Px, and CAT in LD, liver, and serum of finishing pigs, and also significantly increased SOD in liver, while MDA in LD, liver, and serum was significantly reduced. Usually, the activity of antioxidant enzymes is regulated by the expression of antioxidant-related genes. Recent studies have shown that protection against mammalian oxidative stress is mediated by nuclear factor (Nrf2), a master regulator of the antioxidant defense system, and Keap1, a cellular sensor of ROS that targets Nrf2 for proteasomal degradation. However, Nrf2 can be activated by ROS, translocated into the nucleus, and bound to ARE, leading to the downstream genes encoding antioxidant enzymes (*CAT*, *GPX1*, *GST*, and *SOD1*) being upregulated (He et al., 2018). Excitingly, in the present study we found that 25% ALM diet significantly upregulated *CAT*, *GPX1*, and *GST* gene expression levels by activating *Nrf2* and inhibiting *Keap1*, which in turn increased antioxidant enzyme levels in the body (LD, liver, and serum) and ultimately reduced oxidative stress.

4.3. *Effect of ALM diet on meat quality*

Oxidative processes are the main cause of deterioration in meat quality and oxidative stress is directly related to muscle tenderness in the early postmortem stages of animals. Oxidative stress destroys the normal muscle structure and accumulates harmful by-products, such as lipid and protein by-products, and ultimately leads to deterioration of meat quality. Lipid peroxidation leads to the peroxidative loss of unsaturated lipids, which leads to the reduction of lipid content and disruption of cell membrane structure. Furthermore, MDA is a metabolite of lipid peroxidation, which is generated by oxygen radical-induced lipid oxidation reactions in tissues, while MDA can cause further oxidation of OxyMb to form MetMb, promote protein carbonylation, enhance the muscle ROS-generating system, and promote the extent of protein oxidation (Wang, He, Emara, Gan & Li, 2019). The results of the latter study show that the contents of IMF and meat quality were significantly positively correlated with antioxidant capacity of LD, liver and serum, and negatively correlated with MDA. Moreover, lipid synthesis and transport genes were significantly positively correlated with antioxidant genes, and negatively correlated with *Keap1*. This coincides with the fact that when the antioxidant capacity was enhanced, lipid

peroxidation would be reduced (Catalá, 2006). The expression of lipid transport (*CD36*, *FATP4*) and lipid synthesis-related genes (*ACCα*, *PGC-1α*, *PPARα*, *PPARδ*, *SREBP1-c*) was upregulated in muscle, thus promoting IMF deposition (Wu et al., 2022; Nickerson et al., 2009). Meanwhile, in the present study, 25% ALM diet significantly increased a*, cooked meat percentage, and IMF in fattened LD, but had no significant effect on marbling, drip loss, shear force, muscle fiber properties, moisture, and crude protein. Meat color affects the perception of freshness of meat products and is one of the most important factors for customers in their assessment of meat quality, where a* is the key to the desired meat color, and an increase in a* within a certain range indicates an improvement in muscle color quality. Steaming loss is the loss of moisture from meat during cooking, and spoiled meat can lead to greater cooking losses. IMF content and fatty acid composition are key factors for meat quality. It is generally accepted that IMF content has a positive effect on sensory quality characteristics, including meat flavor, juiciness, and tenderness, while low fat content leads to poor meat flavor. Thus, we can speculate that 25% ALM diet had a favorable impact on meat quality of finishing pigs.

4.3.1. Effect of ALM diet on muscle lipid metabolism

Consumers' perception of meat quality is influenced heavily by their socio-demographic background, and thus on cultural factors and health expectations. As society has evolved, consumer concerns about meat quality have shifted from fat quantity to fat quality (i.e., fatty acid composition) in order to reduce dietary ingestion of saturated fatty acids, total cholesterol, and the risk of cardiovascular disease. Regardless of food source, the relationship between dietary fat and its effect on various lifestyle diseases, including cardiovascular disease, is well established. According to certain research, adding alfalfa in the diet results in an increased production of SCFAs. SCFAs may act as starting materials for the body's synthesis of functional fatty acids (Wang et al., 2018). In the present study, we found no significant effect of 25% ALM diet on the content of saturated, polyunsaturated, n-6 polyunsaturated, and n-3 polyunsaturated fatty acids, but monounsaturated fatty acids tended to increase, and increased the content of decanoic acid and ALA in LD of finishing pigs. The medium-chain fatty acid decanoic acid is reported to be a powerful bactericidal agent, possessing antibacterial properties against a number of Gram-positive and Gram-negative microorganisms, and also possessing both antifungal and antiviral properties (Huang et al., 2014). Palmitoleic acid also has some antibacterial properties, especially for Staphylococcus aureus. In animals, ALA may contribute to the regulation of inflammation and liver metabolism and may be used to treat metabolic disorders associated with cardiovascular disease (Jordao et al., 2019). In addition, saturated fatty acids such as heptadecanoic acid, searic acid (C18:0), and arachidic acid were reduced in muscle, thus lowering the risk of cardiovascular disease induced by dietary saturated fatty acids. We therefore concluded that 25% ALM diet increased functional fatty acids, including decanoic acid, palmitoleic acid, and ALA, which have particularly positive effects on meat quality, and thus may increase the muscle preservation capacity of finishing pigs and the positive health effects on humans after pork consumption.

The diet containing 25% ALM diet increased IMF content in the muscles of finishing pigs, indicating that it may have altered lipid metabolism. In the present study, we found that 25% ALM diet significantly increased plasma levels of TG and INS, while decreasing the level of FFA in plasma. Although plasma TG was up-regulated in a certain range, the analysis of growth status and physiological status of various tissues and organs of finishing pigs showed that 25% ALM diet did not affect the indices of heart, skin thickness, liver, kidney, backfat thickness, and carcass length of finishing pigs.

Whereas lipolysis promotes the mobilization of metabolic fuels from adipose to surrounding tissues in response to appropriate energy demands, lipolysis occurs primarily in adipose tissue and fat accumulates primarily as subcutaneous fat (SAT), which is a major contributor to plasma FFA (Mittendorfer, Magkos, Fabbrini, Mohammed & Klein, 2009). Like carbohydrates, insulin promotes lipid synthesis and inhibits its degradation (Saltiel & Kahn, 2001). Increased plasma insulin stimulates hepatic fatty acid synthesis, which is mediated by increased transcription of genes encoding ACC and FAS, and this stimulation is responsible for elevated TG in plasma (Shimomura et al., 1999). In the present study, consistent with our expected results, 25% ALM diet similarly altered molecular regulation regarding lipid metabolism in LD. 25% ALM diet significantly upregulated the expression of lipid transport genes *CD36* and *FATP4* in muscle, enhancing fatty acid uptake in LD and reducing plasma FFA levels. Meanwhile the expression of related lipid synthesis genes *ACC*, *PGC-1α,* and *SREBP1-c* were also upregulated, thus promoting the accumulation of IMF in LD.

4.3.2. Effect of ALM diet on muscle amino acid composition

The animal body's most important form of protein storage is found in the muscle, and muscle protein metabolism determines muscle mass (Lv et al., 2022). The availability of amino acids is an effective regulator of muscle protein synthesis (Wolfe, 2002). Amino acids are typically categorized as either nutritionally necessary or non-essential for animals based on growth or nitrogen balance (Wu et al., 2014). In the present study, we found that 25% ALM diet significantly increased the essential amino acid content of Phe, Thr, Lys, Val, Ile, and Leu, as well as the conditionally essential amino acids Glu, arginine (Arg), and Gly, in LD. There is growing evidence that amino acids have roles beyond protein synthesis. Some studies introduced the concept of functional amino acids, which are those amino acids that take part in and regulate important metabolic pathways to promote animal growth, development, and reproduction. (Wu, 2010). Functional amino acids can be essential, nonessential, and conditionally essential (Hou et al., 2012). In many mammals, including sheep, rats, and pigs, Arg supplementation can increase litter weight (Satterfield, Dunlap, Keisler, Bazer & Wu, 2013). Glu is involved in many anabolic and oxidative pathways in animals and can be used as a major energy substrate and excitatory neurotransmitter in the intestine, which regulates the release of certain hormones, improves digestion and absorption in the intestine, and is a functional amino acid used in animal production (Wu et al., 2014; Baj et al., 2019). The two most abundant proteins in animals, collagen and elastin, are primarily made up of Gly. Gly is also the precursor of various low molecular weight important metabolites, such as creatine, glutathione, purine, and heme, and is an inhibitory neurotransmitter and antioxidant in the central nervous system (Moura et al., 2014; Razak, Begum, Viswanath & Rajagopal, 2017; Wang et al., 2013). Branched-chain amino acids can provide individual amino acids for the synthesis of Ala (a major sugar-producing amino acid) and glutamine (an abundant amino acid in physiological fluids, including milk), and Leu, Ile, and Val are all branched-chain amino acids; increased circulating levels of these amino acids may in turn activate cellular signaling pathways that stimulate protein synthesis (Rezaei, Wu, Hou, Bazer & Wu, 2016). Under physiological conditions, supplementation of grain-based diets with Leu increases the rate of protein synthesis in skeletal muscle of neonatal pigs (Murgas et al., 2010). As the total amount of Val in the sow diet increases from 0.85% to 1.15%, piglet growth increases (Lei et al., 2012); and when the piglet diet is supplemented with Ile, glucose uptake into muscle is enhanced, potentially increasing piglet muscle and intestinal development (Zhang et al., 2016). In the current study, when finishing pigs were fed 25% ALM diet, muscle content of essential amino acids, conditionally essential amino acids, functional amino acids, fresh amino acids, sweet amino acids, and total amino acids increased, so we speculated that 25% ALM diet improved the amino acid nutritional structure and quality of pork.

4.4. Effect of ALM diet on muscle metabolome

To understand the effect of 25% ALM diet on metabolic processes in LD and to characterize the metabolites in muscle tissue, we used

untargeted metabolomics analysis and found that 25% ALM diet significantly altered the metabolic profile in LD of finishing pigs. The results of the analysis of variance showed that 25% ALM diet significantly increased muscle stachydrine, L-carnitine, 2-methylbutyrylcarnitine, oleyl alcohol, inosine 2′-phosphate, and *cis*-5- tetraallyl ketone, and decreased the level of (R)-3-hydroxypropene in muscle. It was shown by previous studies that stachydrine is a free radical scavenger with protective effects on myocardial ischemia and plays a beneficial role in hypoxia reoxidation-induced injury and LPS-induced inflammation in endothelial cells (Cheng et al., 2020). L-carnitine is a powerful antioxidant that protects against oxidative damage to tissues, and L-carnitine is advantageous in reducing neurological damage caused by oxidative stress since it can across the blood–brain barrier (Ribas, Vargas & Wajner, 2014). Through this experiment, we additionally found that stachydrine and L-carnitine may promote growth and development and improve muscle quality in finishing pigs, and these functions are likely related to the antioxidant properties of stachydrine and L-carnitine. It was also found that 2-methylbutyrylcarnitine may have antioxidant capacity and promote amino acid production. Meanwhile, both inosine 2′-phosphate and *cis*-5-tetraallyl ketone were associated with the promotion of amino acid synthesis and improvement of muscle quality. (R)-3-hydroxypropene may be associated with oxidative damage in the body, slowing growth, reducing amino acid production, and decreasing meat quality of LD in finishing pigs. Finally, KEGG pathway enrichment analysis of the differential metabolites revealed that endocannabinoid signaling, Phe metabolism, glycophospholipid metabolism, and glutathione metabolism were involved in 25% ALM diet on metabolism in LD of finishing pigs, and functional regulation of the beneficial effects of ALM diet on LD of finishing pigs was achieved.

5. Conclusions

In this study, we used the new protein feed ALM in finishing pigs' diet to replace SBM, with an optimum replacement ratio of 25%. ALM diet promoted the growth parameters and improved the antioxidant capacity of finishing pigs. ALM diet also improved meat quality by changing the color redness (a*) and rate of cooked LD meat and increasing the content of IMF. At the same time, it improved the metabolic profile of fatty acids and amino acids in LD and increased the content of functional fatty acids beneficial to the human body. Metabolomic analysis revealed that ALM diet mainly affected amino acid and lipid metabolic pathways. The improvement in meat quality of finishing pigs was attributed to the modulation of ALM diet on lipid metabolism and antioxidant capacity. These findings help us to better understand the effects of ALM diet on growth parameters and meat quality of finishing pigs, and contribute to the use of ALM diet as a partial replacement of traditional SBM diet as a protein feedstuff for high-quality pork production.

CRediT authorship contribution statement

Ming Guo: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Zhichang Wang:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – review & editing. **Zimin Gao:** Methodology. **Jixiang Ma:** Methodology. **Weikang Huangfu:** Methodology. **Jiakuan Niu:** Methodology. **Boshuai Liu:** Methodology. **Defeng Li:** . **Xiaoyan Zhu:** Methodology. **Hao Sun:** Methodology. **Sen Ma:** Methodology. **Yinghua Shi:** Funding acquisition, Methodology, Resources, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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