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Effect of dietary *Sophora alopecuroides* supplementation on differential expression of intramuscular fat-related genes of Ningxia Tan sheep as determined by transcriptome sequencing

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Abstract: The present study investigated the effect of different dietary levels (0%, 0.25%, 0.5%, 1%, 2%) of *Sophora alopecuroides* (*S. alopecuroides*) on intramuscular fat (IMF) content in Ningxia Tan sheep. Candidate genes affecting IMF deposition were screened by comparing the RNA-Seq profiles of test (2% *S. alopecuroides*) and control (no *S. alopecuroides*) groups. The results showed that the intramuscular fat content of the diet supplemented with *S. alopecuroides* was significantly lower than that of the control group, and the intramuscular fat content decreased significantly with the increase of the dietary content of *S. alopecuroides* ($P < 0.05$). The highest IMF content of the control group (group A) was 4.21%, which was significantly higher than that of the other four experimental groups (B, C, D, E) ($P < 0.05$). Moreover, 66 genes were significantly upregulated and 202 genes were significantly downregulated in the test group compared to the control. Gene Ontology functional annotation revealed that the differentially expressed genes were closely related to plasma lipoprotein particle assembly, plasma lipoprotein particle organization, protein-lipid complex subunit organization, and other biological processes. KEGG pathway analysis showed that the differentially expressed genes belonged to 147 metabolic pathways. Finally, four differential genes which may be related to IMF deposition were screened out in PPAR signalling pathway, fatty acid biosynthesis, fatty acid degradation, and fatty acid metabolism. The quantitative analysis of four differential genes including *ACSL3, PLIN2, ABCA1* and *ANGPTL4* was carried out by RT-qPCR method, and the results were basically consistent with those of RNA-seq. Among them, the quantitative results of *PLIN2* gene were slightly different from the sequencing results, but the difference was not significant, and they may be false positive. This study lays the foundation for understanding the molecular mechanism regulating mutton quality, and provides a theoretical basis for the study of *S. alopecuroides* as a feed additive to improve mutton meat quality and impact the gene function.

Keywords: *S. alopecuroides*; RT-qPCR; IMF deposition; RNA-Seq

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Mutton has become favoured as a meat by consumers due to its relatively low fat and cholesterol content, and high protein, calcium, potassium, and vitamin B1 levels. This has led to an increased demand for mutton over the years. Meat quality is affected by many factors including breed, environment, and nutrition etc., but dietary regulation is a relatively easy means to improve it (Guo et al. 2019). Therefore, many studies have sought to improve the quality and safety of animal products through dietary nutritional regulation. *S. alopecuroides*, known as 'Bu Ya' by ethnic minorities in China, belongs to the family *Leguminosae* and is distributed widely in the arid and semi-arid regions of northwestern China, and which is rich in protein during all stages of its growth, and all kinds of amino acids necessary for animals are also contained in its leaves and seeds. Additional components of interest include alkaloids, flavones, organic acids, polysaccharides, volatile oils, and other biologically active substances. In recent years, researchers have paid increasing attention to the possibility of using *S. alopecuroides* as a highly nutritious ground feed additive to improve meat quality. Intramuscular fat (IMF) deposition is one of the most critical factors affecting meat quality as it positively correlates with the tenderness and juiciness of meat (Ruth et al. 2012). Animal muscle fat deposition occurs as a result of a balance between fat synthesis and catabolism, and the increase or decrease of fat deposition can affect meat quality and flavour (Chen et al. 2021).

RNA-Seq is based on high-throughput sequencing technology, which can comprehensively analyze changes in type, structure and expression level of transcripts (Owens et al. 2019). Such approach can reveal the molecular regulatory mechanism underlying specific biological processes. Over the past decade, transcriptome analysis based on high-throughput sequencing has become the main method for functional gene research (Wang et al. 2009) in plants, animals, microbiology and medicine. The present study applied RNA-Seq to investigate gene expression of *longissimus dorsi* muscle in Ningxia Tan sheep whose feed was supplemented with or without *S. alopecuroides*. The aim of the study was to identify candidate genes related to mutton IMF deposition. The obtained findings are expected to contribute to the improvement in the quality of mutton meat using *S. alopecuroides* as a feed additive. Moreover, functional gene analysis is expected to provide new information for the genetic improvement of Ningxia Tan sheep.

MATERIAL AND METHODS

Animals and experimental design

Animal tests were carried out at the Xiang Xin Tai Breeding Professional Cooperative of Helan County from 21 October 2018 to 22 December 2018, and they were approved by the animal ethics committee of Ningxia University. The trial feeding period was 60 days, including a pre-test period of 10 days and a subsequent test period of 50 days. Fifty 6-month-old castrated Ningxia Tan sheep with similar body condition and weight (28.02 ± 1.32 kg, mean ± SD) were used. A single-factor completely randomized trial design was applied and the animals were randomly divided into five groups of 10 animals each. The experimental basal diet was formulated according to sheep requirements from NRC (2007) and fed to the control group (group A) without *S. alopecuroides* addition, while experimental groups were fed a similar diet further supplemented with *S. alopecuroides* at 0.25%, 0.5%, 1% and 2% for groups B, C, D and E, respectively [Table S1 in electronic supplementary material (ESM) (for the supplementary material see the electronic version)]. The animals were fed twice a day at 07:00 and 17:00, and water was available *ad libitum*. As the feeding trial was completed, five sheep from each group were randomly selected for slaughter. They were subjected to a 24 h fasting period and received no water 2 h prior to slaughter, and neck exsanguination was used. Approximately 15 cm of the *longissimus dorsi* muscle from behind the 12th rib were collected and stored at –20 °C for IMF measurement and –80 °C for transcriptome sequencing, individually.

Determination of meat quality traits

The determination methods of meat quality traits including pH value, shear force, cooking yield, pressing loss, dripping loss, fatty acids and amino acids followed Chen and Sui (2018), and meat colour determination was evaluated according to Olfaz et al. (2005).

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Total RNA extraction

Three sheep were randomly selected from the control group and group E, respectively, for transcriptome sequencing, and total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and genomic DNA elimination by treatment with DNase I (Invitrogen, Carlsbad, CA, USA) and RNA quality and quantity determination were performed, RNA purity was assessed by measuring optical density (OD) at 260 and 280 nm on a NanoDrop ND-2000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). If the extracted RNA displayed OD260/OD280 = 1.8–2.1, OD260/OD230 ≥ 2, 28S/18S ≥ 1, and RNA complete value (RIN) ≥ 6.5, the extracted RNA was considered to meet library construction sequencing standards.

cDNA library construction and RNA sequencing

After the total RNA sample was assessed for quality and quantity, a cDNA library was constructed. Briefly, oligo (dT) magnetic beads were applied for the separation and enrichment of purified mRNA, followed by enzymatic RNA fragmentation, cDNA synthesis using the reverse transcription PrimeScript RT Reagent Kit (NEB, Ipswich, MA, USA), sequencing adapter connection, and PCR amplification. The size and purity of the cDNA library were determined using the Agilent DNA 1000 Kit (Agilent Technologies, Santa Clara, CA, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). An ABI StepOnePlus real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) was used to accurately quantify the effective concentration of the cDNA library (> 2 nmol/l) and ensure its quality. Finally, each cDNA library was sequenced on an Illumina HiSeq 2000 system (Illumina, San Diego, CA, USA) using double-end sequencing to obtain raw reads.

Quality control of sequencing data

After total RNA samples of Ningxia Tan sheep passed the quality inspection, first-strand cDNA was synthesized with mRNA from enriched samples as template. Then, second-strand cDNA was synthesized with dNTPs and DNA polymerase I. After purification, the terminal was repaired, a poly-A tail was added, and the sequencing connector was attached. Based on the size of the fragment, the cDNA library was enriched by PCR and sequenced on an Illumina HiSeq 2000 system (Illumina, San Diego, CA, USA). The image data file obtained by sequencing was transformed into raw reads by base recognition analysis. Then, low-quality reads were filtered out according to the quality control value to obtain clean reads. Clean reads were compared with the reference genome sequence, and the transcriptome data of mapped reads was analyzed according to the annotated text. DESeq software (Love et al. 2014) was used to normalize mRNA counts in each sample, calculate the multiple of differences, and test for significance.

RNA-Seq correlation analysis and principal component analysis

Given that biological repetition is necessary for any biological experiment including high-throughput sequencing, horizontal correlation of gene expression between samples was applied to test the reliability of the experiment and whether sample selection was reasonable or not. The closer the correlation coefficient was to 1, the higher was the similarity of expression patterns between samples. R^2 between biological repetition samples should be at least > 0.8.

Differential gene screening and functional analysis

The Fragments per Kilobase Million (FPKM) method was used to calculate transcript expression. DESeq software (Love et al. 2014) was used to normalize mRNA counts in each sample, calculate the multiple of differences, and test for significance. Genes expressed differentially between the experimental group and the control group were identified by default screening criteria with $P < 0.05$ and $\log_2$(fold change) > 1.

Functional analysis of differentially expressed genes included cluster analysis of FPKM values, GOseq software to annotate Gene Ontology (GO)

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functions and reveal biological functions, and Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis to identify signalling pathways. Pathway enrichment analysis was performed using KOBAS v2.0 (http://kobas.cbi.pku.edu.cn) and significance was determined for a false discovery rate ≤ 0.05.

Verification of differentially expressed candidate genes

To confirm the accuracy of sequencing, actin beta (*ACTB*) was selected as the internal reference gene, and the levels of candidate differentially expressed genes were verified by quantitative real-time PCR (qPCR). The primer sequences used to amplify candidate genes and *ACTB* were designed with Primer v5.0 software (Biosoft International, San Francisco, CA, USA) and are listed in Table 1. The primers were synthesized by Beijing Orson Gene Technology Co., Ltd (Beijing, China). The PCR reaction program included denaturation at 95 °C for 3 min, denaturation at 95 °C for 10 s, and annealing at 60 °C for 30 s for a total of 39 cycles. Three replicates of qPCR were conducted for each sample during the amplified experiment.

Statistical analysis

One-way ANOVA was performed by SPSS v25.0 software (Chicago, IL, USA) with ANOVA program, and Duncan's test was used for multiple comparisons. The relative expression of candidate differentially expressed genes between samples was calculated by the $2^{-\Delta\Delta CT}$ method, and statistical analysis was performed by *t*-test. $P < 0.05$ was considered significant differences, and $P > 0.05$ was considered no significant difference.

RESULTS

Determination of meat quality

Effect of different levels of S. alopecuroides on meat quality. As can be seen in Table 2, the addition of different amounts of *S. alopecuroides* to the diet had a significant impact on meat redness, pressing loss, and cooking yield ($P < 0.05$), but not on pH value, meat yellowness, meat whiteness, and drip loss ($P > 0.05$). Redness was significantly higher in group C than in the other four groups ($P < 0.05$). Water loss rate was significantly higher in groups C and D compared to groups A and B ($P < 0.05$). Cooking yield displayed a downward trend with increasing *S. alopecuroides* dosage, with the value of group A being significantly higher than those of groups C, D, and E ($P < 0.05$). No significant difference in shear force was observed between groups ($P > 0.05$), although a downward trend was recorded for groups B, C, D, and E.

Effect of different levels of S. alopecuroides on fatty acid and amino acid composition. As shown in Table 1, the amount of polyunsaturated fatty acids (PUFA) was significantly higher in group E (3.663 g/kg) than in groups A, B, and C ($P < 0.05$). The ratio of PUFA/saturated fatty acids, an important index when evaluating the nutritional value of meat, was significantly higher in group E compared to all other groups ($P < 0.05$). The highest n-6 PUFA value was achieved in group E (3.459 g/kg) and was significantly higher than in groups A, B, and C ($P < 0.05$). In contrast, no significant differ-

Table 1. Composition of fatty acids in the *longissimus dorsi* muscle of Tan sheep supplemented with different levels of *S. alopecuroides* (g/kg)

Parameters	Treatments					SEM	*P*-value
	A	B	C	D	E		
MUFA	7.243	7.832	5.663	6.298	5.676	0.372	0.233
PUFA	2.808^{a}	2.633^{a}	2.591^{a}	3.086^{ab}	3.663^{b}	0.113	0.009
PUFA/SFA	0.223^{a}	0.260^{ab}	0.319^{b}	0.341^{b}	0.462^{c}	0.020	< 0.001
n-3 PUFA	0.170	0.156	0.155	0.154	0.187	0.005	0.240
n-6 PUFA	2.625^{a}	2.469^{a}	2.431^{a}	2.926^{ab}	3.459^{b}	0.110	0.010

MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; SFA = saturated fatty acids

^{a,b}Different lowercase letters in the same row indicate a significant difference ($P < 0.05$)

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Table 2. Effects of different levels of *S. alopecuroides* on meat quality traits of Ningxia Tan sheep

Parameters	Treatments					SEM	*P*-value
	A	B	C	D	E		
pH_{45min}	6.48	6.50	6.48	6.47	6.60	0.044	0.908
pH_{24h}	5.90	5.70	5.78	5.84	5.80	0.046	0.737
Meat colour A	16.46^{a}	15.44^{a}	19.44^{b}	14.94^{a}	17.13^{a}	0.452	0.006
Meat colour B	12.68	13.49	15.48	14.33	13.68	0.346	0.106
Meat colour L	31.78	30.02	33.91	29.03	32.84	0.337	0.342
Pressing loss (%)	33.41^{a}	33.94^{a}	37.72^{b}	37.81^{b}	35.64^{ab}	0.008	0.018
Drip loss (%)	3.01	3.11	3.10	2.95	2.69	0.122	0.847
Cooking yeild (%)	65.43^{a}	64.20^{ab}	62.68^{abc}	60.70^{c}	61.55^{bc}	0.009	0.032
Shear force (N)	18.82	17.86	15.04	16.60	16.92	0.703	0.511

Treatment A = without added *S. alopecuroides*; Treatment B = 0.25% *S. alopecuroides* in the diet; Treatment C = 0.5% *S. alopecuroides* in the diet; Treatment D = 1% *S. alopecuroides* in the diet; Treatment E (2% *S. alopecuroides* in the diet; Meat colour A = A > 0 indicates the degree of red and A < 0 indicates the degree of green; Meat colour B = B > 0 indicates the degree of yellow and B < 0 indicates the degree of blue; Meat colour L = 100 is white and 0 is dark

$^{a–c}$Different lowercase letters in the same row indicate a significant difference ($P < 0.05$)

ence between groups was recorded for n-3 PUFA ($P > 0.05$). As reported in Table 3, the total amino acid content giving a fresh, sweet, and aromatic taste was similar among groups and corresponded to 10.14% (A), 10.31% (B), 10.62% (C), 10.4% (D), and 10.83% (E). This similarity reflected also the lack of significant differences between groups with respect to the particular amino acids.

Effect of different levels of S. alopecuroides on common nutrients. As shown in Table 4, IMF decreased gradually with the increase of *S. alopecuroides* supplementation in diet, and the content (4.21%) in group A was significantly higher than that in all other groups ($P < 0.05$). The initial water content was significantly higher in group D (72.48%) than in groups A and B ($P < 0.05$) and that of the latter group was significantly higher than in group E ($P < 0.05$). Addition of *S. alopecuroides* to the diet had no significant effect on muscle crude protein and crude ash ($P > 0.05$).

Total RNA electrophoresis results

Gel electrophoresis revealed three clear 28S, 18S, and 5S bands without significant degradation.

Table 3. Analysis of amino acid content in the *longissimus dorsi* muscle of Tan sheep at different levels of *S. alopecuroides* (%)

Amino acid		A	B	C	D	E	SEM	*P*-value
Umami	Glu	2.76	2.80	3.06	2.88	2.76	0.076	0.755
	Asp	1.67	1.54	1.80	1.79	1.67	0.048	0.338
	Arg	1.13	1.12	1.10	1.03	1.20	0.037	0.816
	subtotal	5.56	5.46	5.96	5.7	5.63	0.168	1.917
Sweet	Ser	0.71	0.72	0.82	0.89	0.81	0.041	0.681
	Ala	1.28	1.42	1.22	1.18	1.34	0.046	0.846
	Gly	0.71	0.71	0.63	0.64	0.76	0.027	0.749
	subtotal	2.7	2.85	2.67	2.71	2.91	0.117	2.275
Aromatic	Tyr	0.82	0.88	0.82	0.85	0.90	0.026	0.938
	Phe	1.06^{a}	1.12	1.17^{a}	1.14^{a}	1.39^{b}	0.032	0.008
	subtotal	1.88	2	1.99	1.99	2.29	0.058	0.939

^{a,b}Different lowercase letters in the same row indicate a significant difference ($P < 0.05$)

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Table 4. Effect of different levels of *S. alopecuroides* on nutrient composition of Ningxia Tan sheep meat

Parameters	Treatments					SEM	*P*-value
	A	B	C	D	E		
Initial moisture (%)	69.15^{a}	68.33^{a}	70.19^{ab}	72.48^{b}	70.76^{ab}	0.457	0.028
Crude protein (%)	22.14	22.11	22.35	21.30	22.69	0.238	0.453
Intramuscular fat (%)	4.21^{a}	3.75^{b}	3.34^{bc}	3.14^{bc}	2.78^{c}	0.162	0.018
Crude ash (%)	1.61	1.72	1.56	1.58	1.61	0.028	0.288

$^{a–c}$Different lowercase letters in the same row indicate a significant difference ($P < 0.05$)

The RNA OD260/OD280 ratio was between 1.8 and 2.2, and RNA exhibited good integrity (RIN ≥ 6.5), indicating its suitability for subsequent experiments (Figure S1 in ESM).

Quality control of raw sequencing data

Accuracy and reliability of the obtained sequencing results were determined by assessing the quality of the original sequencing data. Based on the above IMF data, muscle samples from three animals of the control group (A1, A2, and A3) and three animals of the experimental group (E1, E2, and E3) were sequenced and analyzed. The number of raw reads obtained was 25 151 533 from A1, 19 732 058 from A2, 27 688 954 from A3, 25 918 107 from E1, 25 114 182 from E2, and 20 639 924 from E3. While the number of clean reads obtained by removing low-quality reads was 24 757 542 for A1, 19 425 858 for A2, 27 152 386 for A3, 25 707 092 for E1, 24 741 971 for E2, and 20 468 290 for E3. In addition, the percentage of GC bases was above 52% for all samples, Q20 and Q30 were above 97% and 93%, respectively. Alignment of sequencing data with the Ningxia Tan sheep reference genome revealed more than 81% correspondence.

RNA-Seq correlation analysis and principal component analysis

Correlation analysis of mRNA expression between experimental and control groups (Figure S2A in ESM) revealed $R^2 \geq 0.8$ for biological repetitions and high similarity of expression patterns between samples. Principal component analysis of samples revealed large differences and good repeatability between the test group and the control group (Figure S2B in ESM), indicating that the sequencing results of the transcriptional group were reliable and could be used for follow-up analysis.

Screening and analysis of differentially expressed genes

The results of differential expression of genes in the *longissimus dorsi* muscle of Tan sheep revealed a total of 268 genes that were significantly differentially expressed between the control and experimental groups (Figure 1A), and 66 genes were significantly upregulated while 202 genes were significantly downregulated (Table S3 in ESM). Cluster analysis (Figure 1B) revealed that genes with high and low expression levels in samples from both the experimental and control groups clustered together, suggesting a similar expression pattern.

Differentially expressed genes and co-expression network analysis

The identified differentially expressed genes were analyzed by GO functional annotation, and the first 20 significantly enriched terms were selected (Figure 2). In the Biological Process category, plasma lipoprotein particle assembly, protein-lipid complex assembly, and protein-lipid complex subunit organization were the most enriched, the nucleoplasm part was the most enriched term in the Cellular Components category. Kinase binding was the most enriched term in the Molecular Function category.

Mapping the screened differentially expressed genes to the KEGG database (Figure 3A), it is found that the differential genes are enriched into a total of 147 pathways, and the pathways that enrich the most genes are signalling path-

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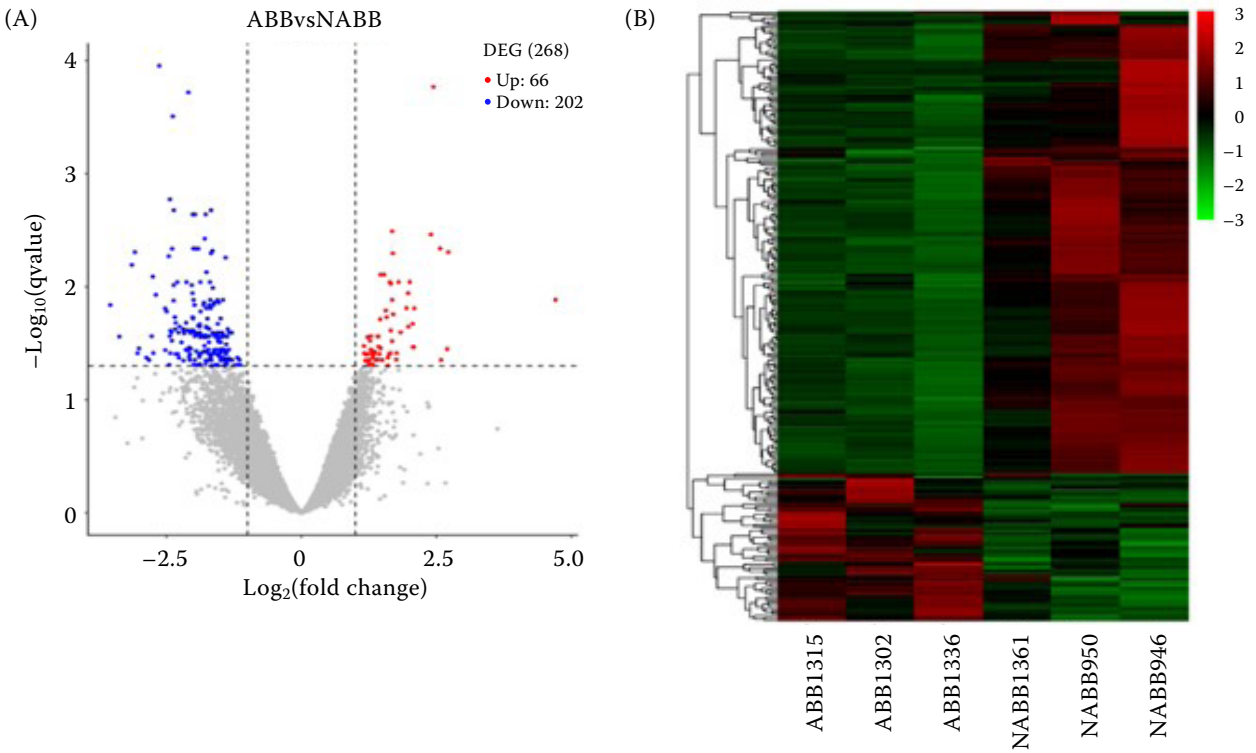

Figure 1. Volcano map and heat map

(A) Differentially expressed genes between the experimental and control groups. Upregulated genes are marked in red; downregulated genes are marked in blue; genes without significant differential expression are represented by grey dots. (B) Red indicates high-expression genes and green indicates low-expression genes; the colour ranges from red to green, indicating that $\log_{10}$ (FPKM+1) goes from large to small

ways regulating the pluripotency of stem cells, Mitophagy-Animal, hepatocellular carcinoma, transcriptional misregulation in cancer, cell cycle, starch and sucrose metabolism, PPAR signalling pathway, as well as neomycin, kanamycin and gentamicin biosynthesis. Among them, it was significantly enriched in the signalling pathways regulating the pluripotency of the stem cell pathway ($P < 0.05$). Four differentially expressed genes, such as *ACSL3* (acyl-CoA synthetase long-chain family member 3), *ANGPTL4* (angiopoietin-like 4), *PLIN2* (perilipin 2), *ABCA1* (ATP binding cassette subfamily A member 1), which may be related to intramuscular fat, were screened out in PPAR signalling pathway, fatty acid biosynthesis, fatty acid degradation and fatty acid metabolism (Table 5).

The screened differentially expressed genes were analyzed by interaction network (Figure 3B). The circle (node) represents the differential genes, in which *ACSL3, ANGPTL4, PLIN2* and *ABCA1* interact with the candidate gene *LPL* (lipoprotein lipase) related to intramuscular fat deposition.

RT-qPCR verification of differentially expressed candidate genes

Four differentially expressed candidate genes were selected for verification by RT-qPCR as shown in Figure 4. The mRNA expression levels of *ABCA1* and *ACSL3* genes in the *longissimus dorsi* muscle of the control group were significantly higher than those in the experimental group (E group) ($P < 0.05$). The mRNA expression of *ANGPTL4* and *PLIN2* genes in the experimental group was higher than that in the control group, and the difference of *ANGPTL4* gene was extremely significant ($P < 0.01$), but there was no significant difference in *PLIN2* gene ($P > 0.05$).

DISCUSSION

Ningxia Tan sheep are an excellent native breed adapted to the northwest of China, and they are very popular among consumers for their tender meat, less muttony odour, delicious taste, and even

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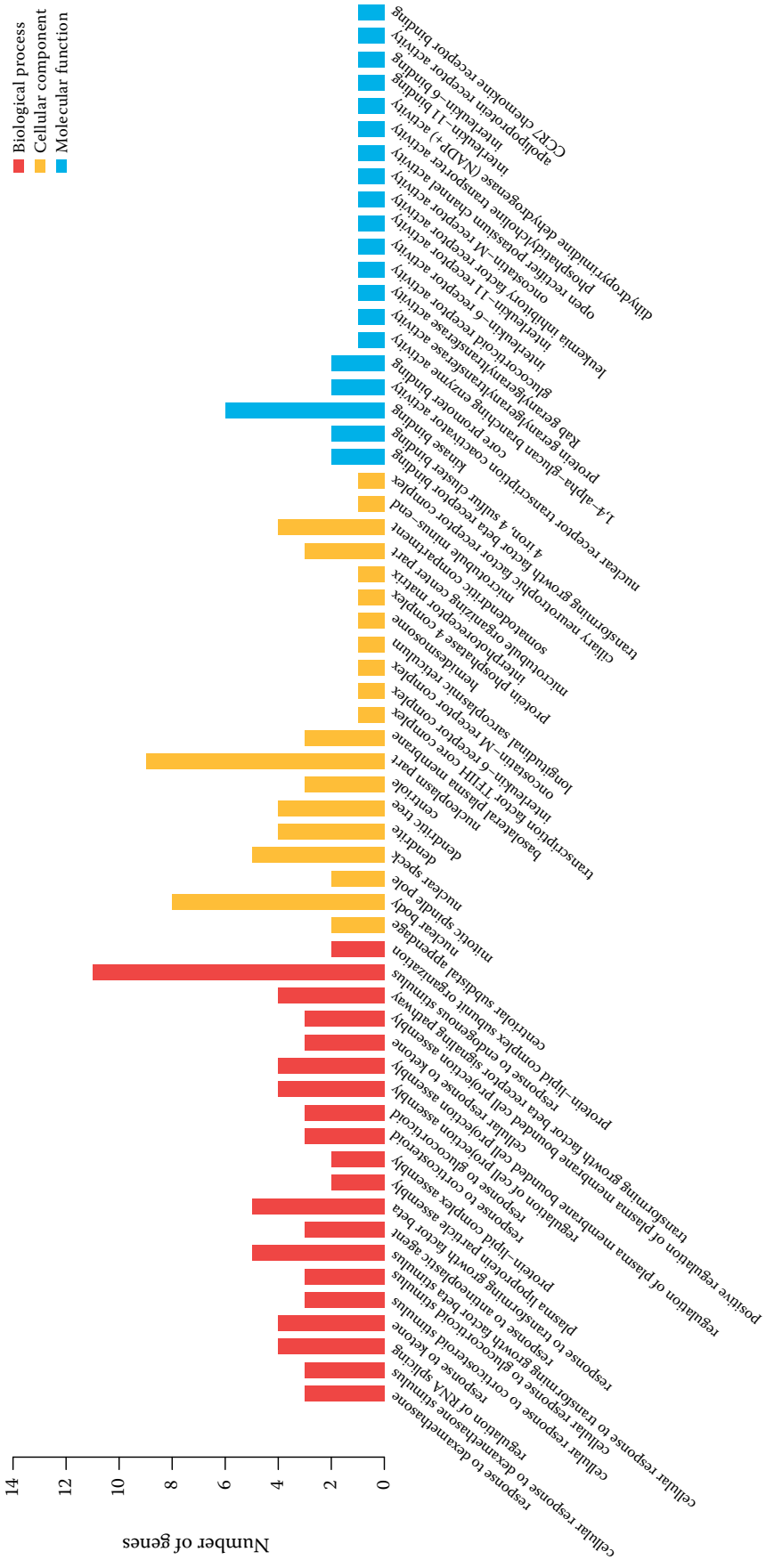

Figure 2. Gene Ontology (GO) enrichment histogram of differentially expressed genes
The ordinate indicates the number of enriched genes; the abscissa lists the GO terms; and different colours represent different GO categories

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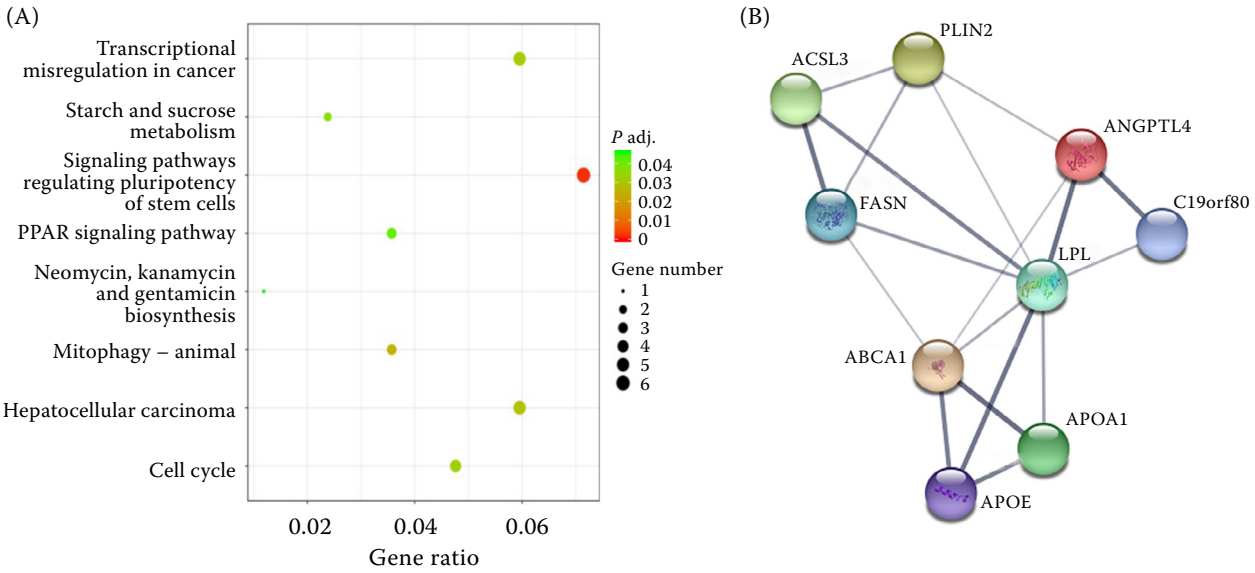

Figure 3. KEGG enrichment and scatter plot of differentially expressed genes

(A) The vertical axis lists the name of the pathway; the horizontal axis indicates the size of the enrichment factor; each dot indicates the number of source genes in the pathway; and the colour of the dot corresponds to different Q value ranges. (B) Cross-expression gene co-expression interaction network

fat distribution. With greater attention to healthy living standards, the requirements of consumers for quality livestock and poultry meat are increasing. Meat quality traits including meat colour, tenderness, cooking yield, amino acids, fatty acids, and other nutrients (initial moisture, crude protein, crude ash and IMF) are common but very important indicators for meat quality evaluation (Olfaz et al. 2005; Chen and Sui 2018; Chen et al. 2021). Cooking yield analysis showed that there was a decreasing trend with the increase of *S. alopecuroides* supplementation in diet, but pressing loss variations were reverse, and the negative relationship of cooking yield and pressing loss was consistent with the reports of Chen et al. (2016). Further, the study also indicated that a higher dosage of *S. alopecuroides* in diet might affect some meat quality traits of Ningxia Tan sheep. However, the addition of *S. alopecuroides* to diet can greatly improve the content of PUFA (particularly n-6 PUFA), PUFA/saturated fatty acid ratio, and Phe level ($P < 0.05$), and group E with 2% *S. alopecuroides* presented a higher PUFA content (particularly n-6 PUFA), PUFA/saturated fatty acid ratio, and Phe level, which also indicated that group E had the highest specific function for nutritional value and meat flavour. In addition, the total content of umami, sweet, and aromatic amino acids was also higher in group E, which further showed that meat quality of Ningxia Tan sheep could be improved by adding *S. alopecuroides* to diet.

Muscle fat content is the most important determinant of meat flavour and is closely related to its nutritional value (Hocquette et al. 2012). The present study showed that IMF content was significantly lower in the 2% *S. alopecuroides* group compared to the control one, and presented a downward trend with increasing addition of *S. alopecuroides*, furthermore it suggested that its supplementation in the diet could improve meat quality. Zhang et al. (1997) analyzed and identified 16 amino acids in

Table 5. KEGG metabolic pathways of intramuscular fat differentially expressed genes

Pathway	Pathway ID	Gene numbers	Corrected *P*-value	Differentially expressed genes
PPAR signaling pathway	oas03320	3	0.041 800 204	ANGPTL4, ACSL3, PLIN2
Fatty acid biosynthesis	oas00061	1	0.164 505 186	ACSL3
Fatty acid degradation	oas00071	1	0.359 368 591	ACSL3
Fatty acid metabolism	oas01212	1	0.460 152 692	ACSL3
Fat digestion and absorption	oas04975	1	0.353 254 168	ABCA1

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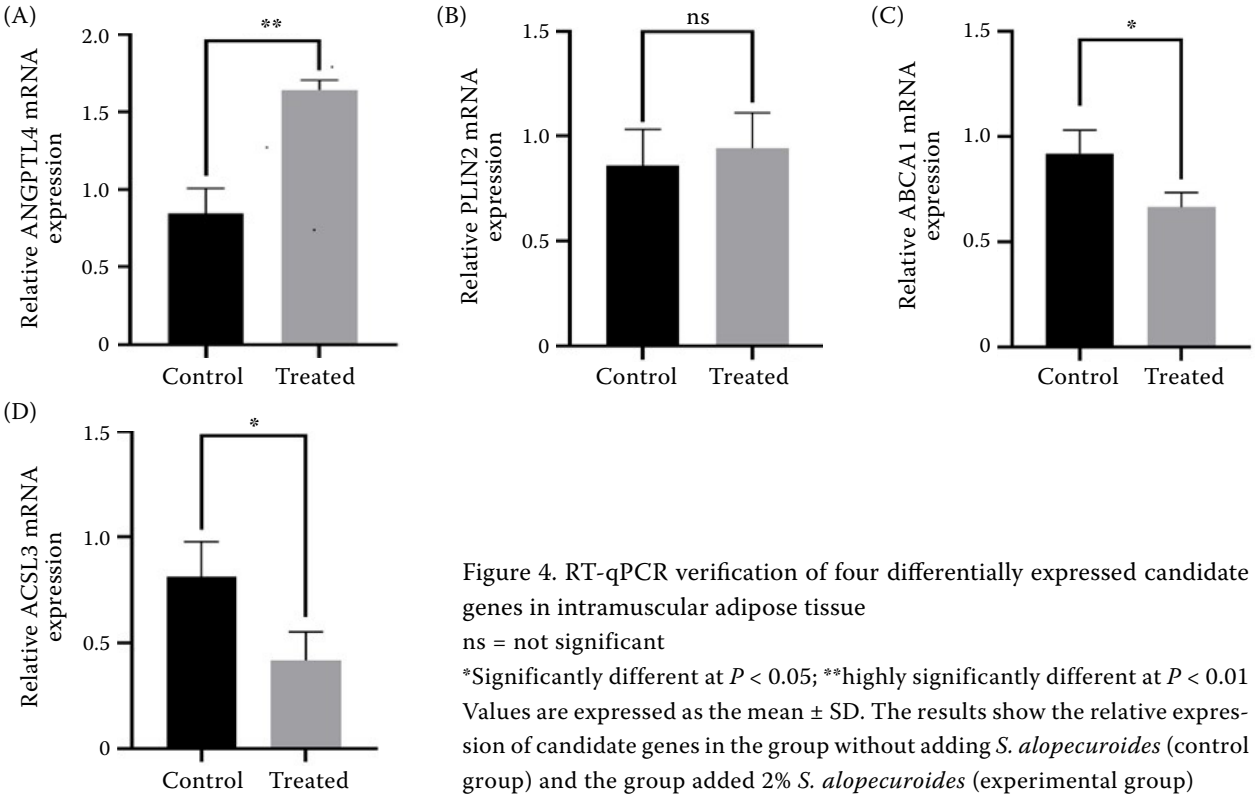

Figure 4. RT-qPCR verification of four differentially expressed candidate genes in intramuscular adipose tissue

ns = not significant

*Significantly different at $P < 0.05$; **highly significantly different at $P < 0.01$

Values are expressed as the mean ± SD. The results show the relative expression of candidate genes in the group without adding *S. alopecuroides* (control group) and the group added 2% *S. alopecuroides* (experimental group)

S. alopecuroides, of which glutamic acid, arginine, threonine, and cysteine were particularly abundant. Glutamic acid and arginine are all savory amino acids related to meat quality, which suggests that feeding *S. alopecuroides* could improve meat quality. Sequencing analysis of the *longissimus dorsi* muscle identified a total of 268 genes expressed differently between the experimental and control groups, including 66 upregulated genes and 202 downregulated genes. Q20, Q30, and alignment with the reference genome indicated that the sequencing data were accurate and reliable, and library coverage and utilization were high. KEGG analysis showed that the differentially expressed genes were enriched in a total of 147 pathways and significantly so in the signalling pathways regulating the pluripotency of stem cells. Among the pathways related to IMF, such as PPAR signalling pathway, fatty acid biosynthesis, fatty acid degradation, fatty acid metabolism, and peroxisome, thereafter four candidate genes were singled out including *ACSL3, PLIN2, ABCA1*, and *ANGPTL4*. The interaction network map of these genes showed that all four interacted with *LPL*, which is related to IMF deposition. This finding suggests that *ACSL3, PLIN2, ABCA1*, and *ANGPTL4* might have similar functions to *LPL* and might contribute to IMF deposition.

ACSL3 encodes long-chain acyl-CoA synthetase 3, which promotes the synthesis of lecithin, the formation of intracellular lipid droplets, and participates in fatty acid oxidation (Eck et al. 2020). These metabolic pathways help maintain cellular lipid homeostasis. In addition, *ACSL3* plays an important role in fatty acid metabolism in diseases such as cancer (Lv et al. 2019). Studies on the effects of lipopolysaccharide endotoxin on milk fat and milk protein synthesis in dairy cows showed that *ACSL3* was closely related to milk fat metabolism. Moreover, *ACSL3* expression was upregulated in Simmental cattle cells cultured *in vitro*, and a change in the relative abundance of *ACSL3* mRNA was closely related to the accumulation of cytoplasmic lipid droplets. Lv et al. (2015) reported that *ACSL3* had the highest gene expression among the ACSL family members in the mammary glands of lactating sows, and that mRNA expression increased significantly with lactation. *ACSL3* regulates mainly the conversion of fatty acids into lipid esterases, which has directed *ACSL3* research towards fatty acid oxidative metabolism, lecithin synthesis, and intracellular fat droplets (Poppelreuther et al. 2012). Studying the role of *ACSL3* gene in regulating fat metabolism in mutton may improve the quality and flavour of meat.

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PLIN2, also known as adipogenic differentiation-related protein, was originally found in fat and steroid-producing cells, and is involved mainly in lipid metabolism. Davoli et al. (2011) found that the *PLIN2* gene was widely expressed in pig muscle and significantly affected pig growth, as well as carcass and meat quality traits. Gandolfi et al. (2011) showed that PLIN2 is localized within myofibres with high lipid content in pig skeletal muscle, and PLIN2 protein and its corresponding gene could play a key role in the regulation of the intramyocellular lipid storage. Sastre et al. (2014) showed that PLIN2 might be involved in storage and utilization of lipids during oocyte maturation. *PLIN2* gene expression has been reported to increase in the later luteal phase of the oestrous cycle and in the conceptus presence (Forde et al. 2013). In addition, in humans, studies have shown that *PLIN2* promotes the formation of internal lipid droplets, thereby protecting triacylglycerol from lipolysis (Carr et al. 2014); Suzuki et al. (2009) showed that peroxisome proliferator-activated receptor gamma (PPARγ) and retinoid nuclear receptor alpha (RXRα) were involved in PLIN2-induced formation of lipid droplets in BeWo human chorionic cancer cells.

Adenosine triphosphate binding cassette transporter A1, which is encoded by *ABCA1*, mediates intracellular lipid outflow and maintains cell lipid homeostasis. Research on *ABCA1* has focused mainly on human liposome transport (Tang et al. 2013). *ABCA1* intracellular transport and other functions have provided new therapeutic targets and approaches to atherosclerosis-related diseases. Brooks-Wilson et al. (1999) showed that mutations in the *ABCA1* gene might affect the transcription and expression of the ensuing protein, thus impacting high-density lipoprotein cholesterol levels in the blood. Yao et al. (2016) reported a correlation between *ABCA1* gene polymorphisms rs2515602, rs2230806, and rs4149313 and blood lipid levels in the adult Kazakh population. Hu et al. (2013) found that intracellular cholesterol efflux mediated by the PPARγ/liver X receptor alpha (LXRα)/ABCA1 signalling pathway played an important role in the accumulation of macrophage lipids.

Angiopoietin-related protein 4, encoded by the *ANGPTL4* gene and known also as fasting-induced adipokine, is a secreted protein factor closely related to angiogenesis, lipid metabolism, glucose metabolism, and insulin sensitivity (Aronsson et al. 2010). Numerous studies have shown that the *ANGPTL4* gene plays a role in regulating lipid and glucose metabolism and in keeping the necessary balance. Legry et al. (2009) found that the rs4076317 polymorphic site on the *ANGPTL4* gene was significantly associated with high fat rates in adolescents. Ren et al. (2014) reported that porcine *ANGPTL4* seemed to be significantly additive in its actions on IMF, and could be potentially considered as a marker for IMF improvement. Mamedova et al. (2010) found that *ANGPTL4* was highly expressed in cattle liver and adipose tissues. Feng et al. (2006) found that porcine *ANGPTL4* was highly expressed in the liver, kidney, and adipose tissue, but slightly expressed in the heart, spleen, and muscles. Moreover, they found that *ANGPTL4* expression was high in obese pigs but slightly lower in lean pigs, and fat pigs had an increased effect on fat deposition. Hence, *ANGPTL4* may be related to fat deposition.

The RT-qPCR verification of *ACSL3, PLIN2, ABCA1*, and *ANGPTL4* showed that mRNA expression of *ANGPTL4* gene was significantly higher following 2% addition of *S. alopecuroides* than without it. *PLIN2* gene expression was higher in the 2% *S. alopecuroides* group than in the control group, but the difference was not significant. *ABCA1* and *ACSL3* gene expression was higher in the control group than in the 2% *S. alopecuroides* group. This result further validates the accuracy of transcriptome sequencing, indicating that *ACSL3, PLIN2, ABCA1*, and *ANGPTL4* can be used as important candidate genes affecting mutton meat quality, however further studies are required. In this study, addition of different amounts of *S. alopecuroides* to the Ningxia Tan sheep diet was found to affect muscle fat deposition. As the amount of *S. alopecuroides* increased, IMF showed a significant downward trend.

CONCLUSION

In this study, addition of different amounts (0.25%, 0.5%, 1% and 2%) of *S. alopecuroides* to the Ningxia Tan sheep diet was found to affect muscle fat deposition. As the amount of *S. alopecuroides* increased, IMF showed a significant downward trend. High-throughput transcriptome sequencing analysis on a total of six individuals in the 2% *S. alopecuroides* and control groups identified 268 differentially expressed genes, of which 202

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were upregulated and 66 were downregulated. Through GO functional annotation and KEGG enrichment analysis, *ACSL3, PLIN2, ABCA1,* and *ANGPTL4* were identified as candidate genes related to IMF. The study reveals that *S. alopecuroides* could be used as a feed additive to improve mutton meat quality by affecting the expression of genes responsible for regulating IMF levels. The study provides basic data and a reference for genetic improvement of Tan sheep meat.

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Conflict of interest

The authors declare no conflict of interest.

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