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Effects of fermented *Caragana korshinskii* on the intramuscular fat content and expression of *FABP3*, *UBE3C*, *ADRB3*, *LIPE*, and *SCD* in different muscles of Tan sheep

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Abstract: The aim of this study was to investigate the effects of fermented *Caragana korshinskii* on the intramuscular fat content and varied expression of the intramuscular fat deposition-related genes *FABP3*, *UBE3C*, *ADRB3*, *LIPE*, and *SCD* among four muscle tissues (*m. psoas*, *gluteus*, *quadriceps*, and *supraspinatus*) of Tan sheep. Twenty-eight male animals of similar age (270 ± 10 days) and weight (24.6 ± 1.06 kg) were randomly divided into a control group (fed the basal diet) and an experimental group (fed the same diet except 10% of corn stalks were replaced with fermented *C. korshinskii*). Soxhlet petroleum-ether extraction and quantitative real-time PCR were applied to evaluate the fat content and gene expression in tissues, respectively. We observed a significant improvement ($P < 0.05$) in the intramuscular fat contents in the *m. gluteus* and *supraspinatus* of treated sheep compared to those of non treated sheep. The *FABP3* mRNA level was markedly higher ($P < 0.05$) in the *m. quadriceps* and *supraspinatus* of treated sheep than in the control sheep. *UBE3C* mRNA levels were significantly decreased in the *m. gluteus*, *quadriceps*, and *supraspinatus* ($P < 0.05$) of treated sheep compared with those of the control sheep. *ADRB3* mRNA levels were significantly lower ($P < 0.05$) in the *m. psoas*, *gluteus*, and *supraspinatus* of sheep fed fermented *C. korshinskii* than in the control group, whereas *LIPE* mRNA levels were significantly increased ($P < 0.05$) in the *m. gluteus* and *quadriceps* of sheep fed fermented *C. korshinskii*. The *SCD* mRNA levels in *m. psoas*, *quadriceps*, and *supraspinatus* of sheep fed fermented *C. korshinskii* were significantly higher than those of the control group ($P < 0.05$). Our results indicated that fermented *C. korshinskii* could partially replace the roughage used in Tan sheep feed, and its substitution affected the intramuscular fat content and altered the expression of intramuscular fat deposition-related genes. The present study lays a solid foundation for further exploring the utilization of *C. korshinskii* in ruminant husbandry.

Keywords: obesity; mRNA; tissue varieties; *Ovis*

Caragana korshinskii has been widely used in the field of feed in recent years. The leafy stalks of *C. korshinskii* grow rapidly, possess high biomass and are extremely rich in various amino acids and

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trace elements. The leaves, flowers, and seeds, and especially the leaves of the stalks, have a high crude protein content. However, owing to the high lignification degree of the stalks and their hard and thick lignin, the thorns can easily make animals feel full when they are fed *C. korshinskii* (Zhong et al. 2014). Microbial fermentation of plants can increase nutrients such as crude protein, reduce mycotoxin contents, and improve palatability of the plant products. Moreover, feeding fermented plants to animals encourages a healthy host-microbiome balance in the animal's digestive system (Wang et al. 2019). Feeding *C. korshinskii* to cattle can enhance meat yields (Yang et al. 2005). However, limited information is available regarding the impact of feeding *C. korshinskii* silage to sheep on mutton.

The content of intramuscular fat is an important factor affecting the quality of meat, and it affects the tenderness, water power, shear force value, flavour and juiciness of meat. The underlying mechanism of intramuscular fat accumulation is utterly complicated and is mainly governed by multiple factors such as animal nutrition, environment and genetics. Among them, gene regulation is one of the fundamental ways to improve intramuscular fat deposition (Hamill et al. 2012).

Fatty acid binding protein 3 (FABP3), also known as heart-type fatty acid binding protein (H-FABP), binds to plasma long-chain fatty acids. Fatty acid binding proteins are low-molecular-weight proteins with a distinct tissue-specific distribution. They play significant roles in fatty acid transport, cell growth, cell signalling transduction, and transcriptional regulation (Kulig et al. 2010). Ubiquitin ligase E3C (UBE3C) belongs to the HECT family of ubiquitin ligases which mediate ubiquitination and play a crucial role in the ubiquitin-proteasome pathway for protein degradation (Supakankul and Mekchay 2016). Activation of the β3-adrenergic receptor (ADRB3) is essential in the process of browning of human adipose tissue, and obese individuals suffer from reduced brown adipose tissue activation (Cao et al. 2018). Hormone-sensitive lipase (LIPE) is an intracellular neutral lipase that can hydrolyze a large variety of esters and plays a critical role in mobilizing fatty acids from diacylglycerols (Goszczynski et al. 2014). Stearoyl-Co A desaturase (SCD) catalyzes the synthesis of monounsaturated fatty acids (MUFAs) from saturated fatty acids (SFAs), including palmitoyl CoA (C16:0) and stearoyl CoA (C18:0). The level of these key enzymes is associated with the composition of fatty acids in milk and adipose tissue and fat deposition in muscles (Xuan et al. 2009). In our study, *C. korshinskii* powder was fermented by brewer's yeast and tannin-degrading and cellulase-producing bacteria and used to partially replace the roughage in Tan sheep feed. Through determining the effects of *C. korshinskii* on the intramuscular fat (IMF) content and the expression of IMF-related candidate genes in Tan sheep, we provide a scientific basis for the rational utilization of *C. korshinskii* as a ruminant feedstuff.

MATERIAL AND METHODS

Experimental diet and management of animals

A total of 196 lambs were with their mothers up to six months of age and were offered a standard feed (Technical Regulations for High-frequency Breeding and Feeding of Stall-fed Sheep (DB64/T 1480-2017). From then and up to 9 months, twenty-eight healthy male Tan sheep from the Chunhao grass industry specialized cooperatives (Ningxia, P.R. China) of similar age (270 ± 10 days) and body weight (24.6 ± 1.06 kg) were randomly divided into two groups: (1) control group (fed the basal diet) and (2) experimental group (fed the same diet except that in the experimental group 10% of corn stalks were replaced with equal amounts of fermented *C. korshinskii*). Each group included 14 sheep, reared in circles. The total mixed ration (Table 1) was manufactured to meet the nutritional requirements of growing sheep with 100 g of weight gain per day. The specific ration was adjusted based on the sheep's body weight, as described by NRC (2007) (Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids). Metabolizable energy was calculated from the ingredient values based on the feeding standard of sheep raised for mutton (NY/T816, 2004) (Agricultural Industry Standard of the People's Republic of China). The pre-feeding period was 10 days, and the experimental period was 50 days.

Animals and sample collection

All animal experiments were managed in accordance with the animal procedures estab-

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Table 1. Ingredients and chemical composition of the basal diet (on dry matter basis)

Ingredients	Diets	
	control group	fermentative *Caragana korshinskii*
Corn (%)	25.00	25.00
Soybean meal (%)	24.00	24.00
Linseed (%)	0.50	0.50
Soybean oil (%)	5.00	5.00
Salt (%)	0.50	0.50
Premix (%)[a]	5.00	5.00
Corn straw (%)	40.00	30.00
Fermentative *Caragana korshinskii* (%)[b]	0.00	10.00
Chemical composition		
DM (%)[c]	83.25	84.65
DE (MJ/kg)[c]	11.09	11.56
CP (%)[c]	14.77	15.30
EE (%)[c]	6.82	6.99
CF (%)[c]	16.03	16.91
Ca (%)[c]	0.23	0.26
P (%)[c]	0.30	0.30

[a]Premix per kg: 100 000 IU vitamin A, 20 000 IU vitamin D_3, 60 IU vitamin E, 1 g Fe, 1 g Mn, 0.78 g Zn, 0.27 g Cu, 0.012 g Se, 0.01 g I

[b]Fermentation of *Caragana korshinskii*: *Caragana korshinskii* (1 t) was crushed to an appropriate length (3~5 cm), then mixed with complex microbial inoculants (cellulase-producing bacteria 3 g, 5.0×10^9 cfu/g; tannin-degrading bacteria 50 ml, 2.0×10^8 cfu/ml; *Saccharomyces cerevisiae* 5 g, 1.2×10^{10} cfu/g, water 100 kg). After mixing evenly, it was put into the fermentation bag, and loading a certain amount of feed each time, the feed should be compacted to avoid air retention. After filling the bag, a vacuum pump was used to draw out the remaining air in the bag and the bag was tightened. Store well and let ferment naturally for 15 days before use

[c]All values are analyzed except metabolizable energy; metabolizable energy content was calculated based on tabular values (NRC 2007)

Ca = calcium; CF = crude fibre; CP = crude protein; DE = digestible energy; DM = dry matter; EE = ether extract; P = phosphorus

lished by the Chinese Ministry of Agriculture and approved by the Animal Protection and Use Committee of North Minzu University. Samples were collected from the *m. psoas*, *gluteus*, *quadriceps*, and *supraspinatus* of Tan sheep. One part of each sample was stored in Ziplock plastic bags at −20 °C for analysis of the IMF content. The other part was placed in liquid nitrogen and stored at −80 °C for total RNA extraction.

Measurement of the IMF content

The IMF contents in the *m. psoas*, *gluteus*, *quadriceps*, and *supraspinatus* were measured via the Soxhlet petroleum-ether extraction method based on Chinese National Standards GB/T 5009.6.2004. The IMF content was expressed as a weight percentage of dry muscle tissue.

Design and synthesis of primers

qPCR primers were designed based on the mRNA sequences of the target genes using Primer Premier 5.0 software (Primer Inc., Canada). *FABP3*, *UBE3C*, *ADRB3*, *LIPE*, *SCD*, and *ACTB* were published in the National Centre for Biotechnology Information (NCBI; GenBank), and *ACTB* was used as the internal reference gene (de Jonge et al. 2007). The geNorm software was used to evaluate the expression stability of *ACTB*. The results showed that *ACTB* was the most stable housekeeping gene and could be used as a reference gene. The primers were synthesized by Sangong Biotechnology Co. Ltd. (Shanghai, P.R. China). The primer sequences, annealing temperatures, PCR product lengths, efficiency and R^2 are shown in Table 2.

Extraction and reverse transcription of RNA

Total RNA was extracted from the *m. psoas*, *gluteus*, *quadriceps*, and *supraspinatus* of the sheep using the AxyPrep total RNA isolation kit (Axygene/Corning Inc., Corning, NY, USA). The total RNA concentration and purity were determined using a Maestro Nanomicro spectrophotometer (MaestroGEN, Las Vegas, NV, USA). The RNA quality was checked using 1% agarose gel electrophoresis followed by staining with 10 μg/ml ethidium bromide. The RNA had an OD260/280 ratio between 1.8 and 2.0. Reverse transcription of 1 μg of total RNA was performed using TransScript one-

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Table 2. Primer sequences used for qPCR

Gene name	Primer sequence (5'~3')	Accession	Product size (bp)	Efficiency	R^2
FABP3	F: TGACCAAGCCTACCACAATCATCG R: CTGTCATCTGCCGTGGTCTCATC	NM_001267884.2	130	1.0246	0.9984
UBE3C	F: AGCCAGCGACTCAGACGAAGAG R: TCAGACACTCCTCGGCGATGTAC	XM_027969069.1	104	0.9988	0.9981
ADRB3	F: GCTGCACCTTCGCCTCCAAC R: GAAGAGCATCACCAGAAGCGGAAG	NM_001159757.1	83	0.9990	0.9971
LIPE	F: CCTCGTGGCTCAACTCCTTCTTG R: TCTGTTGTGTCGCTGCTGTTCC	NM_001128154.1	193	0.9997	0.9997
SCD	F: GCTTCCACAACTACCACCACACC R: CGATGGCAGCCATGCAATCAATG	NM_001009254.1	105	0.9995	0.9997
ACTB	F: TGAACCCCAAAGCCAACC R: AGAGGCGTACAGGGACAGCA	NM_001009784.1	107	0.9998	0.9997

step gDNA removal and cDNA synthesis SuperMix (Transgen, P.R. China). In this reaction, both oligo dT and random hexaprimers were used. The products were then stored at –80 °C until further analysis.

Quantitative real-time PCR (qPCR)

SYBR Green PCR (qPCR) analysis was performed on an iQ5 iCycler iQTM Real-Time PCR Detection System (Bio-Rad, USA). qPCR was performed in a 20-µl reaction system [1.0 µl (50 ng) cDNA, 0.4 µl sense primer (0.2 µM), 0.4 µl anti-sense primer (0.2 µM), 10 µl 2 × TransStart Top Green qPCR SuperMix and 8.2 µl PCR-grade water] using the TransStart Top Green qPCR SuperMix kit (Transgen, P.R. China). The parameters of qPCR were as follows: 95 °C for 30 s, followed by 50 cycles of 95 °C for 5 s, 60 °C for 34 s, and 72 °C for 30 s. Melting curve analysis was performed at 95 °C for 10 s and at 60 °C for 1 min, followed by a temperature decrease of 0.5 °C/10 s to reduce the temperature from 95 °C to 60 °C. The relative expression level of the target genes was determined by the $2^{-\Delta\Delta CT}$ method (Wong and Medrano 2005). Three technical replicates were performed for each sample.

Statistical analyses

The results were subjected to one-way analysis of variance (ANOVA) using SPSS v16.0 software (SPSS Inc./IBM Corp., Armonk, NY, USA), and then Duncan's test was performed to characterize the significance between the results from different muscles of the Tan sheep; $P < 0.05$ was considered statistically significant (Livak and Schmittgen 2001). All statistical analyses were carried out using GraphPad Prism v5.0a (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

IMF content

As displayed in Figure 1, a significant increase ($P < 0.05$) in the IMF content was observed in the *m. psoas*, *gluteus*, and *supraspinatus* of sheep fed fermented *C. korshinskii* compared with those in the control group.

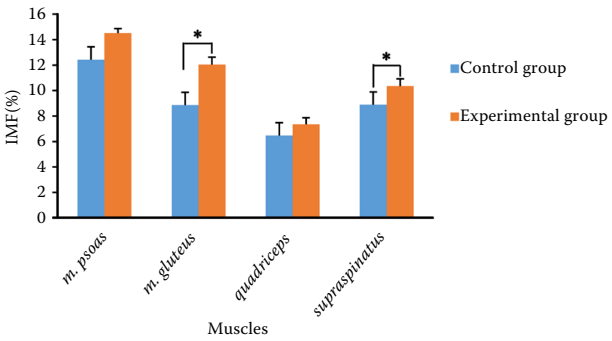

Figure 1. Effects of fermented Caragana on the intramuscular fat in different muscle tissues of Tan sheep
*Indicates a significant difference ($P < 0.05$) between the tested different types of diet according to *t* test

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Expression of IMF-related genes in four muscular tissues of Tan sheep

The expression of genes (i.e., *FABP3*, *UBE3C*, *ADRB3*, *LIPE*, and *SCD*) related to IMF in the *m. psoas*, *gluteus*, *quadriceps*, and *supraspinatus* is shown in Figure 2. The expression of *FABP3* mRNA showed an increasing trend ($P < 0.05$) in *m. psoas*, *quadriceps*, and *supraspinatus* in sheep fed fermented *C. korshinskii* compared with the control animals, whereas its expression in *m. gluteus* showed a decreasing trend ($P < 0.05$). The level of *UBE3C* mRNA was significantly decreased in the *m. gluteus*, *quadriceps*, and *supraspinatus* ($P < 0.05$) in the fermented *C. korshinskii* group compared with that in the control group. The abundance of *ADRB3* mRNA in the *m. psoas*, *gluteus*, and *supraspinatus* was significantly lower ($P < 0.05$) in sheep fed fermented *C. korshinskii* than in the control group.

The expression of *LIPE* mRNA was significantly increased ($P < 0.05$) in the *m. gluteus* and *quadriceps* of sheep fed fermented *C. korshinskii* compared with those fed a basal diet. Moreover, we detected a significantly higher ($P < 0.05$) abundance of *SCD* mRNA expression in the *m. psoas*, *quadriceps*, and *supraspinatus* of sheep fed fermented *C. korshinskii* than in the control group.

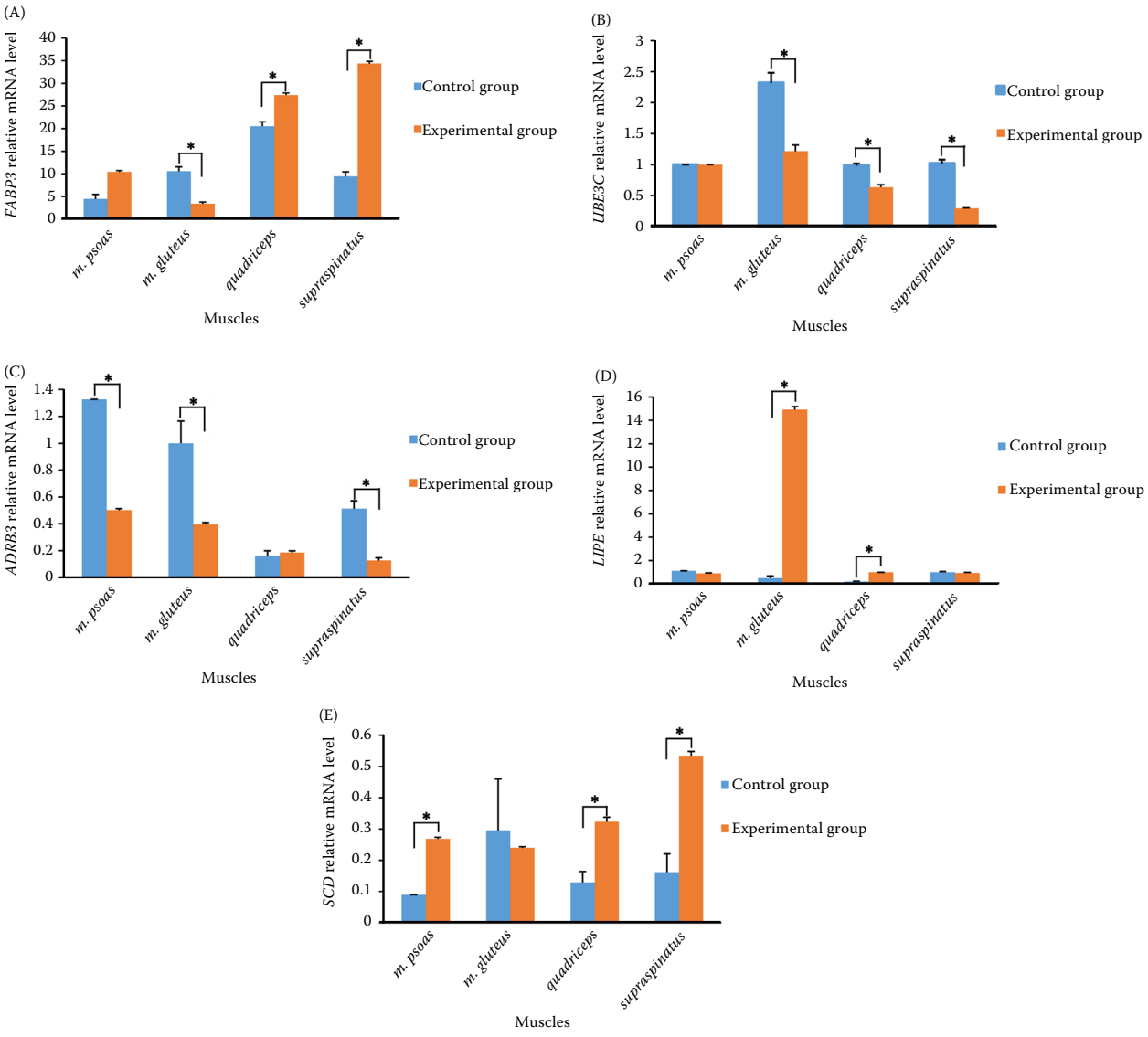

Figure 2. Effects of fermented Caragana on the relative mRNA expression of *FABP3*, *UBE3C*, *ADRB3*, *LIPE* and *SCD* in different muscle tissues of Tan sheep

*Indicates significant difference ($P < 0.05$) between different tested diet types according to *t* test

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DISCUSSION

The value of *C. korshinskii* as a feed ingredient is drawing increasing attention of researchers and farmers aiming to lower the cost of animal rearing and taking full advantage of this region-specific plant. In our study, fermented *C. korshinskii* had a rich acidity and aroma following microbial fermentation. The sheep fed fermented *C. korshinskii* ate normally, and their health condition was excellent. No abnormal conditions were found in water drinking or excrements. Thus, fermented *C. korshinskii* feed had a higher feeding value for Tan sheep. Additionally, it would be feasible to partially replace roughage in the sheep diet with fermented *C. korshinskii*. Fang et al. (2011) reported that *C. korshinskii* was adopted as a part of roughage in the diet of dairy cows, resulting in higher milk yield than in the control. The contents of milk fat, protein, lactose, and dry matter were slightly higher in cows fed *C. korshinskii* than in those without it (Fang et al. 2011).

These results indicated that *C. korshinskii* could be used as a decent substitute for roughage in the dairy cow diet. The roughage of sheep diet has long been dominated by a single corn stalk. In our study, the diet replacing corn stalks with 10% of fermented *C. korshinskii* had an improved nutrient profile compared with the basal diet, and feeding fermented *C. korshinskii* to sheep increased the IMF content, indicating that the diversification of feed composition not only increases feed intake and palatability but also improves meat quality. Fermented *C. korshinskii* could partially replace roughage and increase the IMF content in our study. We also determined its effect on the expression of IMF-related candidate genes. The *FABP3* gene encodes an intracellular protein that plays a vital role in the transport of fatty acids and is involved in lipid metabolism and fat deposition (Chmurzynska 2006). The expression of *FABP3* mRNA in the *m. psoas*, *quadriceps*, and *supraspinatus* of sheep fed fermented *C. korshinskii* showed an increasing trend, but in *m. gluteus,* it showed a decreasing trend in our study. This illustrates that the addition of fermented *C. korshinskii* to partially replace roughage can significantly increase the IMF content in the *m. psoas*, *quadriceps*, and *supraspinatus*. This is in accordance with the research of Hu et al. (2010), in which a correlation between *FABP3* gene expression and intramuscular fat and fatty acid contents in Laiwu pig and Duroc pig muscle was found. However, no significant correlation was detected between *FABP3* expression and the IMF content in Berkshire pigs. Ubiquitin-protein ligase E3C (UBE3C) participates in the ubiquitin-proteasome pathway. Some ubiquitin-protein ligases are crucial for fat deposition and lipid metabolism. We found that the expression of *UBE3C* was significantly decreased in the *m. gluteus*, *quadriceps*, and *supraspinatus* of sheep fed fermented *C. korshinskii* compared with that in control sheep. The results confirm the importance of sheep UBE3C in fat deposition in muscle. *UBE3C* may be used as a candidate marker for the genetic improvement of fat deposition in pigs. The E3 ubiquitin ligase COP1, an enzyme in the fatty acid synthesis pathway, is associated with the pseudokinetic kinase TRB3, which inactivates acetyl-CoA carboxylase, suggesting that E3 ubiquitin-protein ligase is important for fat deposition and energy balance (Qi et al. 2006).

Our results also indicate that *UBE3C* expression in sheep is associated with the IMF content. The beta-3 adrenergic receptor (ADRB3*)* is an R7G superfamily of G protein-coupled receptors and is mainly distributed on the surface of animal adipocytes. It is the main receptor adjusting white adipose tissue decomposition and adaptive heat production of brown adipose tissue. In our study, *ADRB3* expression in the *m. psoas*, *gluteus*, and *supraspinatus* was significantly lower in sheep fed fermented *C. korshinskii* than in control sheep. This means that feeding fermented *C. korshinskii* to sheep can promote the deposition of fat in their *m. psoas*, *gluteus*, and *supraspinatus*. Therefore, *ADRB3* could be selected as a genetic marker for muscle area or IMF of Tan sheep.

The hormone sensitive lipase (LIPE) is involved in free fatty acid mobilization (Miyoshi et al. 2008). The *LIPE* gene is an important resource for pork selection to obtain favourable meat with higher IMF levels and improved porcine fat quality and thus better taste properties. In addition, *LIPE* c.442G>A was found to be significantly correlated with C12:0 and C14:0 contents, and a trend was observed between this gene and the C18:0 content (Xue et al. 2015). *ADRB3*, *FABP3*, *LIPE* and *LPL* gene polymorphisms are correlated with the fat content and fatty acid composition in pig muscle. We found that the mRNA expression of *LIPE* significantly increased in the *m. gluteus* and *quadriceps* of sheep

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fed fermented *C. korshinskii* compared with those in the control group; thus, *LIPE* was significantly associated with the IMF content in the *m. gluteus* and *quadriceps* of Tan sheep. Uncoupling proteins (UCPs) exhibit high specific expression in skeletal muscle, indicating an increased ability to transport, uptake, and oxidize fatty acids and increase systemic fat oxidation (Bezaire et al. 2005). Mostyn et al. (2005) studied the expression pattern of *UCP3* in commercial and Meishan pigs at 0, 4, 7, 14 and 21 days after birth. They found that the expression of *UCP3* in adipose tissue was similar in the two breeds before 7 days of age. *UCP3* expression was significantly higher in Meishan pigs than in commercial pigs after 7 days of age, indicating that *UCP3* expression is species specific (Mostyn et al. 2005). Other researchers also found that the expression pattern of *UCP3* in Hu lambs differed in different muscles, indicating that the expression of *UCP3* has tissue specificity, which was also consistent with previous studies (Mostyn et al. 2005). Some studies have shown that the expression level of *UCP3* in the *m. psoas* at 3 months of age was significantly higher than that at 0 or 5 months, indicating that *UCP3* may be involved in the regulation of fat metabolism (Himms-Hagen and Harper 2001). A significant difference in *SCD* mRNA expression was observed in the *m. psoas*, *quadriceps*, and *supraspinatus* between sheep fed fermented *C. korshinskii* and sheep in the control group in our study. This indicates that *UCP3* may be involved in the regulation of fat metabolism and have significant tissue specificity in mRNA expression in different muscles of Tan sheep.

CONCLUSION

Our results showed that the IMF content of the *m. psoas*, *gluteus*, and *supraspinatus* increased in sheep fed fermented *C. korshinskii* compared with sheep fed the basal diet. Expression of the *FABP3*, *LIPE*, and *SCD* genes had a positive effect on the IMF content in different muscles of Tan sheep, whereas expression of the *UBE3C* and *ADRB3* genes had a negative effect on the IMF content in different muscles. Therefore, the relevant genes appear to partially regulate the deposition of IMF in Tan sheep. These findings lay the groundwork for further research that will unearth the molecular mechanisms of IMF deposition in sheep.

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Conflict of interest

The authors declare no conflict of interest.

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