

Transcriptome analysis reveals candidate genes of the synthesis of branched-chain fatty acids related to mutton flavor in the lamb liver using *Allium mongolicum* Regel extract

Yabo Zhao,¹ Yanmei Zhang, Erdene Khas, Chen Bai, Qina Cao, and Changjin Ao¹

Key Laboratory of Animal Feed and Nutrition of Inner Mongolia Autonomous Region, College of Animal Science, Inner Mongolia Agricultural University, Hohhot 010018, China

¹Corresponding author: changjinao@aliyun.com

Abstract

The objective of this study was to identify candidate genes via which *Allium mongolicum* Regel ethanol extract (AME) affects the synthesis of branched-chain fatty acids (BCFAs) related to mutton flavor by transcriptome analysis in the lamb liver. Thirty male Small-tailed Han sheep (3 mo old; 33.6 ± 1.2 kg) were randomly divided into two groups and fed for 75 d with a basal diet containing no AME (CON, control group) or 2.8 g·lamb⁻¹·d⁻¹ AME (AME group). Twelve sheep, CON (*n* = 6) and AME (*n* = 6), were selected for slaughter at the end of the trial period, and liver samples were subsequently collected. There was no difference in 4-ethyl-octanoic acid content among treatments. The 4-methyl-octanoic acid and 4-methyl-nonanoic acid levels were significantly lower in the AME group than in the CON group (*P* < 0.05). Furthermore, 461 differentially expressed genes (DEGs) were identified between the CON and AME groups, of which 182 were upregulated and 279 were downregulated in the AME group. The DEGs were enriched in three pathways, namely, glutathione metabolism, ECM–receptor interaction, and steroid hormone biosynthesis, as determined by the Kyoto Encyclopedia of Genes and Genomes pathway analysis. Finally, *CYP2B6*, *ACOT12*, *THEM4*, *ACSF2*, *LPIN1*, and *ADCY4* were identified as candidate genes that might be involved in regulating the BCFAs synthesis in the sheep liver.

Lay Summary

Mutton is characterized by a typical species-related flavor named “mutton flavor,” which is mainly associated with branched-chain fatty acids (BCFAs), such as 4-methyl-octanoic acid, 4-ethyl-octanoic acid, and 4-methyl-nonanoic acid. Previous studies demonstrated that *Allium mongolicum* Regel ethanol extract (AME) reduced the concentration of BCFAs in the muscle and adipose tissues of lambs. The liver is a primary metabolic organ in mammals and is involved in the metabolism of BCFAs. In the present study, we investigated the influences of AME on the concentration of BCFAs and the transcriptomic profile in the lamb liver. Supplementation of lamb diet with AME reduced the concentration of BCFAs in the liver. We also found six candidate genes related to the synthesis of BCFAs from differentially expressed genes in the liver. The findings of this work contribute to a better understanding of the effect of AME on the synthesis of BCFAs in the liver of lambs.

Key words: *Allium mongolicum* Regel extract, branched-chain fatty acids, lamb, liver transcriptome, mutton flavor

Abbreviations: ACOT12, acyl-CoA thioesterase 12; ACS, acyl-CoA synthetase; ACSF2, acyl-CoA synthetase family member 2; ACTB, β-actin; ADCY4, adenylate cyclase 4; ADF, acid detergent fiber; AME, *Allium mongolicum* Regel ethanol extract; BCAAs, branched-chain amino acids; BCFAs, branched-chain fatty acids; CP, crude protein; CYP2B6, cytochrome P450 family 2 subfamily B member 6; DE, digest energy; DEGs, differentially expressed genes; EOA, 4-ethyl-octanoic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LPIN1, lipin 1; MF, mutton flavor; MNA, 4-methyl-nonanoic acid; MOA, 4-methyl-octanoic acid; NDF, neutral detergent fiber; PGC-1α, PPARγ coactivator-1α; qRT-PCR, quantitative real-time PCR; START, steroidogenic acute regulatory protein-related lipid transfer; THEM4, thioesterase superfamily member 4

Introduction

Mutton is characterized by a typical species-related flavor named “mutton flavor,” which negatively affects consumer acceptability (Young et al., 2010; Abuzahra et al., 2018). Mutton flavor (MF) is mainly associated with the presence of branched-chain fatty acids (BCFAs) in muscle and adipose tissue, especially 4-methyl-octanoic acid (MOA), 4-ethyl-octanoic acid (EOA), and 4-methyl-nonanoic acid (MNA), which are considered the main contributors to the MF (Watkins et al., 2010; Abuzahra et al., 2018). BCFAs also give a characteristic flavor to goat and sheep milk, which is conspicuously

different from the flavor of cow milk (Salles et al., 2002), and Ha and Lindsay (1993) also suggested that there are no or few BCFAs in cow milk. Therefore, it is necessary to obtain deeper insight into the regulatory mechanism of BCFAs, which would help improve the MF in lambs.

In recent years, researchers have found that the application of natural plants or their extracts as dietary supplements can efficiently reduce the concentration of BCFAs in muscle and fat (Devincenzi et al., 2014; Del Bianco et al., 2020) and thus improve the MF of mutton products. *Allium mongolicum* Regel is a traditional Mongolian medicinal herb belonging

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to the genus *Allium* of the *Liliaceae* family and grows extensively in northwest China (Wang et al., 2019). *Allium mongolicum* Regel contains polysaccharides, flavonoids, polyphenols, and other bioactivators (Li et al., 2019). Our previous studies indicated that *A. mongolicum* Regel ethanol extract (AME) significantly decreased the concentration of BCFAs in the *Longissimus dorsi* muscle and *Dorsal subcutaneous*, *Omental*, and *Perirenal* adipose tissues of Small-tailed Han sheep (Liu et al., 2019; Liu and Ao, 2021). However, the molecular mechanism of this effect has not yet been completely elucidated.

The liver is a primary metabolic organ in mammals that serves as the metabolic center and is the site of multiple metabolic processes of nutrients (Ren et al., 2019). The metabolism of BCFAs occurs mainly in the liver, and the involved regulatory genes have been studied by researchers. Gunawan et al. (2018) found six differentially expressed genes (DEGs) in the liver between low- and high-MF Indonesian fat-tailed sheep by RNA deep sequencing. Listyarini et al. (2018) further verified this view and suggested that the expression of genes related to MF might affect the accumulation of BCFAs in the liver. In recent years, transcriptomic analysis has been extensively applied to identify factors in the lamb liver that affect MF and meat quality. For example, Abuzahra et al. (2018) revealed DEGs in the liver between animals with low- and high-MF by using RNA sequencing in Javanese fat-tailed sheep. An et al. (2021) explored the molecular regulation mechanism of fermented *Broussonetia papyrifera* L. feed influencing sheep meat quality traits by liver transcriptome analysis. Therefore, the transcriptome is a reliable tool for investigating the regulatory effect of AME on the expression of genes associated with MF in the liver.

We hypothesize that AME changes the expression of genes related to the formation of BCFAs in the sheep liver to reduce the “mutton flavor.” Thus, the aim of this study was to evaluate the effect of AME on the concentration of BCFAs, and identify candidate genes of AME affecting the synthesis of BCFAs related to MF in the liver of Small-tailed Han sheep by transcriptome analysis.

Materials and Methods

Animals used in this trial were cared for and handled in accordance with the recommendations of the Instructive Notions Concerning Caring for Experimental Animals, Ministry of Technology of China. All experimental procedures involving animals were evaluated and approved according to the guidelines of the Animal Care and Use Committee of Inner Mongolia Agriculture University (Hohhot, China).

Animals and samples

This study was conducted at a commercial farm in Bayan-naoer, Inner Mongolia Autonomous Region, China (latitude 40°13′–42°28′; longitude 105°12′–109°53′). Thirty 3-mo-old, male, Small-tailed Han sheep (33.60 ± 1.23 kg) were selected and randomly assigned to two groups and received a basal diet (CON, control group) or the same basal diet supplemented with 2.8 g of AME (AME group). Within each group, the sheep were reared in three different pens (each pen with five sheep). *Allium mongolicum* Regel powder was purchased from Hao Hai Biological Company (Alxa League, Inner Mongolia, China), and the ethanol extraction process was previously described by Ding et al. (2021).

The AME product primarily contains 26.43% flavonoids, 18.57% organic acids and derivatives thereof, 14.43% nucleotides and derivatives thereof, 11.14% amino acids and other compounds (Ding et al., 2021). The dose of the ethanol extract (2.8 g·lamb⁻¹·d⁻¹) in the diet has been proven to be most beneficial for the lamb based on our previous studies (Zhao et al., 2021). The extract was incorporated into a concentrate to feed lambs. The basal diet met the requirements for sheep, as described by the National Research Council (NRC, 2007), and its composition and nutritional level are shown in Table 1.

The experimental feeding trial lasted for 75 d, including a period of 15 d to adapt to the experimental environment and diet and a 60-d experimental feeding period. We provided the diet to the lambs twice a day, at 7:00 and 18:00. Lambs were given free access to feed and drinking water. At the end of this trial, we randomly selected 12 lambs that 6 from CON group and 6 from AME group. These lambs were fasted overnight and transported to a commercial abattoir, where they were stunned and slaughtered by exsanguination. Samples of left hepatic lobe tissues were collected and put into liquid nitrogen until transcriptome analysis and measurement of the three BCFAs.

Measurement of BCFAs in the liver

Three BCFAs, namely, MOA, MNA, and EOA, in the liver were analyzed by gas chromatography–mass spectrometry (GC-MS). Before the GC-MS measurements, fatty acids in the liver needed to be converted to the corresponding methyl esters, which was carried out according to the method of Del

Table 1. Composition and nutrient levels of the basal diet (dry matter basis %)

Item	Content (%)
Chinese wild rye	31.00
Caragana	17.80
Whole corn silage	23.60
Wheat bran	3.15
Sunflower seed meal	17.35
Pea stem and leaf	2.64
Red jujube	2.04
CaHPO ₄	0.74
NaCl	0.68
Premix ^a	1.0
Total	100.00
Nutrient level ^b	
DE (MJ/kg)	13.46
CP	16.87
NDF	38.72
ADF	27.51
Ca	1.33
P	0.53

^aNutritional composition of premix per kilogram: Mn 30.00 mg, Fe 25.00 mg, Zn 29.00 mg, Cu 8.00 mg, Co 0.10 mg, I 0.04 mg, VA 3200 IU, VD 1200 IU, VE 20 IU. DE was a calculated value, and the others were measured values.

^bDE, digestible energy; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; Ca, calcium; P, phosphorus.

Bianco et al. (2020). Undecanoic acid (Sigma-Aldrich, USA) was used as an internal standard.

A GC-MS system (Thermo TRANCE GC 1300) equipped with an autosampler was used in EI mode (70 eV). Solutions (1 μ L) were injected in split mode (split ratio 10:1) onto TG-WAX columns (30 m, 0.25 mm i.d., 0.25 μ m film thickness). The GC oven was held at 60°C for 2 min, and then, the temperature was raised from 20°C min⁻¹ to 250°C, holding for 5 min. The flow rate of the helium carrier gas was 1.2 mL/min. The mass spectrometer transfer line was at 250°C. Mass spectra were acquired using an ion source temperature of 220°C. GC-MS analyses were performed in full-scan mode (m/z 50-350).

Library construction and sequencing

Total RNA was extracted from liver tissue using TRIzol reagent (Invitrogen, USA) in accordance with the manufacturer's recommendations. The RNA integrity (RIN) and concentrations of each sample were evaluated on Agilent 2100 (Agilent) and NanoDrop 2000 (Thermo, Waltham, MA, USA) systems. The RNA of liver tissue samples with an RIN \geq 6.5, OD260/280 \geq 1.8, and OD260/230 \geq 2.0 was used to construct a sequencing library. A total of 10 RNA samples (CON, $n = 5$; AME, $n = 5$) were used for library construction with the Illumina TruSeq RNA Sample Prep Kit (Illumina) following the manufacturer's instructions. Subsequently, the paired-end RNA-seq sequencing library was sequenced using the Illumina HiSeq 2500 platform.

Raw reads were trimmed by removing linker sequences, low-quality reads (quality scores lower than 20), and reads with N contents of at least 10%, after which the clean reads were obtained. The clean reads were aligned to the *Ovis aries* reference genome (Oar v 3.1) using HISAT2 software, in which the default parameter settings were used. The mapped reads of each sample were assembled by StringTie (v2.1.2) via a reference-based approach. The raw data can be accessed in the NCBI BioProject database under the identifier PRJNA794958.

Differential gene expression analysis

The expression levels of genes were quantitatively analyzed by RSEM ver.1.3.3 software (Dewey and Li, 2011). Normalized expression levels of genes were expressed in fragments per kilobase per million reads. The DEGs between the CON and AME groups were identified by DESeq2 software (Love et al., 2014), which follows the negative binomial distribution model and read counts of genes. The corrected P -value was used to filter DEGs between the two groups. The false discovery rate (FDR) was used to correct the P -value for multiple tests. DEGs were selected with the criteria of fold change \geq 1.5 and corrected P -value $<$ 0.05 (Xue et al., 2021). The FDR was performed on the APT-BioCloud platform (APT BIO Co., Ltd, Shanghai, China; website: cloud.aptbio.com). The corresponding annotation information of DEGs was obtained by comparing the DEG sequences with the Nr, Swiss-Prot, and Gene Ontology (GO) databases using Blast2Go software.

GO and KEGG pathway analyses

GO was used to functionally annotate the identified DEGs, which were categorized into the biological process, cellular component, and molecular function categories, by Blast2Go software ver.2.5 (<https://www.blast2go.com/>). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses

were carried out to predict biological processes and pathways enriched for the identified DEGs using KOBAS software ver.2.1.1 (<http://kobas.cbi.pku.edu.cn/download.php>) (Xie et al., 2011). Rich factor is the ratio of the number of differential genes in this metabolic pathway to the number of genes annotated to this pathway, and the larger the value is, the greater the enrichment degree is.

Validation of RNA-Seq data by qRT-PCR

We randomly selected seven DEGs to verify the reliability and repeatability of the transcriptome profiling data by quantitative real-time PCR (qRT-PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin (ACTB) were used as reference genes to normalize target genes. The sequences of the primers were designed by Primer 5 software and are shown in Table S1 available in Supplementary Material 1. One microgram of total RNA was used to synthesize cDNA by reverse transcriptase using the PrimeScript RT reagent Kit with gDNA Eraser (Takara). PCR was conducted using TB Green Premix Ex Taq II Kit (Takara) in a 20 μ L reaction on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems Inc., USA) with the following program: 95°C for 30 s and 40 cycles of 95°C for 10 s and 60°C for 34 s. Each sample was tested three times, and the relative gene expression values were calculated by $2^{-\Delta\Delta CT}$ method (Silver et al., 2006).

Statistical analysis

One-way ANOVA was conducted to evaluate the differences in the concentration of BCFAs between the CON and AME groups using SPSS Statistics 26.0. The results are expressed as the mean \pm SEM. A P -value $<$ 0.05 was considered statistically significant.

Results

Effect of AME on the BCFA concentration in the sheep liver

The results for three BCFAs are shown in Figure 1. Compared to those in the CON group, the concentrations of MOA and MNA were significantly decreased in the AME group ($P <$ 0.05). No significant difference in the EOA concentration was detected between the groups.

Analysis of RNA-Seq data

After quality control and filtering, the sequencing results are shown in Table S2 available in Supplementary Material 1. The number of clean reads for liver tissues ranged from 40.83 to 50.12 million, with an average of 44.10 million. With regard to the livers from the CON group, 87.78% to 90.22% of the clean reads were aligned to the reference genome, and for the livers from the AME group, 88.50% to 90.12% of the clean reads were aligned to the reference genome. Approximately 58.82% to 75.67% of clean reads were singly aligned to the reference genome. The Q30 values of sequencing library were 92.67% to 92.92% for CON group and 92.26% to 93.13% for AME group, which indicated good sequencing quality. The GC content of the liver tissues was within 48.64% to 50.24%, which was consistent with the base composition rules.

DEG analysis

A total of 461 genes were identified as DEGs that met the criteria of corrected P -value $<$ 0.05 and fold change \geq 1.5 (Figure 2).

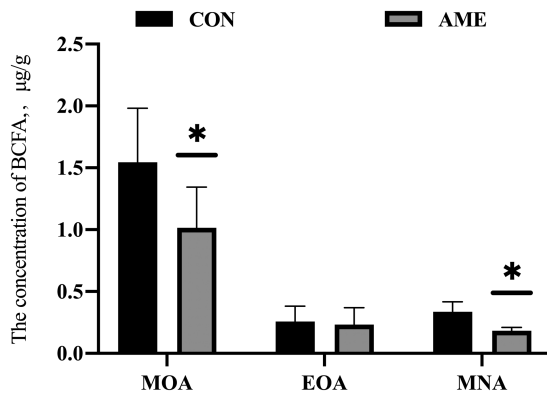


Figure 1. The concentration of BCFAs in the lamb liver between the CON group and AME group ($\mu\text{g/g}$). Each bar represents the mean \pm SD ($n = 6$); * indicates P -value < 0.05 . The x-axis indicates three branched-chain fatty acids and the y-axis is their concentration level.

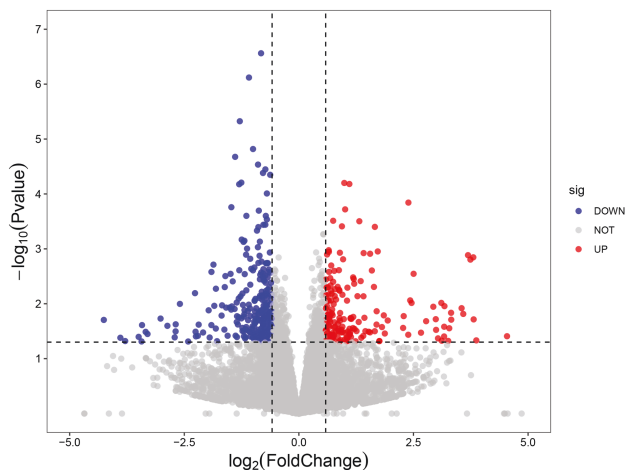


Figure 2. Volcano plot of differentially expressed genes in the liver between the CON and AME groups. Each point represents a gene. A total of 182 significantly upregulated genes are represented in red, and 279 significantly downregulated genes are represented in blue. Not significantly expressed genes are represented in black. The x-axis and y-axis are the \log_2 (fold change) and base mean expression values, respectively.

Furthermore, 182 genes were upregulated, whereas 279 genes were downregulated in the AME group compared with the CON group. Genes with similar expression patterns usually have functional correlation. Clustering analysis was conducted to illustrate the DEGs of liver samples from the CON group and AME group. The results indicated that up- and downregulated DEGs of liver samples from the CON group and AME group were clustered into a single class (Figure 3). Four hundred sixty-one DEGs between the CON group and AME group were presented in Supplementary Material S2.

To identify candidate genes related to the synthesis of BCFAs from 461 DEGs, we performed GO functional classification for DEGs between the CON and AME groups. The corresponding annotation information of DEGs was obtained by comparing the DEG sequences with the Nr, Swiss-Prot, and GO databases using Blast2Go software. Through the annotation information of DEGs, we searched for the genes related to the synthesis process of BCFAs, and then, the function of genes was confirmed in NCBI. Finally, the genes were

identified as candidate genes. Six candidate genes related to the synthesis of BCFAs were identified in the liver tissues and are listed in Table 2.

Function and pathway analysis of DEGs

GO and KEGG pathway analyses were conducted to provide functional annotation and identify the enriched pathways of DEGs between the CON and AME groups in this study. The identified GO terms were in the molecular function, cellular component, and biological process categories (Figure 4; Supplementary Material S3). Molecular functions regulating the metabolism of MF were related to binding, catalytic activity, transporter activity, and molecular function regulation. In the cellular component category, cell part, membrane, membrane part, organelle, and organelle part represented the primary proportion of genes. The identified biological processes were mainly related to cellular processes, biological regulation, metabolic processes, responses to stimuli, developmental processes, and cellular component organization or biogenesis. DEGs were enriched in three pathways, including glutathione metabolism, ECM–receptor interaction, and steroid hormone biosynthesis (Figure 5; Supplementary Material S4).

Validation of DEGs by qRT-PCR

To verify the reliability and repeatability of the RNA-Seq data, we randomly selected seven genes (*GSTCD*, *CYP2B6*, *THEM4*, *ACSF2*, *ELOVL6*, *ACOT12*, and *LPIN1*) from the DEGs and quantified them by qRT-PCR. *GAPDH* and *ACTB* were used as reference genes to normalize the target genes. The results showed that the mRNA expression levels from qRT-PCR were consistent with the RNA-Seq data (Figure 6).

Discussion

Effect of AME on the BCFA concentration in the sheep liver

The liver is an important target tissue in animals, playing a central role in numerous metabolic processes (Liu et al., 2019). BCFAs, such as MOA, EOA, and MNA, have been reported to be responsible for MF (Kaffarnik et al., 2015). Therefore, the effect of AME on the BCFAs concentration in the liver of Small-tailed Han sheep was evaluated in the present study. As hypothesized, AME significantly decreased ($P < 0.05$) the concentrations of MOA and MNA in the liver. Our findings were similar to the results of Liu et al. (2019) and Liu and Ao (2021), who found that supplementation with flavonoids and ethanol extract in the diet of Small-tailed Han sheep significantly decreased the concentrations of MOA and EOA in the *Longissimus dorsi* muscle and *Dorsal subcutaneous*, *Omental*, and *Perirenal* adipose tissues. In the present study, there was no difference in the concentration of EOA in the livers of lambs between the CON group and the AME group. This probably occurred because AME did not affect the carboxylation of butyryl-CoA in the liver. Butyryl-CoA was carboxylated to produce ethylmalonyl-CoA, which is a precursor of EOA biosynthesis. AME improved the flavor of sheep meat, and polyphenolic and flavonoid compounds from extracts may regulate the expression of genes related to MF in the liver to reduce the synthesis of BCFAs and then BCFA deposition in muscle and fat (Wang et al., 2019).

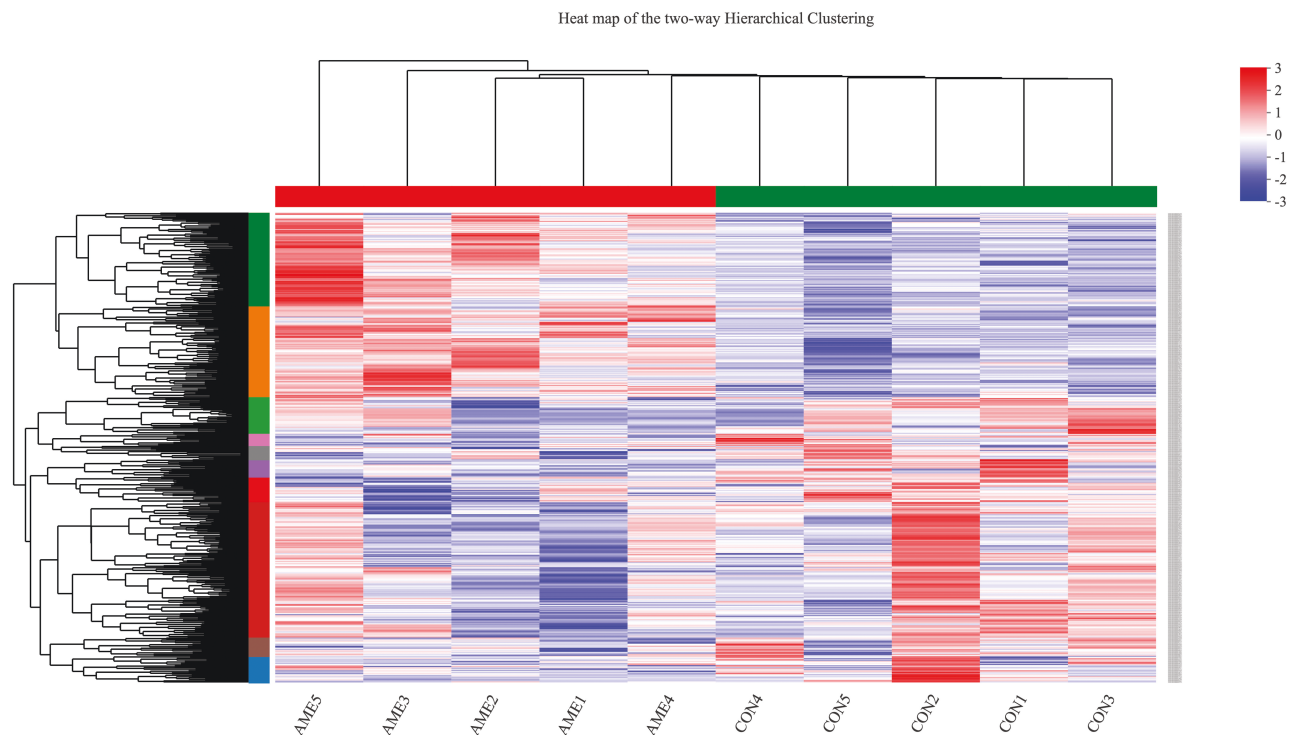


Figure 3. Heat map of differentially expressed genes in the liver between the control (CON) group and *Allium mongolicum* Regel ethanol extract (AME) groups. The column and row represent liver samples and genes, respectively. The left side is the dendrogram of gene clustering and the module diagram of subclustering; the closer two gene branches are to each other, the closer their expression levels are. The red block represents high expression of the gene, and the blue block represents low expression of the gene in the samples.

Table 2. Candidate genes related to mutton flavor from differentially expressed genes

Gene ID	Gene	Annotation	Log2 FC	Corrected P-value
ENSOARG00000007274	CYP2B6	Cytochrome P450 family 2 subfamily B member 6	1.10	0.0020
ENSOARG00000015301	ACOT12	Acyl-CoA thioesterase 12	0.71	0.0249
ENSOARG00000021072	THEM4	Thioesterase superfamily member 4	0.64	0.0473
ENSOARG00000016144	LPIN1	Lipin 1	-1.14	0.0053
ENSOARG00000019102	ADCY4	Adenylate cyclase 4	-1.00	0.0163
ENSOARG00000004315	ACSF2	Acyl-CoA synthetase family member 2	-0.73	0.0058

Abbreviations: ACOT12, acyl-CoA thioesterase 12; ACSF2, acyl-CoA synthetase family member 2; ADCY4, adenylate cyclase 4; CYP2B6, cytochrome P450 family 2 subfamily B member 6; LPIN1, lipin 1; THEM4, thioesterase superfamily member 4.

DEG analysis

In the current study, 461 genes were differentially expressed in liver samples between the CON and AME groups. Compared with the CON group, 182 genes were upregulated, whereas 279 genes were downregulated, in the AME group. MOA, MNA, and EOA are de novo synthesized by the use of methylmalonyl-CoA in chain lengthening with acetyl-CoA using propionate or butyrate as a precursor (Vlaeminck et al., 2006; Kaffarnik et al., 2015). Through the annotation information of DEGs, we searched for the genes related to the synthesis process of BCFAs, and then, the function of genes was confirmed in NCBI. Finally, six candidate genes (*CYP2B6*, *ACOT12*, *THEM4*, *LPIN1*, *ADCY4*, and *ACSF2*) were identified as likely to be involved in synthesizing BCFAs in the sheep liver (Table 2).

Among them, *CYP2B6*, *ACOT12*, and *THEM4* were significantly upregulated in the AME group. Cytochrome P450

family 2 subfamily B member 6 (*CYP2B6*) is an isozyme of the cytochrome P450 2B subfamily that metabolizes a wide variety of endogenous and xenobiotic compounds (Reed and Hollenberg, 2003). Cytochrome P450 isoenzymes, such as *CYP2A6*, degrade skatole to several intermediate products to improve the odor and flavor of pig meat (Robic et al., 2007). Gunawan et al. (2018) also suggested that *CYP2A6* was overexpressed in the liver of Javanese fat-tailed sheep with strong odor and flavor. *CYP2B6* is mainly expressed in the liver and belongs to the same family as *CYP2A6*. Acyl-CoA thioesterase 12 (*ACOT12*) contains a catalytic thioesterase domain at the N-terminus and a steroidogenic acute regulatory protein-related lipid transfer (*START*) domain at the C-terminus (Khandokar et al., 2017); this protein can hydrolyze the thioester bond of acetyl-CoA in the cytosol in the liver (Horibata et al., 2013). The latest evidence suggests the important role of ACOTs in maintaining metabolic homeostasis and diverse biological responses in the

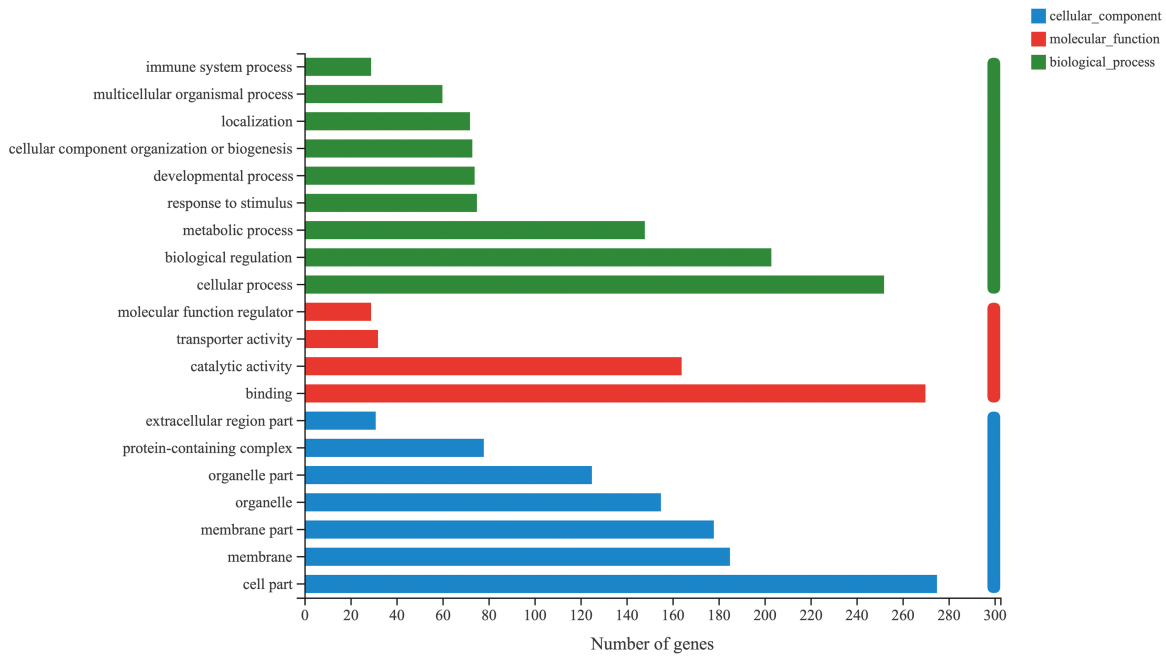


Figure 4. Gene Ontology (GO) annotation of differentially expressed genes in the liver between control (CON) group and *Allium mongolicum* Regel ethanol extract (AME) groups. The results are classified into three categories: molecular function, cellular component, and biological process. The x-axis represents the number of genes, and the y-axis shows the GO terms.

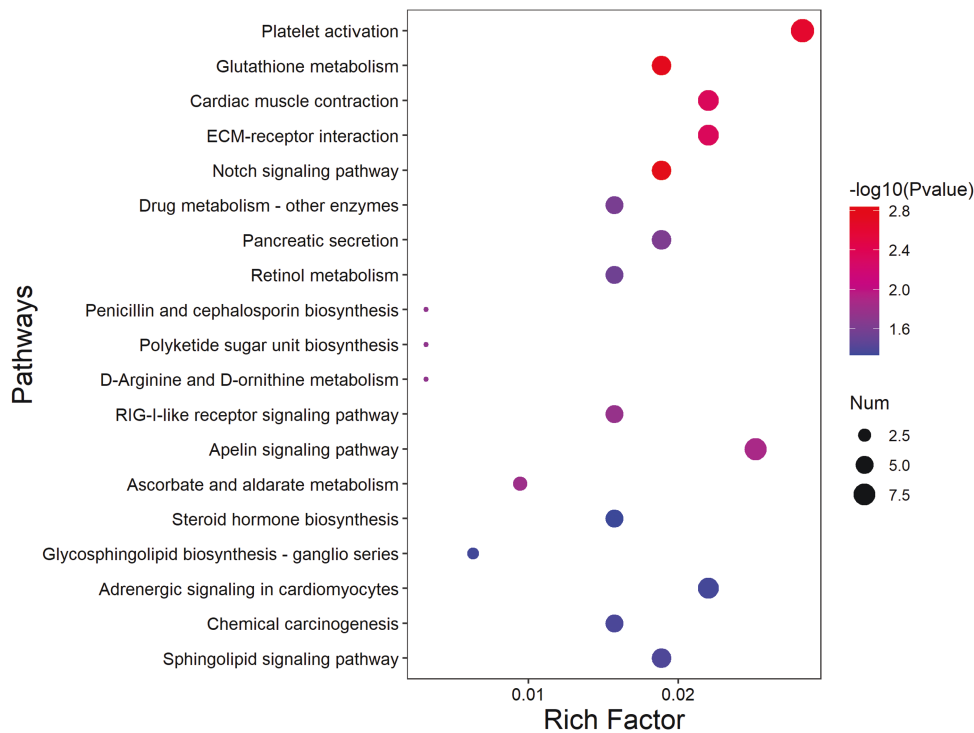


Figure 5. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes in the liver between control (CON) group and *Allium mongolicum* Regel ethanol extract (AME) groups. The x-axis is a rich factor that indicates the ratio of the sample number of genes/transcripts enriched in this pathway and the background number of annotated genes/transcripts. The y-axis is pathways. The P-value of all pathways is less than 0.05.

liver (Park et al., 2021). Thioesterase superfamily member 4 (*THEM4*) is a mitochondrial protein that acts synergistically with *ACOT12* to preferentially metabolize medium- to long-chain acyl-CoA thioesterases (Parcellier et al., 2009; Brocker et al., 2010). A previous study has shown that monomethyl-sub-

stituted fatty acids, such as MOA and MNA, were synthesized de novo by the use of methylmalonyl-coenzyme A in chain lengthening with acetyl-CoA (Vlaeminck et al., 2006). Therefore, *CYP2B6*, *ACOT12*, and *THEM4* might participate in the synthesis of BCFAs in the sheep liver.

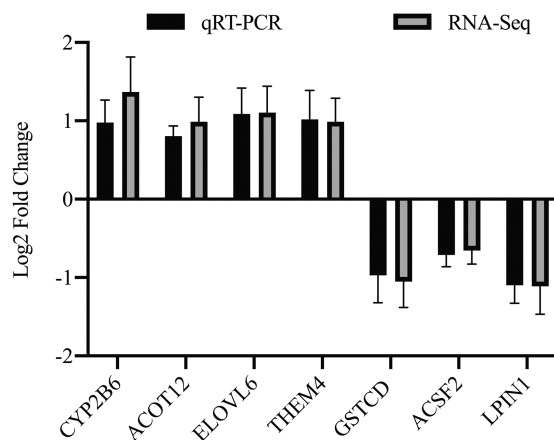


Figure 6. Expression levels of seven randomly selected differentially expressed genes by qRT-PCR. The x-axis indicates the seven DEGs, and the y-axis indicates the relative fold change. The values are shown as the mean \pm SD ($n = 6$).

On the other hand, acyl-CoA synthetase family member 2 (*ACSF2*) was significantly downregulated in our study. *ACSF2* is a mitochondrial matrix protein that belongs to the acyl-CoA synthetase (*ACS*) family, activating fatty acids by forming a thioester bond with CoA (Yu et al., 2016). *ACS*s are responsible for acyl-CoA synthesis from nonpolar hydrophilic fatty acids, and CoA and ATP are involved in various metabolic pathways, including fatty acid and cholesterol synthesis and the tricarboxylic acid cycle (Tian et al., 2018). In the process of fatty acid activation, *ACS* enzymes catalyze the ATP-dependent reaction to form an intermediate acyl-AMP, which is then converted to acyl-CoA (Ellis et al., 2010). As was previously reported, *ACSF2* robustly activated the eight-carbon medium-chain fatty acid octanoate in *ACSF2*-overexpressing cells (Watkins et al., 2007). Moreover, lower expression of *ACSF2* was suggested to reduce the formation of acetyl-CoA (Yu et al., 2016). Among other members of this family, *ACSF1* catalyzes a single condensation of acyl-CoA and malonyl-CoA to acetoacetyl-CoA (Matsumoto et al., 2011), and *ACSF3* serves as a mitochondrial malonyl-CoA synthetase to generate intramitochondrial malonyl-CoA from malonate in mammals (Lombard and Zhao, 2017).

Similar RNA-Seq data showed that the expression levels of *LPIN1* and *ADCY4* were significantly downregulated in the sheep liver. Lipin 1 (*LPIN1*) is a member of the lipid family, playing a crucial role in lipid homeostasis and metabolism (Kok et al., 2012). It can interact with *PPARG* coactivator-1 α (*PGC-1 α*) to regulate fat differentiation and lipid synthesis (Han et al., 2019). Li et al. (2020) demonstrated that *LPIN1* is involved in muscle growth and intramuscular fat deposition in the developmental stage of yellow broilers. Wang et al. (2020) also indicated that *LPIN1* affected muscle growth traits in Romney sheep. Adenylate cyclase 4 (*ADCY4*) encodes one of the adipocyte adenylate cyclase isoforms that synthesize cAMP upon Gas binding (Serazin-Leroy et al., 2001). The cAMP/PKA pathway is well known to act downstream of G-protein-coupled receptors, such as β -adrenergic receptors and prostaglandin E receptors, and to stimulate lipolysis via hormone-sensitive lipase activation (Kempe-Teufel et al., 2019). Based on these observations, we thought that *ACSF2*, *LPIN1*, and *ADCY4* can

be considered as candidate genes affecting the metabolism of BCFAs in the liver.

Function and pathway analysis of DEGs

DEGs were significantly enriched in three signaling pathways, namely, ECM–receptor interaction, glutathione metabolism, and steroid hormone biosynthesis, by KEGG pathway analysis in the liver of sheep. ECM–receptor interactions play an important role in maintaining cell structure and function and can directly or indirectly influence cellular activities, such as cell adhesion, proliferation, differentiation, and migration (Kim et al., 2011; Jiang et al., 2013). Glutathione metabolism is involved in the metabolism of amino acids. French et al. (2012) reported that branched-chain amino acids (BCAAs), such as Leu, Ile, and Val, are incorporated into BCFAs intracellularly via deamination and decarboxylation to the acyl-CoA esters 3-methylbutyryl-CoA, 2-methylbutyryl-CoA, and isobutyryl-CoA, followed by elongation to BCFAs. Steroid hormone biosynthesis is associated with lipid metabolism. The candidate gene *CYP2B6* belongs to this pathway, which may be involved in fatty acid oxidation. Gunawan et al. (2018) also found that DEGs were enriched in the steroid hormone biosynthesis pathway in the livers of sheep with high and low MF.

Conclusions

In this study, the effect of AME on the concentration of BCFAs and the transcriptomic profile were characterized in the lamb liver. Our results indicated that AME can reduce the concentration of BCFAs and cause differential expression of genes in the lamb liver. In addition, six DEGs (*CYP2B6*, *ACOT12*, *THEM4*, *ACSF2*, *LPIN1*, and *ADCY4*) were identified as candidate genes that might be involved in regulating the synthesis of BCFAs in the lamb liver. The findings of this study will help us to understand the molecular mechanism by which AME reduces the “mutton flavor” in lamb.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of interest statement

The authors declare no conflict of interest.

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