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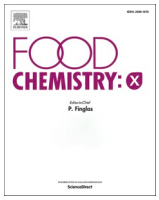

Insight into the differences in meat quality among three breeds of sheep on the Qinghai-Tibetan plateau from the perspective of metabolomics and rumen microbiota

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ABSTRACT

Mutton is one of the most popular meats among the general public due to its high nutritional value. This study evaluated the differences in meat quality among Chaka (CK), Black Tibetan (BT) and Oula (OL) sheep and investigated the metabolic mechanisms affecting meat quality using targeted and untargeted metabolomics and 16S rRNA. The results showed that the meat quality of CK sheep was superior to that of BT and OL sheep in terms of meat color, muscle fiber characteristics and nutritional quality. *Pseudobutyrivibrio*, *Alloprevotella, Methanobrevibacter*, *unidentified_Christensenellaceae*, *and unidentified_Bacteroidales* were key microbes involved in regulating meat color, muscle fiber characteristics, amino acid and fatty acid content. Protein digestion/absorption, pentose phosphate metabolism, carbon metabolism, and glyoxylate and dicarboxylate metabolism were important metabolic pathways involved in meat quality regulation. Our study is important for the development of sheep breeding strategy and sheep meat industry in Qinghai-Tibetan Plateau.

1. Introduction

Mutton is a popular meat around the world because of its unique taste and nutritional value (Sun, Guo, Wei, & Fan, 2011). Meat quality is a multifaceted concept involving organoleptic, nutritional, and microbiological properties regulated by both intrinsic and extrinsic factors (Boughalmi & Araba, 2016). Several studies have confirmed that meat quality can be influenced by animal breed and sex, age and weight at slaughter, and degree of fatness. (Ekiz et al., 2013; Monsón, Sañudo, & Sierra, 2005). Research shows significant differences in the fatty acid content of different types of lambs (Hajji et al., 2016). In Cloete's study, sensory analysis revealed a significant difference between Dormer sheep and Dohne Merinos in terms of initial juiciness and sustained juiciness of meat (Cloete, Hoffman, & Cloete, 2012).

The rumen is an important fermentation organ in the digestive tract of ruminants, where a large number and variety of microorganisms live (Jin et al., 2023). The diversity and composition of rumen microorganisms have an impact on the degradability of the diets and their functions in the rumen (Xiang et al., 2022;), which in turn significantly affects the meat quality of ruminant animals. It has been suggested that microbiota may be influenced and shaped by diet, age, gender, health, and lineage (Sasson et al., 2017).

As substrates, intermediates or end products of metabolism, metabolites play a central part in cellular biochemistry. In meat science, metabolites influence the texture, taste and color stability of meat (Mao et al., 2023). Metabolites of meat, including amino acids (AA) and their derivatives, are vital to human heath and contribute significantly to the nutritional value of meat (Khan, Jo, & Tariq, 2015). An association between rumen bacteria and metabolite deposition has been demonstrated in several studies. Wang et al. found a negative correlation between *Bacteroidales_UCG-001* and linoleic acid and arachidonic acid. *Ruminococcaceae_UCG-004* positively associated with L-lysine, DHA, and EPA (Wang et al., 2021). Modifying the rumen bacterial community can improve the nutritional quality of lamb meat (Xiang et al., 2022).

The Qinghai-Tibet Plateau is an important base of grassland animal husbandry in China and a major exporter of meat products (Wang & Zhao, 2023). Among them, Black Tibetan sheep (BT), Chaka semi-fine wool sheep (CK), and Oula sheep (OL) are important breeds (T. Guo

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et al., 2022; S. Li et al., 2023; Wu, Liu, Yan, Dong, & Wu, 2023). As an endemic livestock species distributed on the Tibetan Plateau, they have very strong physiological adaptability to the harsh alpine natural environment of the plateau (Wang et al., 2022). As local specialties of the Tibetan Plateau, there are few systematic comparative studies on the meat quality of CK, BT, and OL, and even fewer studies on the differences in their meat quality regarding rumen microorganisms and muscle metabolites.

Firstly, the aim of this study was to assess carcass traits, meat quality, the diversity of the rumen bacterial community and the metabolome between CK, BT and OL sheep. Then, we analyzed the relationship between rumen microbiota, muscle metabolites and meat quality to further elucidate the possible mechanisms influencing meat quality. Our study can greatly facilitate evaluating carcass traits and meat quality in sheep from differing genetic sources. It can also serve as an excellent guideline for the use of native Chinese breeds in the future.

2. Materials and methods

2.1. Sample collection

Nine CK, BT, and OL sheep, weighing approximately 10 kg, male, and 5 months old, were selected and grazed in a highly saline environment for 6 months (Chaka Town, Wulan County, Haixi Mongol and Tibetan Autonomous Prefecture, Qinghai Province, China) (Fig.1). The pasture started at 8:00 am and ended at 6:00 pm, and the experimental animals were given free access to water. The main species in the pasture were *Potentilla multicaulis* Bunge, *Chrysanthemum morifolium* Ramat, *Carex capillifolia* (Decne.), *Poa crymophila* Keng, *Oxytropis caerulea (Pallas)* Candolle, *Achnatherum splendens (Trin.)* Nevski, *Allium mongolicum* Regel, *Kalidium foliatum* (Pall.) Moq., *Stipa capillata* L. Dietary nutrient composition is shown in **Table S1**. 27 sheeps (9 of each breed) were selected for sampling at the end of the experiment. Slaughter was performed at Upland Farm (a commercial breeding farm with an exclusive slaughter and sampling room) by trained personnel using special butcher knives. The experimental sheep were slaughtered by the decapitation-bleeding method, which involves cutting the carotid artery in the sheep's neck near the throat with a sharp knife and completely bleeding the sheep with its head down. Rumen fluid was placed in freezer tubes and immediately stored in liquid nitrogen after being filtered through four layers of gauze. *Longissimus dorsi* muscle samples were collected from the left side of the sheep, vacuum packed and frozen instantly for compositional and metabolomic analysis. Nutrients, microbes and metabolites were determined in six replicates.

2.2. Analysis of meat edibility and myofiber properties

Defrosted specimens were taken and cut into standard sizes of 1 cm × 1 cm × 1 cm. Hardness, elastic, viscous, cohesive, chewability, and adhesion of specimens were determined using a TPA Texture Analyzer with probe model TA-3/100 and fixture model TA-RT-KIT (Beijing Jinyang Wanda Technology Co.). The muscle color was measured using an OPTO-LAB meat color analyzer (MATTHAUS, Germany). The conventional nutrients (moisture, crude protein, ether extract, and total ash) in the muscle were determined according to Cohen's method (Cohen, 1971). Drop loss, steam loss, defrost loss, thaw loss, and cooked meat rate of samples were evaluated according to the Zhang et al. (Zhang et al., 2022a). Muscle fiber characteristics (number, area, density, diameter) were measured by hematoxylin-eosin (H&E) staining. Image acquisition was performed with CaseViewer 6.0 software.

Fig. 1. The physical pictures of three breeds of sheep.

2.3. Measurement of amino acid and fatty acid content

An amino acid analyzer (Sykam S—433D) was used to determine the amino acids. 25 mg of the sample was hydrolyzed by hydrochloric acid at 113 °C, 0.5 mL of the sample solution was evaporated in a test tube concentrator, 5 mL of specimen diluent was added, after mixing, 1 mL of the mixture was pipetted with a syringe into the injection vial, and the injection sequence was edited to analyze the types and contents of amino acids in the sample.

The targeted metabolomics was used to determine fatty acids and their derivatives in the samples. After thawing, 0.05 g of the sample was diluted with 140 μL MeOH, and 40 μL 36% phosphate solution. The sample was spun at 2500 rpm for 3 min, and centrifuged at 12000 rpm for 6 min at 5 °C. 200 μL upper liquid was transferred to a new centrifuge tube, 300 μL methanol solution of 15% boron trifluoride was centrifuged and baked at 60 °C for 30 min. Allow to cool to room temperature, 600 μL n-hexane and 300 μL saturated sodium chloride solution were added in an accurate volume, 100 μL of the n-hexane layer solution was transfered to the GC–MS. The analytical conditions were the following: Carrier gas: high-purity helium; the heating temperature started from 40 °C (2 min), 35 °C/min to 200 °C (1 min), 15 °C/min to 245 °C (1 min), 5 °C/min to 280 °C (4 min); flow rate: 1.0 mL/min; injection volume: 1.0 μL.

2.4. Quantification and diversity analysis of rumen microbiota

Total genomic DNA was extracted from samples by CTAB method according to Zheng et al. (Zheng et al., 2023). Purity and concentration of extracted DNA were checked by 1% agarose gel electrophoresis. The 16S rRNA gene of different regions (16S V3-V4) was amplified. Qubit@ 2.0 fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system were used to detect the PCR products. The DNA fragments were subjected to high-throughput sequencing on the Illumina NovaSeq sequencing platform after passing the assay.

2.5. Analysis of non-targeted metabolomics

Thawed samples were homogenized with a 30 HZ grinder for 25 s. 400 μL 70% methanol solution was added and shaken at 1500 rpm for 5 min. Samples were placed on ice for 15 min followed by centrifugation at 12,000 rpm for 8 min at 5 °C. A supernatant of 300 μL was stored at −20 °C for 30 min. The samples were spun at 12,000 rpm for 3 min (4 °C). For LC-MS analysis, 200 μL aliquots of the sample supernatant were transferred. An aliquot was analyzed in positive ion mode using solvent A (0.1% formic acid solution) and solvent B (0.1% formic acid acetonitrile solution) in the gradient: 5%–20% (2 min), increased to 60% in 3 min, increased to 99% in 2.5 min, and returned to 5% mobile phase B in 0.1 min and held for 2.4 min. Parameters were as follows: column temperature, 36 °C; injection volume, 3.5 μL; flow rate, 0.4 mL/min; negative ion conditions were used. Data were collected using Analyst TF 1.7.1 software (Sciex, Concord, ON, Canada) in IDA mode.

2.6. Statistical analysis

The statistical analysis was performed using SPSS software version 17.0. One-way analysis of variance (ANOVA) was applied to analyze the nutritional components of muscle. Duncan's test was used to analyze the multiple comparisons of the mean values. The correlation between

rumen microbes, muscle metabolites and meat quality was assessed by Pearson's correlation analysis. PCoA analysis was performed with the phylosEq. (v1.40.0) package in R software (v4.2.0), and OPLS-DA algorithm was implemented with MetaboAnalystR (v1.0.1) in R. LEfSe (LDA Effect Size) analysis was conducted using LEfSe software (v1.1.2). Network analysis was carried out with Cystoscope v3.10.2 software.

3. Results

3.1. Slaughtering performance and myofiber properties

BT sheep had the largest liveweight, CK sheep the next largest, and OL sheep the smallest. Carcass weight and slaughter rate were not significantly different between the three experimental groups (Table 1). Regarding myofiber characteristics, the area, total number, and density of myofibers were significantly higher in CK compared to BT and OL, while the diameter of myofibers was higher in OL compared to CK and BT ($P < 0.05$) **(**Fig. 2**)**. The hardness, stickiness, elasticity, cohesion, masticity, and adhesion of the *longissimus dorsi* muscle of CK sheep were higher than in BT and OL sheep ($P < 0.05$) **(**Table 1**)**.

3.2. Meat edibility and nutritional quality

The nutritional and eating quality of the sheep are shown in Table 2. The L and c values in CK were higher than in OL and BT ($P < 0.05$), while the b value of BT was significantly higher than CK and OL ($P < 0.05$). The crude protein level in BT was higher than that in CK and OL ($P < 0.05$). But the thawing losses and the percentage of cooked meat were highest in OL sheep ($P < 0.05$).

3.3. Determination of amino acid and fatty acid content

The results of the amino acid content are shown in Table 3. Methionine content was higher in OL than in CK and BT ($P < 0.01$). The level of essential amino acids (EAA) was highest in OL sheep, followed by CK and lowest in BT sheep. The levels of total amino acids (TAA) and non-essential amino acids (NEAA) were higher in OL and CK sheep than in BT sheep ($P < 0.01$).

The fatty acid content is shown in Table 4, the concentration of C10:0, C22:4, C16:1 T, C20:5n3, C8:0, C22:5, C20:4n6, C20:3n6, C12:0, C13:0, C14:0, C15:0, C15:1, C16:0, C16:1, C18:1n9c, C18:2n6c, C20:3n3 was higher in CK than in BT and OL ($P < 0.05$). The levels of saturated fatty acids (SFA), polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), and n-6 fatty acids were highest in CK and lowest in OL ($P < 0.05$).

Table 1
Slaughtering performance and muscle fiber characteristics of different breeds of sheep.

Items	Groups			SEM	*P*-value
	OL	BT	CK		
live weight (kg)	23.833c	30.200a	27.600b	0.966	0.001
carcass weight (kg)	9.133	10.783	9.733	0.332	0.107
slaughter rate (%)	37.850	35.630	36.167	0.534	0.224
hardness (g)	3477.333b	3857.000b	8115.000a	770.470	<0.001
resilience (mm)	7.587b	6.873b	9.893a	0.511	0.009
tackiness (mJ)	0.400	0.433	0.467	0.037	0.813
cohesion	0.377b	0.433b	0.553a	0.028	0.004
chewability (mJ)	80.900b	104.767b	274.167a	32.016	0.001
agglutinative (g)	538.000c	812.667b	1069.000a	93.998	0.038

Note: a − c means within a row with different subscripts differ when *p*-value <0.05.

3.4. Rumen microbiota

We conducted 16S rRNA sequencing of rumen bacteria, and the analysis of rank abundance curves indicated that the sequencing depth obtained was sufficient and feasible for further analysis (Fig. 3a). A total of 2411 ASVs were detected in three groups, with 2814, 3189, and 2805 specific ASVs identified in CK, BT, and OL (Fig. 3b). The PCoA plots showed that the rumen bacterial community of CK, BT and OL groups clustered separately from each other ($p < 0.05$) (Fig. 3c). Anosim analysis indicated significant differences between groups (R-value>0) (**Supplementary Fig. 1**). The Chao1, Shannon, and Simpson indices of bacterial abundance and diversity were not significantly different between the comparison groups ($P > 0.05$) (Fig. 3d, e, f).

To better evaluate the total rumen bacterial community makeup in the three comparison groups, phylum and genus abundance percentages were analyzed. At the phylum level, Bacteroidetes and Firmicutes were the dominant bacterial communities (Fig. 4a). The relative abundance of *Spirochaetota* was significantly higher in BT compared to CK and OL ($P < 0.05$) (**Table S2**). *Methanobrevibacter* abundance was higher in CK than in BT and OL at the genus level ($P < 0.05$) (Fig. 4b). The abundance of *unidentified_Bacteroidales* was higher in BT than in CK and OL ($P < 0.05$) (**Table S3**).

To identify the biomarker of each group, the bacterial composition was analyzed using linear discriminant analysis (LDA). The LDA effect size results indicated six genera discriminated among the samples from the three groups. The dominant rumen bacteria enriched in CK were *gut_metagenome* and *Selenomonas_ruminantium*. The key rumen bacteria including *c_Bacilli* and *o_Erysipelotrichales* were identified in BT. The *human_gut_metagenome* and *Alloprevotella_sp* were significantly enriched in OL (Fig. 4c). The ternary phase diagram analysis based on ASV at the phylum (a) and genus (b) levels are shown in **Supplementary Fig. 2**. The results of the functional annotation showed that the microorganisms in each comparison group were mainly annotated for metabolism (**Supplementary Fig. 3)**.

3.5. Untargeted metabolomic analysis

To show the differential metabolites of the three groups, score plots were made using OPLS-DA. The R^2Y values of OPLS-DA models in BT vs. OL (Fig. 5a), CK vs. BT (Fig. 5b), and CK vs. OL (Fig. 5c) were 0.916, 0.953, and 0.944, respectively. The Q2 values of the models >0.5 demonstrated a good efficacy model. Score plots showed distinct separation and discrimination, indicating that the OPLS-DA model can identify differences between the three groups.

We used volcano plots to show the differences of metabolites in the three groups of samples. In BT vs. OL, there were 412 differential metabolites, in CK vs. BT, 173 metabolic biomarkers were identified, and 438 metabolic biomarkers were detected in the CK and OL groups (Fig. 6). The top 10 up- and down-regulated metabolites in BT vs. OL, CK vs. BT, and CK vs. OL are shown in **Table S4**.

The top 20 pathways in different comparison groups were identified by KEGG pathway enrichment analysis (Fig. 7). The metabolites in BT and OL groups were mainly enriched in the pathways of Phenylalanine, tyrosine, and tryptophan biosynthesis, 2-Oxocarboxylic acid metabolism, Tryptophan metabolism, and Aminoacyl-tRNA biosynthesis (**Table S5**). The key metabolic pathways in the CK and BT groups were Sulfur relay system, Protein digestion and absorption, Aminoacyl-tRNA biosynthesis, D-Amino acid metabolism, and Mineral absorption. Glycine, serine and threonine metabolism, AMPK signaling pathway, Carbon metabolism, and Glycolysis/Gluconeogenesis were the major metabolic pathways in CK and OL groups.

3.6. Correlation analysis

We performed a correlation analysis of rumen microbes and muscle differential metabolites at the genus level (Fig. 8a, c), and found a

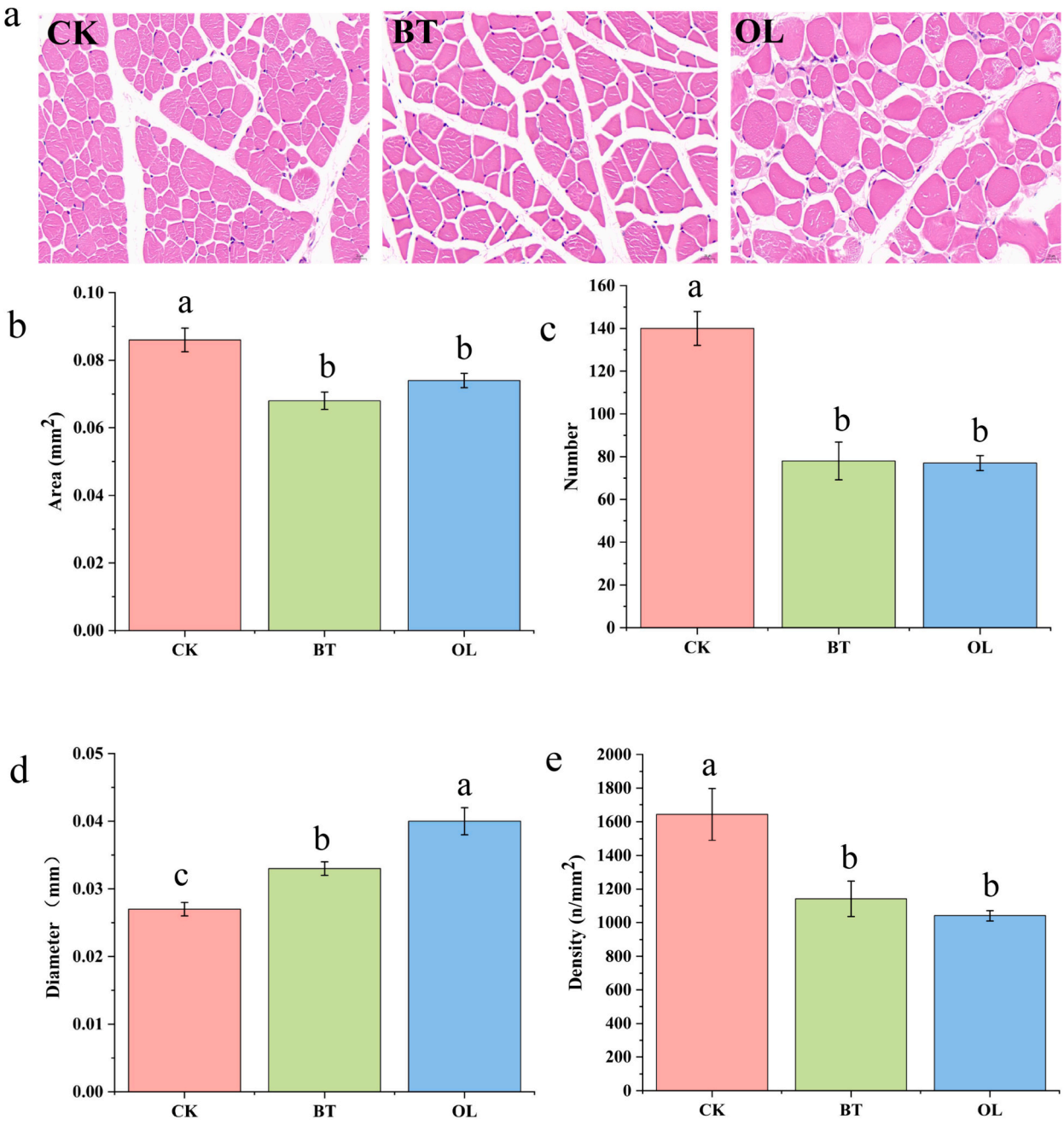

Fig. 2. H&E staining image of the *longissimus dorsi* muscle in CK, BT, and OL sheep (a). Total area (b), total number (c), the diameter (d), and the density (e) of muscle fibers in selected fields of view.

Note: In the picture above, CK is Chaka Sheep, BT is Black Tibetan Sheep and OL is Oula Sheep. a-c means within a row with different subscripts differ when *p*-value <0.05.

significant positive correlation between *Methanobrevibacter* and 2-phospho-D-glyceric acid. *Unidentified_Bacteroidales* showed positive correlations with L-tryptophan, S-adenosylmethionine, and phenylpyruvic acid, and positive correlations with pyruvic acid. *Unidentified_Christensenellaceae* was positively correlated with L-Tyrosine and Phenylpyruvic acid. *Alloprevotella* was positively associated with L-Alanine and Phenylpyruvic Acid. The *unidentified_Gracilibacteria* was negatively correlated with L-Tyrosine. We found a positive correlation between *unidentified_Bacteria* and Dihydroxyacetone phosphate. Between *Pseudobutyrivibrio* and L-Alanine, we found a significantly positive correlation. There was a positive correlation between the *ruminococcus* and the dihydroxyacetone phosphate.

We investigated the relevance analysis between key metabolites and meat quality (Fig. 8**b** and **c**). Our results showed that *L*-phenylalanine had a positive correlation with L and methionine, and a negative correlation with B and crude protein. L-alanine was positively correlated with MUFA and negatively correlated with muscle fiber diameter. S-adenosylmethionine correlates positively with L-value and methionine and negatively with b-value and crude protein. We found significantly positive correlations of glyceric acid with C20:4n6, MUFA, PUFA, n-6, and muscle fiber density. The results showed that both 3-phosphoglyceric acid and 2-phospho-D-glyceric acid were positively correlated with C20:4n6, PUFA, n-6. Phenylpyruvic acid correlated negatively with b-value and crude protein and positively with methionine. Phosphoenolpyruvate was positively correlated with MUFA, PUFA, and n-6 and negatively correlated with methionine.

Table 2
Nutritional and edible qualities of the *longissimus dorsi* muscle in different breeds of sheep.

Items	Groups			SEM	*P*-value
	OL	BT	CK		
L	64.000b	48.667c	69.667a	3.209	<0.001
a	34.333	33.667	33.667	0.564	0.888
b	14.000b	17.333a	13.667b	0.707	0.031
c	54.333b	47.667c	59.333a	1.832	0.003
moisture(g/100 g)	74.975	74.297	74.606	0.221	0.520
ash (g/100 g)	1.143	1.153	1.078	0.027	0.552
ether extract(g/100 g)	0.425	0.964	1.196	0.162	0.126
crude protein(g/100 g)	22.071b	23.370a	22.504b	0.230	0.030
drip loss (%)	8.783	8.217	10.277	0.397	0.065
steaming loss (%)	47.400	47.633	47.246	0.316	0.909
thawing loss (%)	10.910a	5.360b	8.250ab	0.945	0.022
cooked meat rate (%)	69.173a	65.953b	65.117b	0.664	0.002

Note: a – c means within a row with different subscripts differ when *p*-value <0.05.

Table 3
The amino acid content in the *longissimus dorsi* muscle of three different breeds sheep (%, DM basis).

Items(%)	Groups			SEM	*P*-value
	OL	BT	CK		
Asparticacid	5.743	5.487	5.510	0.122	0.698
Threonine	2.957	3.047	2.903	0.043	0.452
Serine	2.447	2.327	2.499	0.026	0.176
Glutamicacid	12.027	11.310	11.690	0.135	0.068
Glycine	3.773	3.147	3.847	0.140	0.051
Alanine	4.207	3.843	4.287	0.093	0.098
Cystine	0.407	0.317	–	–	–
Valine	3.243	2.973	3.317	0.070	0.085
Methionine	1.343a	1.043b	1.143b	0.049	0.006
Isoleucine	3.170	3.247	3.133	0.179	0.783
Leucine	5.303	4.957	5.337	0.122	0.425
Tyrosine	2.087	1.537	2.010	0.140	0.241
Phenylalanine	2.587	2.263	2.463	0.068	0.141
Histidine	2.483	2.507	2.670	0.045	0.195
Lysine	5.103	5.057	5.027	0.077	0.938
Arginine	4.463	4.230	4.540	0.059	0.051
Proline	2.473	2.290	2.810	0.128	0.269
EAA	26.190a	25.093b	26.020a	0.613	0.028
NEAA	37.627a	34.487b	37.093a	0.540	0.007
TAA	63.817a	59.580b	63.113a	0.712	0.003

Note: a – c means within a row with different subscripts differ when *p*-value <0.05.
EAA, essential amino acids. NEAA, non-essential amino acids. TAA, total amino acids. EAA = Threonine + Valine + Methionine + Isoleucine + Leucine + Phenylalanine + Histidine + Lysine + Arginine; NEAA = TAA-EAA. Percentage indicates the amount of amino acids per milligram of muscle sample.

4. Discussion

The organoleptic quality of meat influences the consumer's willingness to buy to a certain extent, and the color of meat is an intuitive factor for consumers to judge the quality of meat(Eom et al., 2024). Meat color is generally expressed in terms of L-value (brightness), a-value (redness), b-value (yellowish), and c-value (chroma) (Joseph, Suman, Rentfrow, Li, & Beach, 2012). Significant differences in L, b, and c values were found between all three sample groups($P < 0.05$), with CKs having significantly higher L and c values than OLs and BTs, and CKs having lowest b values. The L-value represents the lightness or darkness of meat, and the higher the L-value, the more popular it will be with the consumer (Yakan et al., 2024). The b-value is related to the freshness of the meat and increases with storage time (Su et al., 2024).The c-value indicates color purity and saturation, with a higher c-value indicating better color and saturation (Bueno, Massingue, Ramos, Ferreira, & Ramos, 2024). Based on this, we judged that the meat color in CK was better than in other two groups and might be more preferred by consumers during selling. Muscle fiber properties are associated with meat quality (Jeong et al., 2010). The muscle fiber properties can be related to factors including breed, age, sex, and location of the animal (Ryu & Kim, 2006). In our study, the mean myofibrillar area, number, and density were higher in CK than in BT and OL. The muscle fiber diameter, perimeter, and area were higher in CK sheep than BT sheep in Guo's study (Guo et al., 2022). In comparison, we found that our test animals were about 1 year old, while theirs were about 3 years old. Therefore, we hypothesized that the reason for the inconsistent results of the muscle

Table 4
The fatty acid content in the *longissimus dorsi* muscle of three different breeds sheep (μg/g Tissue).

Items (μg/g)	Groups			SEM	*P*-value
	OL	BT	CK		
C10:0	0.728c	2.297b	3.638a	0.440	0.001
C22:1n9	2.728	2.815	3.853	0.332	0.350
C22:4(cis-7,10,13,16)	7.933b	10.846ab	16.073a	1.398	0.021
C22:6n3	10.028	15.351	19.566	1.782	0.064
C23:0	4.726	4.653	4.663	0.020	0.278
C24:0	5.047	4.961	5.051	0.034	0.534
C6:0	0.095	0.141	0.281	0.036	0.057
C9:0	0.226	0.205	0.272	0.024	0.582
C16:1 T	3.964b	4.579b	10.871a	1.167	0.001
C19:1(cis-10)	3.104	3.591	3.517	0.154	0.429
C22:0	2.292	2.302	2.878	0.231	0.562
C20:5n3	32.976c	40.888b	59.977a	4.443	0.007
C8:0	0.120c	0.405b	0.673a	0.085	0.002
C14:1	–	–	1.995	–	–
C22:5(cis-7,10,13,16,19)	47.895c	55.658b	81.230a	5.835	0.017
C21:0	2.260	2.257	2.276	0.022	0.947
C20:4n6	79.471c	114.831b	180.201a	17.668	0.028
C20:3n6	8.475b	11.236ab	17.419a	1.541	0.018
C11:0	0.015	0.027	0.321	0.004	0.105
C12:0	0.671c	1.382b	1.959a	0.200	0.003
C13:0	11.288a	8.933b	1.861c	1.149	0.001
C14:0	6.721c	15.253b	19.785a	1.982	<0.001
C15:0	4.906b	7.987ab	8.442a	0.641	0.015
C15:1	6.400b	6.490b	63.327a	9.870	<0.001
C16:0	269.599c	317.695b	350.328a	12.675	0.003
C16:1	11.717c	21.620b	24.680a	2.058	0.001
C17:0	21.162	26.801	23.138	1.299	0.212
C18:0	259.352	291.112	316.689	11.824	0.131
C18:1n9c	191.030c	288.510b	362.047a	25.716	<0.001
C18:1n9t	26.462	35.178	33.487	2.080	0.204
C18:2n6c	140.667c	250.906b	317.041a	30.864	0.029
C18:3n3	33.173	61.005	54.901	5.465	0.065
C18:3n6	1.606	1.982	2.930	0.248	0.051
C19:0	2.710	4.051	2.373	0.340	0.080
C20:0	4.760	4.902	6.642	0.596	0.409
C20:1(cis-11)	3.968	4.305	8.100	1.149	0.297
C20:1 T	1.162	1.039	1.287	0.096	0.638
C20:2	2.450	2.640	2.919	0.164	0.566
C20:3n3	24.589a	20.302b	1.950c	4.073	0.020
SFA	596.679c	695.363b	750.982a	26.158	0.017
MUFA	250.534c	368.127b	513.161a	39.366	<0.001
PUFA	338.325b	518.561ab	658.591a	55.928	0.031
n-3	57.762	81.307	56.851	6.323	0.215
n-6	230.218b	378.955ab	517.591a	49.170	0.024

Note: a – c means within a row with different subscripts differ when *p*-value <0.05.
SFA: C6:0 + C8:0 + C9:0 + C10:0 + C11:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C19:0 + C20:0 + C21:0 + C12:0 + C23:0 + C24:0;
MUFA: C22:1n9 + C16:1 T + C19:1(cis-10) + C14:1 + C15:1 + C16:1 + C18:1n9c + C18:1n9t + C20:1(cis-11) + C20:1 T;
PUFA: C22:4(cis-7,10,13,16) + C22:6n3 + C20:5n3 + C22:5(cis-7,10,13,16,19) + C20:4n6 + C20:3n6 + C18:2n6c + C18:3n3 + C18:3n6 + C20:2 + C20:3n3;
n-6 PUFA: C20:4n6 + C20:3n6 + C18:2n6c + C18:3n6;
n-3 PUFA: C22:6n3 + C20:5n3 + C18:3n3 + C20:3n3.
SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

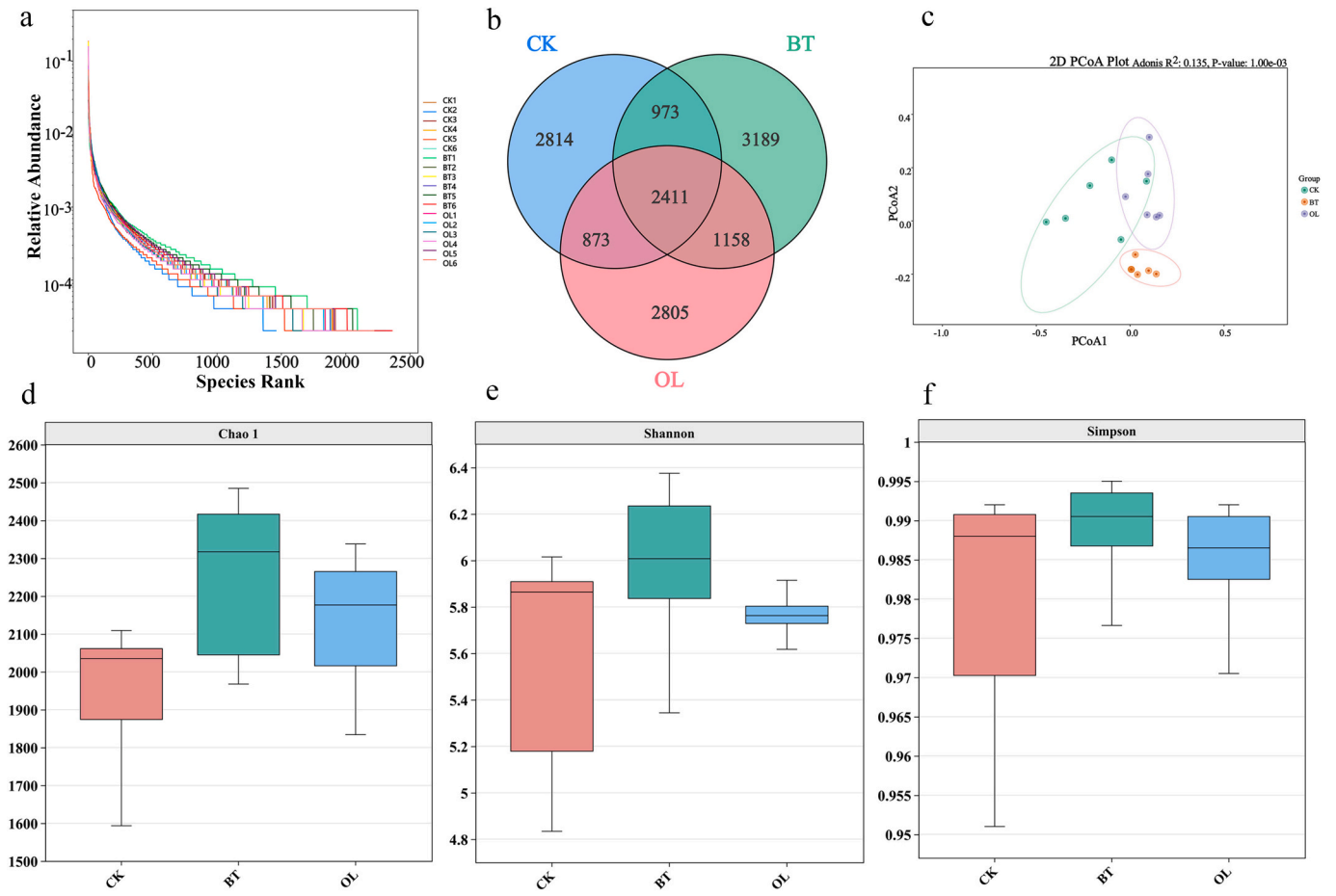

Fig. 3. Rarefaction curve (a). Venn diagram illustrating the overlaps of microbial ASVs between the three groups (b). PCoA of taxonomical classifications of ruminal bacteria communities (c). Alpha diversity of the ruminal bacteria between the three groups (d, e, and f).
Note: In the picture above, CK is Chaka Sheep, BT is Black Tibetan Sheep and OL is Oula Sheep.

fiber properties was related to the age of the test animals. Studies have shown that the specific properties of muscle fibers affect meat color, water retention and texture (Pette & Staron, 2000), and our results are consistent with these findings. In our study, the meat color and edible characteristics including hardness, elasticity, tenderness, cohesion, and adherence of the CK were better than BT and OL. Skeletal muscle myofibers are typically classified as type I, IIA, IIX, and IIXB, and different types of muscle fibers have varying metabolic characteristics (Schiaffino & Reggiani, 1996). The three groups of samples may have been of different muscle fiber types, resulting in differences in muscle fiber properties that further influenced meat quality. The muscle fiber types of these three comparison groups require further investigation.

The nutritional value is also an important indicator of the quality of the meat (Xu et al., 2023). Lamb is an excellent source of protein, with skeletal muscle containing many essential amino acids (Jia et al., 2021). In our study, we found significant differences in the levels of amino acids and fatty acids among the three breeds of sheep. Cao et al. have shown that the amount and type of amino acids determine the nutritional value of muscle (Cao et al., 2021). In our study, the OL group had the highest EAA, NEAA, and TAA, followed by the CK group and the BT group had the lowest. The level of methionine was also higher in OL than in CK and BT. Methionine is an amino acid that is essential for the formation of proteins, and it also affects the metabolism of lipids and oxidative status (Chen, Chen, Zhang, & Zhou, 2013). From this point of view, it can be judged that the amino acid value of lamb in the CK and OL groups was superior to the BT group. As consumers become more health conscious, there is a greater demand for nutritious foods with health-promoting properties. The distribution of fatty acids (FA) and PUFA has received increased attention in recent years (Hajji et al., 2016). Ruminants fed fresh pasture have higher levels of PUFA in their meat than those fed grain concentrates, according to several studies (Sanudo et al., 1998). In our study, the CK group had the highest total PUFA content, followed by the BT group, and the OL group had the lowest total PUFA content. The levels of most of the PUFA's like C22:4 (cis-7, 10, 13, 16), C20:5n3, C22:5 (cis-7,10,13,16,19), C20:4n6, C20:3n6, C18:2n6c, and C20:3n3 were consistent with the expression of total PUFA's in our study, whereas the levels of C22:6n-3, C18:3n-3, C18:3n-6, and C20:2 did not differ significantly among the three comparison groups. It has been shown that individual PUFA was not affected by breed (Hajji et al., 2016). This view is supported by the results of our study. PUFAs are susceptible to oxidation, and too much oxygen can easily lead to oxidative meat rancidity, creating a distinctive odor and decreasing meat quality (D'Alessandro, Palazzo, Petrotos, Goulas, & Martemucci, 2015). Although SFAs are commonly considered to be unhealthy, some of them are beneficial. For example, lauric acid (C12:0) has anti-bacterial, anti-oxidant, and anti-viral qualities (Hinton Jr. & Ingram, 2006). It was also highest in the CK group in this study. It has been shown that breeds affect the levels of C10:0, C14:0, and C16:0, confirming the influence of genotype on fatty acid profiles (Yousefi, Kohram, Shahneh, Nik-Khah, & Campbell, 2012). Our findings were consistent with this point. In summary, we found that the CK was significantly better than BT and OL in terms of fatty acid content, and the fatty acid expression profiles differed significantly between sheep breeds, which may be related to genetic factors.

Comparing the overall composition of the rumen microbiota in the three groups revealed that Bacteroidetes and Firmicutes were the

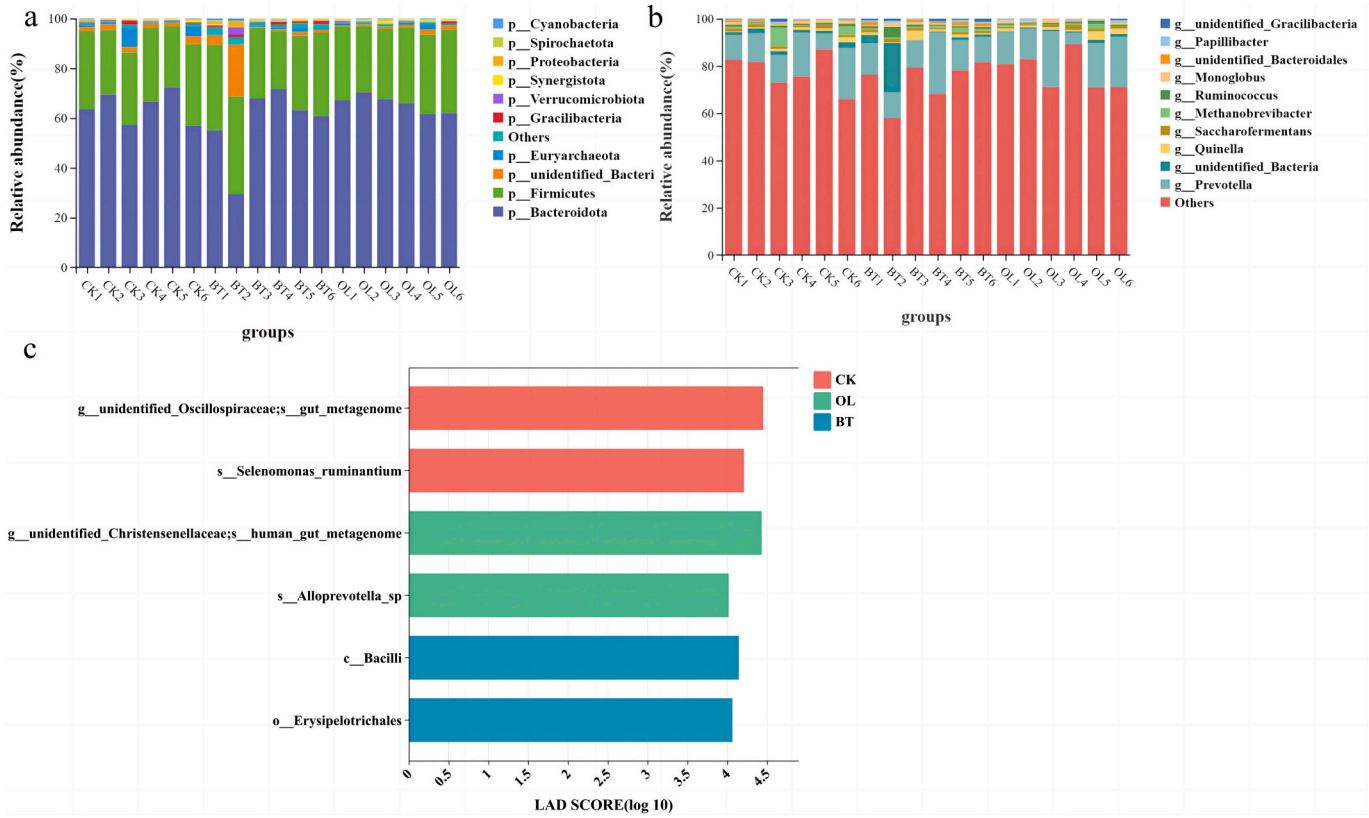

Fig. 4. Relative abundance of ruminal bacteria at the phylum (a) and genus (b) levels among the experimental treatments. Histogram of the linear discriminant analysis (LDA) effect among the three groups (C), and the LDA score (log10) > 4 were shown.
Note: In the picture above, CK is Chaka Sheep, BT is Black Tibetan Sheep and OL is Oula Sheep.

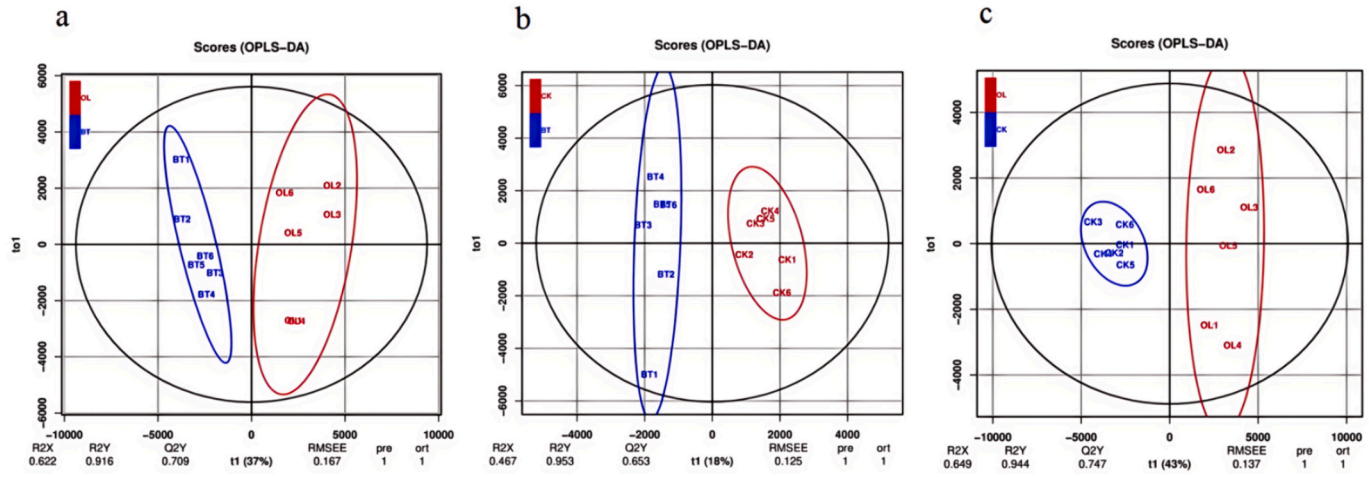

Fig. 5. Scatter plots of the OPLS-DA model based on all metabolites in the *longissimus dorsi* muscle between the BT and OL groups (a), CK and BT groups (b), and CK and OL groups (c).
Note: In the figure above, OPLS-DA predicts sample grouping by modeling the relationship between metabolite expression and grouping to provide information about differences between groups.

dominant phylum level bacterial communities. Ma et al. also showed that they are the most dominant bacterial communities in black and white Tibetan sheep (Ma et al., 2023). The relationship between the deposition of intramuscular fat and the proportion of Firmicutes: Bacteroidetes ratio in livestock has been the subject of much research. Guo et al. studied the gut microbiota of feedlot swine and found that fat deposition was correlated with changing relative Firmicutes abundance and Firmicutes/Bacteroidetes ratio in the gut (X. Guo et al., 2008). Wang et al. hypothesized that rumen microbes may have an indirect effect on metabolite deposition by interacting with the host (Wang, Luo, et al., 2021). Xiang et al. found a significant association between the rumen microbiota and the nutrients in the meat, especially free fatty acids and amino acids (Xiang et al., 2022). Our findings were consistent with this point. We found that colonies like *methanobrevibacter*, *unidentified_Bacteroidales*, *unidentified_Christensenellaceae*, and *Alloprevotella* were primarily associated with metabolites, such as 2-phospho-D-

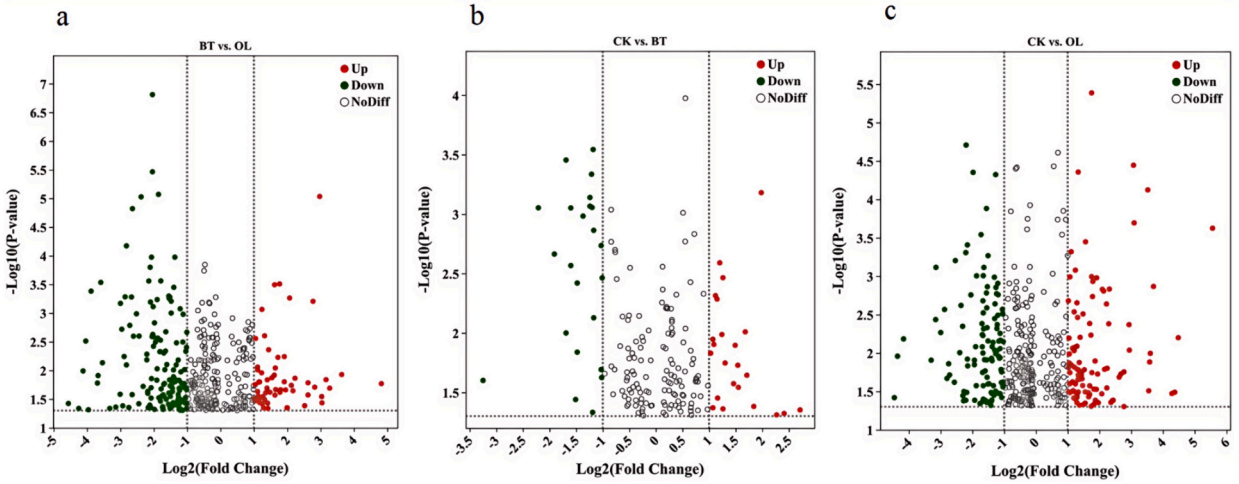

Fig. 6. The volcano plot of the comparison between the BT and OL groups (a), CK and BT groups (b), and CK and OL groups (c).
Note: The volcano plot above reflects the overall differences in metabolites between the comparison groups, and the up- and downregulated top 10 metabolites in BT vs. OL, CK vs. BT, and CK vs. OL are shown in Supplementary Table S4.

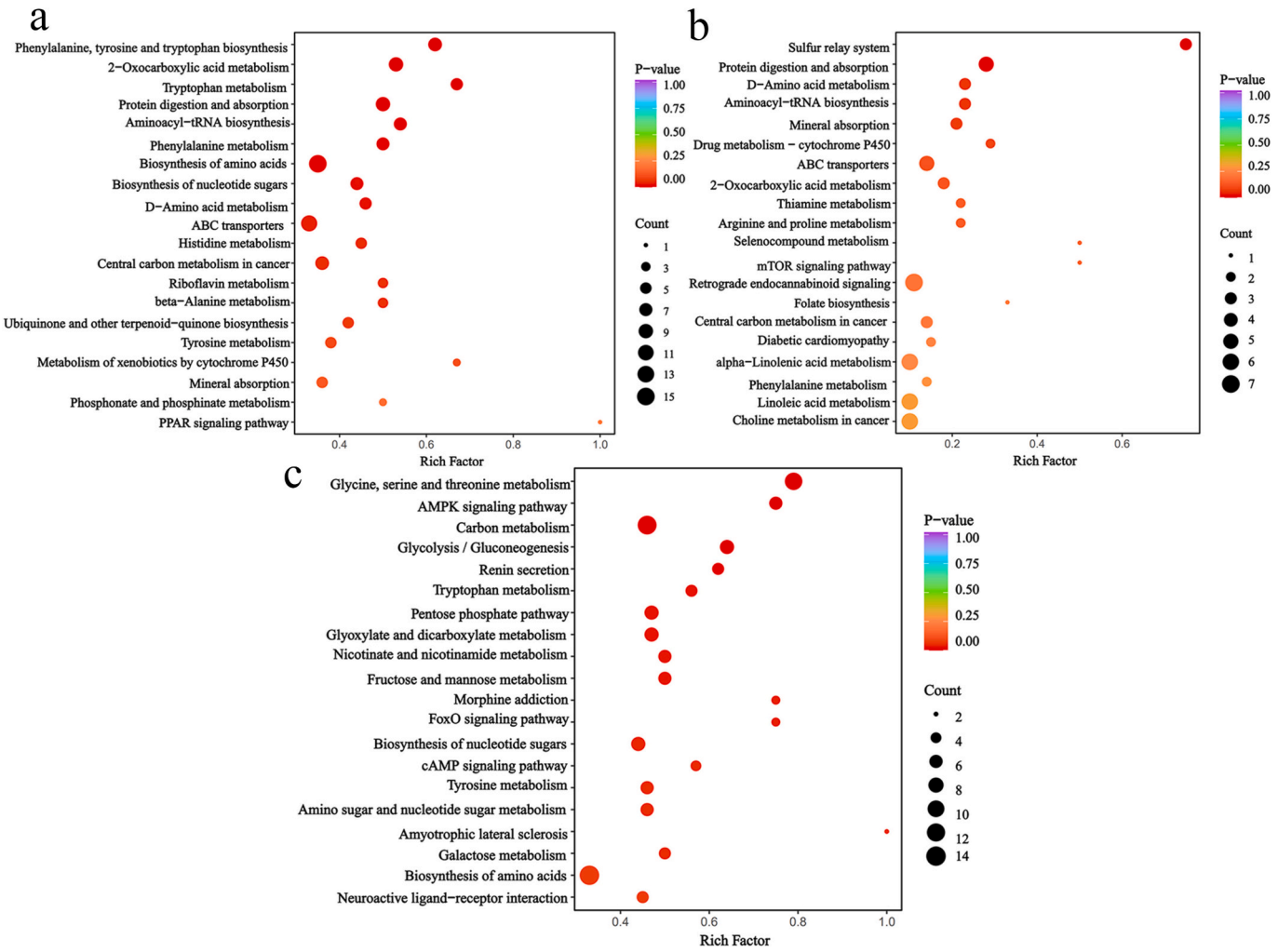

Fig. 7. Top 20 enriched KRGG pathways of the comparison between BT and OL groups (a), between CK and BT groups (b), and between CK and OL groups (c) in the *longissimus dorsi* muscle.

glyceric acid, L-tryptophan, S-adenosylmethionine and phenylpyruvic acid, and L-tyrosine. *Alloprevotella* has been shown to be involved in the production of short chain fatty acids (SCFAs), which bind to ligands for the free fatty acid receptor 2 (FFAR-2) and free fatty acid receptor 3 (FFAR-3), playing a regulatory role in glucose metabolism (Zhang et al., 2024). Therefore, we hypothesized that *Alloprevotella* may further

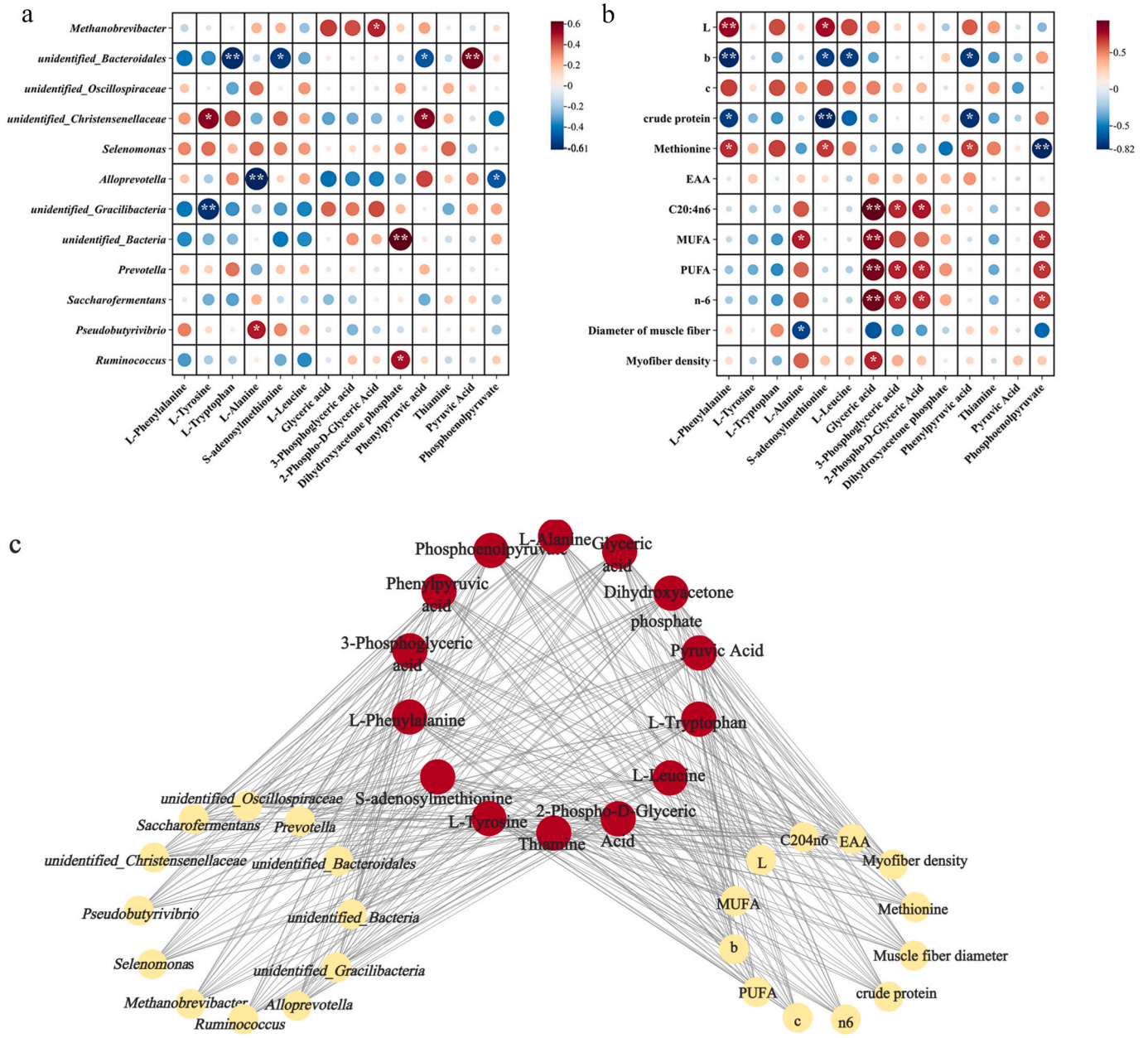

Fig. 8. Pearson correlation analysis between rumen microbes and muscle metabolites (a). Pearson correlation analysis between muscle metabolites and meat quality (b). Network diagram for correlation analysis between rumen microorganisms, muscle metabolites and meat quality (c).

influence sheep quality by regulating metabolites related to glucose metabolism. *Unclassified_Oscillospiraceae* has been identified as a characteristic microbiota of the ruminant (Kartzinel, Hsing, Musili, Brown, & Pringle, 2019), but no correlation with metabolites of amino acids, fatty acids were found in our study. *Ruminococcaceae* has been shown to be positively associated with L-lysine, EPA, and oleic acid and primarily involved in amino acid and fatty acid metabolism (H. Li et al., 2020). There was a positive correlation between *Ruminococcus* and dihydroxyacetone phosphate in our study. *Ruminococcus* is associated with carbohydrate metabolites (fructose, isomaltose, D-lactose, and maltose) that have some effect on muscle metabolism, as shown by Zhang et al. (Zhang et al., 2022b). *Ruminococcus* is a host-beneficial catabolic bacterium, and studies have shown that it is more prevalent in obese patients with higher saturated fat and simple sugar intakes (Sun et al., 2021). Therefore, we hypothesized that *Ruminococcus* may have additional effects on meat quality through the regulation of metabolites involved in sugar metabolism and lipid metabolism. *Unidentified_Christensenellaceae* was positively associated with glyoxylate and dicarboxylate metabolism (Gao et al., 2023). *Unidentified_Christensenellaceae* was significantly and positively correlated with L-Tyrosine and Phenylpyruvic acid in our study. However, further studies are expected to confirm the mechanism by which rumen microbiota affect mutton.

Using untargeted metabolomics, we found that the different metabolites in the BT and OL groups were primarily enriched in the pathways of Phenylalanine, tyrosine, and tryptophan biosynthesis, 2-Oxocarboxylic acid metabolism, Tryptophan metabolism, and Aminoacyl-tRNA biosynthesis. L-Phenylalanine, L-Tyrosine, L-Tryptophan, 4-Hydroxyphenylpyruvic acid, and Phenylpyruvic acid were major metabolites enriched in these pathways. Amino acids are the basic building blocks of proteins and are important for the flavor of meat. They are a source of energy and can synthesize other building blocks in muscle (Watkins, Frank, Singh, Young, & Warner, 2013). In our study, *L*-phenylalanine correlated positively with L-value and methionine and negatively with

b-value and crude protein. The downregulated expression of *L*-phenylalanine in BT resulted in significantly lower L-value and methionine content in the BT group than in the OL group, while the b-value and crude protein content were higher in BT than in OL. Guo et al. have shown that L-tryptophan is enriched in the tryptophan pathway, which can synthesize melatonin and is critical for regulating body fat content and energy metabolism (Tongqing Guo et al., 2024). Phenylpyruvic acid correlated negatively with b-value and crude protein and positively with methionine. In BT, the expression of phenylpyruvic acid was downregulated. This resulted in higher b-value and crude protein content in BT than in OL, whereas the methionine content was lower in BT than in OL. The metabolites in CK and BT were primarily enriched in Sulfur relay system, Protein digestion and absorption, Aminoacyl-tRNA biosynthesis, D-Amino acid metabolism and Mineral absorption. The key metabolites enriched in these pathways were: L-Alanine, L-Phenylalanine, L-Leucine, Thiamine, and S-adenosylmethionine. Amino acids are the major metabolites that influence meat quality and are crucial for flavor, such as phenylalanine, which gives a bitter taste, and alanine, which gives a sweet taste (Xiao, Ge, Zhou, Zhang, & Liao, 2019). Wang et al. have shown that an increase in the levels of these amino acids is essential in high quality mutton (Wang & Zhao, 2023). Leucine may increase muscle texture and nutrition by binding to myogenic control genes through the AKT/TOR-FoxO3a signaling pathway (Yang, Li, Rahman, & Leng, 2023). In our study, L-Alanine was positively correlated with MUFA and negatively correlated with muscle fiber diameter, and L-Leucine had a significant negative correlation with meat color value b. Both L-alanine and L-leucine were upregulated in the CK group. As a result, MUFA content was higher in CK than in BT, while muscle fiber diameter and b-value were lower in CK. Glycine, serine and threonine metabolism, AMPK signaling, carbon metabolism, glycolysis/gluconeogenesis and renin secretion were the main metabolites enriched in the CK and OL groups. 3-Phosphoglyceric acid, L-Serine, Glyceric acid, Pyruvic Acid, Phosphoenolpyruvate, Dihydroxyacetone phosphate, and 2-Phospho-D-Glyceric acid were the main enriched metabolites. Pyruvic acid, a central biochemical node in glycolysis, is a cellular metabolic product that is an essential meat biomarker and strongly correlates with drip and cooking loss (Sieczkowska et al., 2010). In our study, pyruvate was involved in sugar and some amino acid metabolism, but correlation analysis showed no significant correlation with amino acids, fatty acid content, meat color, and other meat qualities. We hypothesize that pyruvate may be an important intermediate metabolite in the metabolism pathway. Our results showed that Glyceric acid, 3-phosphoglyceric acid and 2-phospho-D-glyceric acid were positively correlated with C20:4n6, PUFA, n-6. Glyceryl, 3-phosphoric, and 2-phospho-D-glyceryl acids were all upregulated in the CK group, resulting in significantly higher levels of C20:4n-6, PUFA, and n-6 in CK than in OL. Phosphoenolpyruvate was positively correlated with MUFA, PUFA, n-6 and negatively correlated with methionine. The expression of phosphoenolpyruvate was upregulated in the CK group, resulting in significantly higher levels of MUFA, PUFA, and n-6 in CK than in OL, and lower levels of methionine than in OL.

The bacteria in the rumen have been identified as playing a key regulatory function in the nutrition and health of ruminants (Zeineldin et al., 2018). The microbiome of the rumen is correlated with the amino acid and fatty acid content of meat (Nagaraja, 2016). Based on correlation analysis, we hypothesize that microbes may further control meat quality by influencing metabolites. According to Zhang et al., an increase in protein digestibility and absorption is a promising indicator of increased tenderness and water retention, as well as protein and fat deposition (Zhang et al., 2022b). Studies have shown that the distribution and levels of protein and fat in meat are closely related to the number and strength of muscle fibers and connective tissue (Font I Furnols et al., 2009). Our hypothesis is consistented with this view. Our study suggests that *Pseudobutyrivibrio* and *Alloprevotella* regulate L-alanine, which further regulates muscle fiber properties and fatty acid content through D-amino acid metabolism, aminoacyl-tRNA biosynthesis, and protein digestion/absorption pathways. Kong et al. argued that amino acids influenced the carcass and meat quality by participating in the tricarboxylic acid cycle, and that reducing the process of muscle glycolysis/gluconeogenesis and enhancing the tricarboxylic acid cycle and pentose phosphate pathway can enhance meat quality (Kong et al., 2023). Previous reports indicated that increased carbohydrate metabolism may provide the host with more energy and substrate to synthesize muscle fat (Smith et al., 2018). We found that *Alloprevotella* was negatively correlated with phosphoenolpyruvate, which may affect muscle fatty acid content through metabolic pathways such as carbon metabolism and glycolysis/gluconeogenesis. *Methanobrevibacter* was positively correlated with 2-phospho-D-glyceric acid, which may regulate muscle fatty acid content through the glyoxylate and dicarboxylate metabolism and pentose phosphate pathway. Furthermore, we found that *unidentified_Christensenellaceae* and *unidentified_Bacteroidales* are associated with phenylpyruvic acid and further regulate muscle amino acid content and meat color via phenylalanine metabolism and amino acid biosynthesis. The *unidentified_Bacteroidales* also showed a negative correlation with S-adenosylmethionine, which may influence the meat color and muscle amino acids via sulfur relay, arginine, and proline metabolism (Fig. 9).

5. Conclusions

Meat quality varies among different sheep breeds, and in general, the meat quality of CK sheep was superior to that of BT and OL sheep in terms of meat color (higher L and c values, lower b value), muscle fiber characteristics (area, number and density of myofibers, elasticity, tenderness, cohesion, and adherence), and nutritional quality (SFA, PUFA, MUFA, and n-6 fatty acids). Rumen microorganisms and muscle metabolites influenced meat quality through certain correlations. Amino acid metabolism and protein digestion and absorption mainly regulate myofiber properties, while carbon metabolism, glycolysis/gluconeogenesis, and the pentose phosphate pathway are mainly related to fatty acid synthesis. The regulation of amino acid and protein synthesis and flesh color in muscle involved the phenylalanine, arginine and proline pathways and the sulfur relay system. Our results provide strong scientific data to support and establish Chaka sheep as a protected product under the Geographical Indication of Agricultural Products in China. It also provides reference and guidance for future sheep breeding strategies aimed at enhancing the quality of sheepmeat and developing the livestock industry. In future practical work, the protection of the good breed of Chaka sheep should be strengthened. In addition, the performance level of other inferior sheep breeds can be improved through selective breeding and purification or crossbreeding improvement.

Institutional review board statement

The animal procedures used in this study were performed according to the Guidelines for the Care and Use of Laboratory Animals in China. This study was approved by the Experimental Animal Use Ethics Committee of the Northwest Institute of Plateau Biology, CAS (Permit No. NWIPB20160302).

CRediT authorship contribution statement

Xianli Xu: Writing – review & editing, Supervision, Software, Conceptualization. **Hongjin Liu:** Validation, Supervision, Methodology, Formal analysis. **Tongqing Guo:** Software, Resources, Methodology, Conceptualization. **Qian Zhang:** Validation, Resources, Methodology, Investigation. **Xungang Wang:** Validation, Supervision, Software, Resources. **Yalin Wang:** Methodology, Investigation, Formal analysis, Data curation. **Lin Wei:** Supervision, Software, Project administration, Formal analysis. **Yuna Jia:** Software, Methodology, Investigation, Conceptualization. **Linyong Hu:** Project administration, Investigation, Formal analysis, Conceptualization. **Shixiao Xu:** Writing – review &

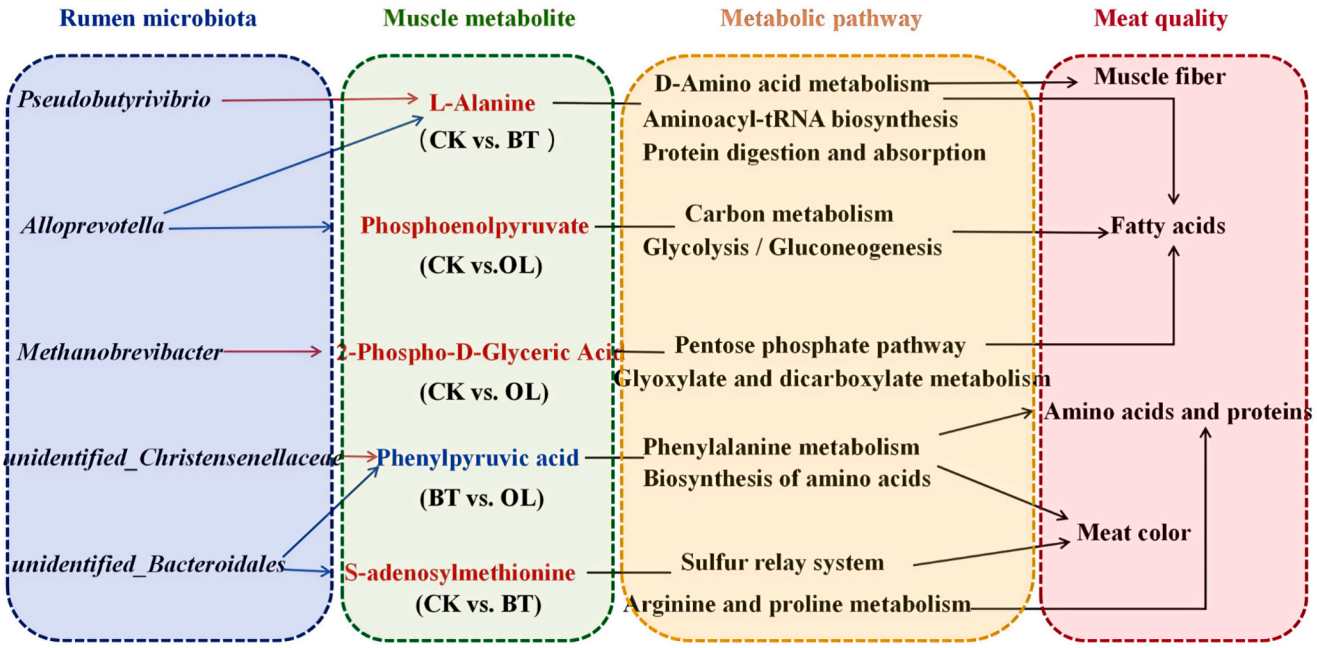

Fig. 9. Possible roles and hypothesized pathways of rumen microbiota and muscle metabolism in regulating meat quality.
Note:Red arrows in the figure indicate positive correlations, blue arrows indicate negative correlations, and red font indicates up-regulated metabolites and blue font indicates down-regulated metabolites. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

editing, Resources, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declared that they have no conflicts of interest with this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Data availability

The data that has been used is confidential.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101731.

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