Food Science of Animal Products

Research Article

Effects of *Limosilactobacillus reuteri* on gastrointestinal microbiota, muscle fiber formation and meat quality of Sunit sheep

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Abstract: *Limosilactobacillus reuteri* is a species of lactobacillus present in the gastrointestinal tract of animals and may contribute to the balance of intestinal flora. This study aimed to investigate the effects and correlations of *L. reuteri* on both intestinal flora and muscle fiber characteristics of Sunit sheep, with the objective of enhancing meat quality. Twelve 8-month-old Sunit sheep were randomly divided into two groups: control (fed without *L. reuteri*) and *L. reuteri* group. The results showed that *L. reuteri* had a significant positive impact on meat quality, particularly improving the tenderness of sheep meat compared with the control group, with values of 69.8 and 76.8 N, respectively ($P < 0.05$). *L. reuteri* group showed significantly more type I muscle fibers (23% vs. 13%) and higher succinate dehydrogenase activity than the control group, while lactate dehydrogenase activity was significantly lower ($P < 0.05$). The *L. reuteri* group showed significantly higher expression of genes encoding colonic epithelial nutrient transport (*GPR43*) and immune barrier function (*Zonulin*) than the control group. At the genus level, *Alistipes* and *Methanobacterium* were significantly different between *L. reuteri* and control groups ($P < 0.05$), with *L. reuteri* group showing an increase of 2.9 times and a decrease of 8.0 times compared to the control group, respectively. At the species level, *Succiniclasticum ruminis* and *Bacteroides* sp. 43_108 were significantly ($P < 0.05$) lower and higher, respectively in *L. reuteri* group than those in the control group. Butyric acid exhibited the highest concentration in *L. reuteri* group, reaching 7.52 mmol/L. In conclusion, supplementation with *L. reuteri* may affect muscle fiber characteristics, enhance intestinal health, and facilitate carbohydrate digestion of Sunit sheep. This study offers novel insights into the use of feed additives for improving meat quality.

Keywords: *Limosilactobacillus reuteri*; gut microbiota; muscle fiber types; meat quality; Sunit sheep

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1 Introduction

Sheep meat is an excellent source of high-quality protein. In recent years, consumer demand and expectations for the quality of sheep meat have increased due to the improved economic situation and quality of life. As a result of national policy and market demand, the feeding practices for sheep have transitioned from traditional natural grazing to intensive-housed or semi-housed systems. This transition has led to varying degrees of decline in both nutritional value and quality of meat[1–2]. Therefore, improving the meat quality of indoor-housed sheep through grain feeding has become a major research goal. *Limosilactobacillus reuteri*, present in the intestinal tract of vertebrates and mammals, plays an essential role in maintaining intestinal microflora balance, improving feed efficiency, and promoting animal growth[3–4]. Tian et al.[5] demonstrated that *Lactobacillus reuteri* can enhance pork quality by reducing muscle water loss and modifying muscle fiber characteristics while also improving flavor through increased free amino acids and inosinic acid content.

Muscle, constituting 45%–60% of the body weight in adult animals, is crucial for sustaining motor and metabolic functions, while serving as a principal source of edible meat[6]. The quantity and quality of muscle fibers, which are the smallest components of muscle, directly influence meat production capacity and quality, ultimately determining the animal's economic value. A significant correlation was found between muscle fiber characteristics (number, diameter, and cross-sectional area) and meat quality (pH, shear force, water retention, protein content, and intramuscular fat content)[7]. Muscle fibers can be classified into slow oxidative (type I), fast oxidative (type IIA), fast glycolytic (type IIB), and intermediate (type IIx) based on the type of metabolism and contractile characteristics. And the diversity of these types is a critical factor that impacts meat quality in meat-producing from animals. Oxidized muscle fibers contain high levels of myoglobin, mitochondria, cytochromes, and glycogen, which rely on aerobic metabolism for energy production. In contrast, enzyme-type muscle fibers predominantly depend on glycolysis as their main energy source. The correlation between meat quality and the proportion of oxidized muscle fibers (types I and IIa) in skeletal muscle tissue suggests a positive association, while the presence of glycolytic muscle fibers (type IIB), which are negatively correlated with meat quality, indicates a potential link to their mode of energy supply[8]. The composition of mammalian skeletal muscle is established prenatally. However, the distribution of muscle fiber

types can be altered in response to changes in external conditions: I↔IIa↔IIx↔IIb[9]. The change in dietary nutrition has been confirmed to regulate muscle fiber type transformation, thereby improving meat quality in livestock and poultry. The change in dietary nutrition has been confirmed to regulate muscle fiber type transformation, thereby improving meat quality in livestock and poultry. Wen et al.[10] found that lycopene could increase the antioxidant capacity of fattening pigs, promote the transformation of muscle fibers from fast to slow, improve redness value (a^*), intramuscular fat and crude protein content, thus increasing the nutritional value of meat. The addition of flaxseed was also found to result in a reduction in the cross-sectional area and diameter of sheep muscle fibers, as well as an enhancement in the formation of type I muscle fibers. Moreover, it contributed to an improvement in the tenderness, color, flavor, and other characteristics of lamb meat[11]. Currently, the "gut-muscle axis" theory provides us with a potential approach to modulate the transformation of muscle fiber type. In germ-free mouse muscle, decreased expression of succinate dehydrogenase (SDH), mitochondrial SDH enzyme activity and dysregulation of muscle energy metabolism were observed[12]. Liu et al.[13] discovered that supplementation with probiotics induced modifications in the activity of oxidation-related myofibril metabolic enzymes, leading to a significant enhancement in meat tenderness.

The gut microbiota metabolizes complex dietary carbohydrates into energy sources for the host, and the hindgut plays a crucial role in influencing meat quality by providing energy. Compared to traditional high-throughput sequencing methods (e.g., 16S ribosomal RNA sequencing), metagenomic analysis offers a more comprehensive understanding of the composition and interactions of gut microorganisms, as well as their molecular-level metabolic pathways and gene functions. Hess et al.[14] integrated host and rumen metagenomic analyses to predict sheep health traits. Guo et al.[15] employed metagenomics to identify *Prevotella ruminosus* as the dominant bacteria in sheep after feeding them with lignocellulolytic bacteria-treated whole-plant corn silage, revealing an enrichment of functional attributes associated with glycolysis/gluconeogenesis and the citric acid cycle. Similarly, Xie et al.[16] using metagenomic sequencing, demonstrated that feeding sheep diets high in grain content altered the abundance of acetate-producing bacteria (e.g., *Akkermansia* spp.) and butyrate-producing bacteria (e.g., *Roseburia* spp.) in their cecum, resulting in damage to the cecal mucosa.

As previously mentioned, *L. reuteri* plays a pivotal role in balancing the intestinal microflora, and prior research has showed its beneficial effects on slaughter and growth performance as well as meat quality in pigs and broilers[17–18]. However, the underlying biological mechanism remains unexplored. The present study hypothesizes that *L. ruteri* improves the meat quality of Sunit sheep by influencing gut microbial communities. Therefore, the study aimed to investigate the impact of *L. reuteri* on Sunit sheep muscle fiber, meat quality and gut microbial communities, thus clarifying the potential mechanisms underlying muscle fiber formation and meat quality improvement. This study will provide a theoretical foundation for dietary regulation to improve sheep meat quality under semi-intensive breeding conditions.

2 Materials and methods

2.1 Animals and experimental design

This study was approved by the Ethics Committee of Inner Mongolia Agricultural University (NND2021072). Animal experiments followed the guidelines for animal experiments of *Welfare criteria for animals to be slaughtered* (GB/T 42304–2023), *Laboratory animal-quality control of reproductive and developmental health* (GB/T 39647–2020) and were approved by Inner Mongolia Agricultural University Ethics Committee (NND2021072). Twelve purebred and healthy 8-month-old Sunit rams from one farm (Rongyu Modern Ecological Animal Husbandry Co., Ltd., Xilingol League, China) were considered. Experimental animals were randomly divided into 2 dietary groups, C group (n = 6, fed the basal diet) and L group (n = 6, fed the basal diet + 4 g/day of *L. reuteri*, viable count of 5 × 10^9 CFU/g per animal). The pre-experimental period lasted 7 days and the experimental period 150 days. Animals grazed together on a sonid right banner pasture, during the evening the experimental diets were individually offered basal diet 3% of body weight. The animals consumed all the feed they received. The composition of the basal diet included corn (55%, *m*/*m*), wheat bran (17%, *m*/*m*), soybean meal (8%, *m*/*m*), rapeseed meal (8%, *m*/*m*), cotton meal (8%, *m*/*m*), and premix (4%, *m*/*m*). At the end of the experimental period and after 12 h of fasting, all rams were slaughtered at an abattoir near the farm.

2.2 Sample collection

Longissimus thoracis muscle samples (12^{th}–13^{th} rib) weighing 3 g, colonic epithelium samples weighing 3 g, and feces samples weighing 1 g were collected from each Sunit sheep within 45 min of slaughter and placed in sterile enzyme-free frozen tubes with a volume of 2 mL. The samples were immediately submerged in liquid nitrogen for gene expression analysis, detection of short-chain fatty acids (SCFAs) levels, metagenomics and enzyme activity. *Longissimus thoracis* muscle tissue weighing at least 50 g was collected. The first portion was stored in polyethylene zip-lock bags at 4 °C for 24 h for meat quality analysis. The second portion was cut into 6 pieces per animal (0.5 cm × 0.5 cm × 1 cm) along the direction of muscle fibers. The muscle pieces were then dehydrated and dried in cooled isopentane. After bleaching, they were frozen in liquid nitrogen for about 10 s and stored in a refrigerator at −80 °C for determination of muscle fiber characteristics.

2.3 Meat quality measurement

The pH of *longissimus thoracis* muscle was measured after 45 min ($pH_{45\ min}$) and 24 h ($pH_{24\ h}$) using a carcass pH direct meter (Matthäus GmbH & Co., Eckelsheim, Germany), with each sample replicated three times. Meat color parameters, brightness value (L^*), a^*, and yellowness values (b^*) were assessed after 1 h muscle surface bloom using a chromatograph (TC-P2A, Beijing Aoike Optoelectronic Instrument Co., Ltd., Beijing, China) under white ceramic tiles, with a 2° observer angle and D65 light source (three measurements for each sample). Cooking loss and shear force were determined based on the method of Dou et al.[19]. The samples were sealed in plastic ziplock bags and heated in a water bath at 80 °C until the core temperature reached 70 °C, then cooled to room temperature. Only one cooking batch was used. The weights before cooking (m_1/g) and after cooking (m_2/g) were recorded separately to calculate cooking loss using the following formula:

$$\text{Cooking loss}\ (\%) = \frac{m_1 - m_2}{m_1} \times 100$$

The cooked samples were sliced into rectangles of 3 cm × 1 cm × 1 cm aligned with the muscle fibers and tested using a muscle tenderness meter (C-LM3B, Northeast Agricultural University,

Harbin, China) equipped with a 500 N load cell, with a minimum of eight repetitions per sample.

2.4 Determination of the histological proportion of muscle fiber

Samples were cut perpendicular to the direction of the muscle fibers in a cryostat microtome (MEV, SLEE, Mainz, Germany) at −30 °C, and adenosine triphosphatase (mATPase) staining was performed according to the method of Brooke et al.[20]. Images with muscle fibers greater than 1 000 were observed with a fluorescence inverted microscope (DM4000B, Leica, Wetzlar, Germany). Muscle fiber characteristics, such as proportion ratio, diameter and cross-sectional area of type I, IIA, and IIB, were analyzed using the software Laica QWin V3 Processing Analysis Software (Leica, Wetzlar, Germany).

2.5 Determination of muscle fiber-related enzyme activities

Longissimus thoracis muscle, 0.5 g, were homogenized with 4.5 mL of 0.86% saline at −80 °C. The homogenate was centrifuged at 4 000 r/min for 10 min at 4 °C. According to the method of Luo et al.[21], the resulting supernatant was analyzed with kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) using enzyme-linked immunosorbent assay to measure total protein (TP), total antioxidant capacity (T-AOC), malate dehydrogenase (MDH), lactate dehydrogenase (LDH), and SDH activities.

2.6 Determination of relative gene expression in colonic epithelium

The mRNA expressions of genes related to intestinal permeability (*Zonulin1*, *Occludins*), intestinal fatty acid absorption and transport (*MCT-1*), and fatty acid receptors (*GPR43*, *GPR120*) were detected by quantitative real-time polymerase chain reaction (qRT-PCR). The sequences of the gene primer are shown in Table 1. The design and synthesis of gene primer sequences were completed by Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China) and *β-actin* was used as the reference gene. Total RNA was isolated from frozen colonic epithelium according to the protocol of the RNAiso Plus kit (Cat# 9108Q; Takara, Dalian, China). The concentration and purity of the total RNA were evaluated with BioDrop μLite (BioDrop, Cambridge, UK). Reverse transcription was conducted using a reverse transcriptase kit (Cat# RR047A; Takara, Dalian, China). Each sample yielded 500 ng of total RNA. Gene expression levels were assessed by qRT-PCR using SYBR Premix Ex *Taq* (Cat# RR820A; Takara, Dalian, China), and relative expression was determined by the $2^{-\Delta\Delta Ct}$ method[22]. Primer efficiencies were verified using the curve obtained from the amplification of serial dilutions of the pooled cDNA.

2.7 Metagenomics

The QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used for DNA extraction. The integrity of the entire DNA sample was assessed using a 1 g/100 mL agarose gel, and then fragmented to a size of 300 bp with the assistance of Covaris M220 (Gene Co., Ltd., Shanghai, China). TruSeq™ DNA Sample Prep Kit (Illumina Inc., San Diego, CA, USA) was used for library preparation. Paired-end sequencing was conducted on the Illumina HiSeq 4000 platform using the HiSeq 4000 PE Cluster Kit (Illumina Inc., San Diego, CA, USA).

Readfq (v8, https://github.com/cjf!elds/readfq) was used to preprocess the raw data obtained from the Illumina HiSeq platform to obtain clean data for subsequent analysis. MEGAHIT software (v1.0.4-beta) was used for assembly and analysis. Alignment of the clean data from each sample to the initial gene catalogue was conducted using Bowtie2 (v2.2.4), and the number of reads on gene alignments in each sample was calculated. Subsequently, basic information statistics, core-pan gene analysis, correlation analysis between samples, and Venn diagram analysis of gene numbers were performed based on the gene abundance information in the gene catalogue for each sample. DIAMOND software (v0.9.9.110, https://github.com/bbuchfink/diamond/) was used to compare unigenes with the NR database from NCBI (https://www.ncbi.nlm.nih.gov/)[23]. The Lowest Common Ancestor (LCA) algorithm was applied to the MEGAN software system classification for annotation information to determine gene sequences based on LCA annotation results and gene abundance tables at each taxonomic level[24]. Dimensionality reduction analyses, including principal coordinates analysis (PCoA) were performed using R software (v2.15.3, ade4 package) based on the relative abundance profiles derived from abundance tables at each taxonomic level[25]. Anosim analysis (R software, v2.15.3, vegan package) was used to test for differences between experimental groups, followed by Metastats and Linear discriminant analysis (LDA) Effect Size (LEfSe) analyses aimed at identifying different species between groups through permutation tests. Functional data comparison was carried out using DIAMOND software with function databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.kegg.jp/kegg/) and Carbohydrate-active enzymes (CAZy) database (http://www.cazy.org/). The raw sequencing reads

Table 1 Primers of qRT-PCR.

Gene	Primer sequence (5′–3′)	Size (bp)	Accession	Primer melting temperature (°C)
β-actin	F: CAGAGCAAGCGTGGCATCCTAAC R: GATCTTCTCCATGTCGTCCCAGTTG	162	XM_004013078.5	58
Occludins	F: ACTCCGCCAATGGTAAAG R: TCCCGTCGTGTAGTCTGTT	117	XM_015101255.4	54
Zonulin1	F: ATCCACTCTGCTGACGCCTC R: GCTCGTGGGATCGACCTGAA	150	XM_060401409.1	55
GPR120	F: CCGACCTGCTCTTCATCAGT R: GCGATCCCTCTGGTGCTGGC	145	XM_012102571.4	60
GPR43 (*FFAR2*)	F: TGCTCTGATCGCCTGGGTC R: GCTCTTGGCTGAAGTTCTCGTA	128	XM_012190178.4	45
MCT1 (*SLC16A1*)	F: ACATAAGCCTATTCAAGCACA R: TCCAACAAGGTCCATCAGT	389	XM_042252642.1	50

were submitted to the NCBI Sequence Read Archive database with the accession number PRJNA1099796.

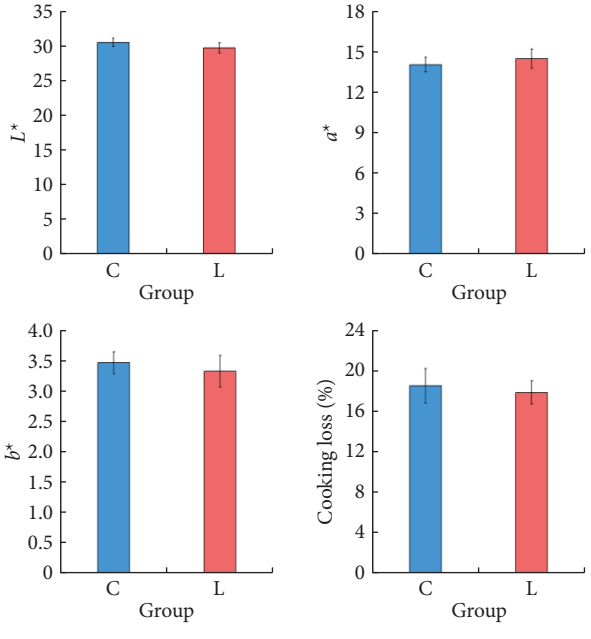
2.8 Statistical analysis

Assessment of meat quality, muscle fiber characteristics, enzyme activity, and gene expression was conducted using one-way ANOVA with dietary group (C and L) considered as a fixed factor in IBM SPSS Statistics v26 (IBM SPSS Inc., Chicago, USA). Tests of homogeneity of variances were performed using the Levene test, and test of normality of data distribution were performed using the Shapiro-Wilks test. Data were represented as means ± standard deviation, and $P < 0.05$ was considered significant difference.

3 Results and discussion

3.1 Meat quality

In Figure 1, a significant increase in the $pH_{24\ h}$ and a decrease in shear force were observed in the *L. reuteri* group compared to the control group ($P < 0.05$). All recorded pH values at 45 min and 24 h were above and below 6.0 respectively, indicating that these pH levels can be considered normal for sheep muscle[26]. Nie et al.[27] observed that probiotics supplementation affected lamb meat quality and muscle fiber composition. Specifically, it was found to increase the moisture content and intramuscular fat contents in the *longissimus thoracis* muscle, as well as enhance the mRNA expression of *MyHCI*. Additionally, probiotics supplementation led to a decrease in shear force, L^*, and b^*. Probiotic supplements in the diet were also found to increase the eye muscle area, intramuscular fat content and $pH_{24\ h}$ of Sunit sheep, while significantly reducing cooking loss and shear force[28]. These findings are in line with the shear force results of our study. In addition, the $pH_{24\ h}$ of the C group was significantly lower than that of the L group ($P < 0.05$), probably due to alterations in glycolysis rate in the lamb's body, resulting in increased lactic acid accumulation in the meat and subsequent decrease in the $pH_{24\ h}$ of *longissimus thoracis* muscle[29].



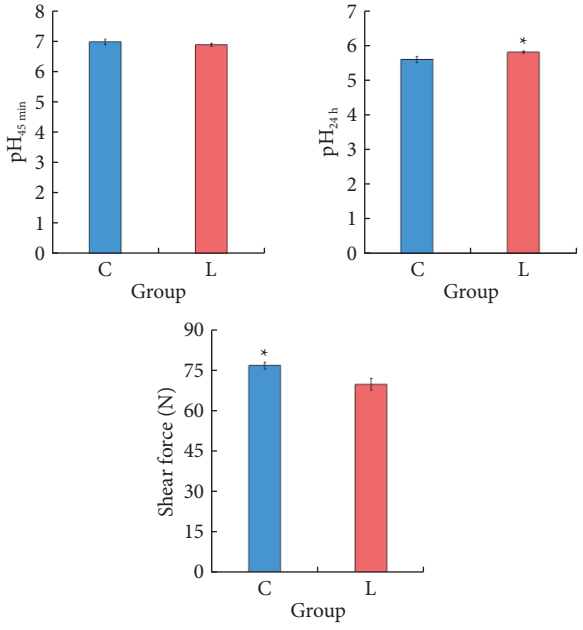
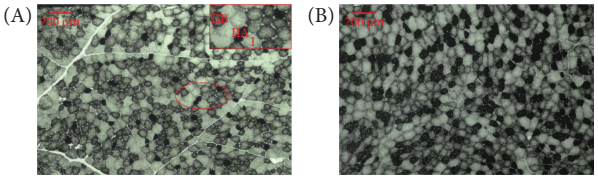


Figure 1 Differences in meat quality between C (animal fed basic diet) and L (animal fed basic diet and *L. reuteri*) group of Sunit sheep. * Represents significant differences ($P < 0.05$).

3.2 Characteristics of muscle fiber

The ultrastructure of muscle fibers was observed by ATPase histochemical staining. It was noted that the muscle fibers were structurally intact, tightly arranged and categorized under a 100× microscope, with no interstitial breakage or enlargement. Muscle fibers can be categorized into three types based on histochemical staining with ATPase: dark type I muscle fibers, light type IIA muscle fibers, and intermediate type IIB muscle fibers. These classifications reflect the varying pH stability ranges of ATPase in different myogenic fiber types. The muscle fiber composition in both the C (Figure 2A) and L groups (Figure 2B) followed a pattern of type IIB > type IIA > type I. Software analysis was used to compute the biological characteristics indices of muscle fibers, as shown in Figure 2. In sheep *longissimus thoracis* muscle, predominantly Type IIB muscle fibers accounted for approximately 50%, with the remaining being oxidized muscle fibers (type I + IIA). The inclusion of *L. reuteri* did not significantly impact the cross-sectional area and diameter of each muscle fiber type (Figures 2C and D). However, as depicted in Figures 2E and F, *L. reuteri* added significantly increased the area and proportion of type I muscle fibers while decreasing type IIB muscle fibers ($P < 0.05$). Additionally, there was a slight increase in muscle fiber density after intake of *L. reuteri*, although it was not statistically significant (Figure 2G).



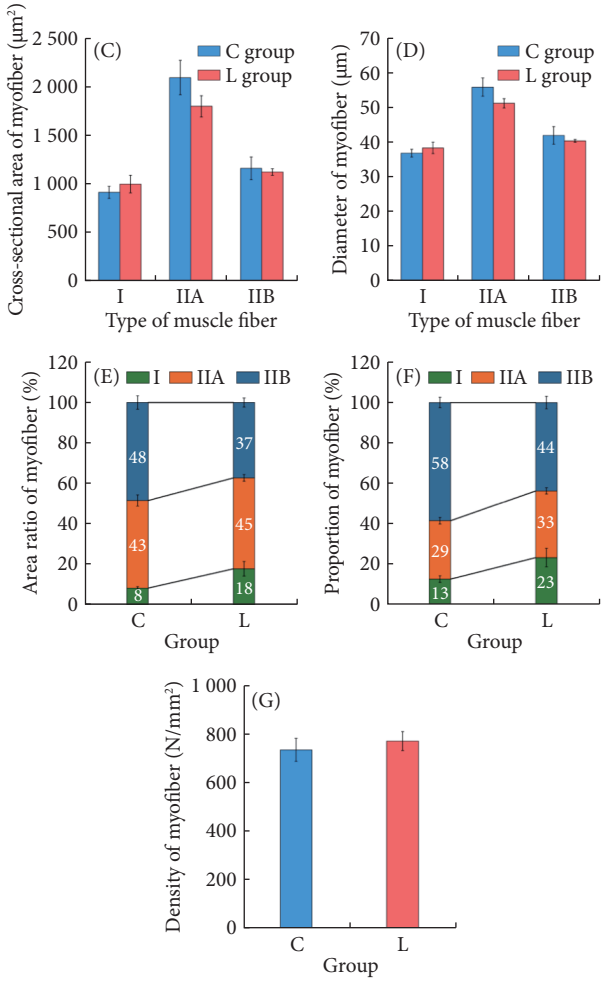


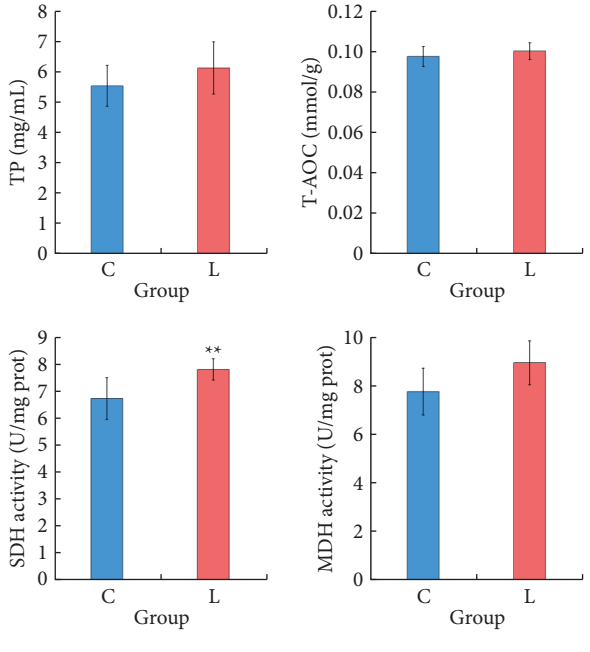
Figure 2 Muscle fiber characteristics of sheep fed basic diet (C group) or fed basic diet and *L. reuteri* (L group). ATPase staining results in *longissimus thoracis* muscle in (A) C and (B) L groups, respectively. (C) The cross-sectional area of muscle fiber; (D) muscle fiber diameter; (E) area ratio of muscle fiber; (F) proportion of muscle fiber; (G) density of muscle fiber. * Represents significant differences ($P < 0.05$).

The histological properties of muscles are crucial in meat quality research. Meat tenderness is directly linked to the diameter and cross-sectional area of muscle fibers. As livestock and poultry grow, an increase in the diameter and cross-sectional area of muscle fibers leads to a decrease in fiber density, an elevation in shear force, and a deterioration in meat tenderness[30]. The present study revealed a slight, albeit statistically non-significant, decreasing trend in the diameter and cross-sectional area of type IIA and IIB muscle fibers in the L group compared to the C group, which may have implications for meat tenderness (Figures 2C and D). Additionally, meat tenderness is associated with the ratio of muscle fiber types. Lyu et al.[31] observed a positive correlation between type II muscle fibers and shear force, noting that the *longissimus thoracis* muscle site with a greater number and area of type IIB muscle fibers in camels exhibited a fast rate of glycolysis, high shear force, and poor tenderness. Kang et al.[32] confirmed a correlation between the proportion of muscle fiber types and tenderness in Bucksia pigs, where a greater proportion of type I muscle fibers led to improved muscular tenderness. Shear force in the L group was significantly lower ($P < 0.05$) than in the C group, probably due to the higher proportion of type I muscle fibers and the lower proportion of type IIB muscle fibers. Furthermore, previous research has indicated that glycolytic-type fibers result in a more rapid decrease in pH value and greater water loss rate, likely due to rapid glycolytic metabolism and reliance on glycogenolysis for energy, resulting in high lactic acid content and rapid decrease in pH[33]. Gil et al.[34] demonstrated that higher *MyHCI* gene expression was associated with higher ultimate muscle pH and slower rate of pH decrease, consistent with our findings.

Regulating the histological properties of muscle to improve meat quality is a key objective for the meat industry. Muscle fiber characteristics are influenced by various factors such as breed, location, age, feeding method, and nutritional intake. *Lactobacillus*, known for their eco-friendly properties, have been used in livestock and poultry diets, with effects on muscle fiber properties potentially dependent on their species and quantity. Feeding pigs with co-fermented feeds containing *Bacillus subtitles* and *Enterococcus faecalis* resulted in increased slow muscle fibers and specific mRNA expressions, potentially linked to improved antioxidant capabilities[35]. A complex diet including *L. casei* and *L. plantarum* reduced the cross-sectional area of some muscles and triggered muscle fiber type conversion[36]. Overall, the consumption of *L. reuteri* in the diet facilitated the transition from glycolytic to oxidative muscle fibers in sheep. The underlying biological mechanism could be related to *L. reuteri*'s influence on the expression of some muscle genes, such as transcriptional peroxisome proliferator-activated receptor α coactivator-1 and myogenic differentiation antigen[5].

3.3 Activity of metabolic enzyme

The results depicted in Figure 3 indicate that TP, T-AOC, and MDH activities showed a slight but statistically insignificant increase in the L group compared with the C group. *L. reuteri* increased SDH ($P < 0.01$) and reduced LDH ($P < 0.001$) activity in Sunit sheep muscles, suggesting a modulating effect of metabolic enzyme activity.



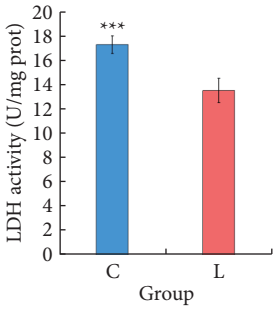


Figure 3 Differences in enzymatic activity between C (animal-fed basic diet) and L (animal-fed basic diet and *L. reuteri*) groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

The energy metabolism of organisms includes processes of aerobic oxidation and anaerobic fermentation. During post-mortem, animals deplete oxygen and undergo mainly anaerobic fermentation. LDH facilitates the reversible conversion of lactate to pyruvate through NAD^+ and nicotinamide adenine dinucleotide (NADH) redox pairs during glycolysis in organisms, reflecting the levels of anaerobic fermentation. LDH is commonly found in glycolytic muscle fibers[37].

In our study, LDH activity was decreased in muscles of the L group ($P < 0.001$), consistent with the decrease in type IIB muscle fiber area and proportion observed in this group ($P < 0.05$). MDH aids fatty acid synthesis, while SDH contributes to the tricarboxylic acid cycle, supplying electrons for eukaryotic cell respiratory chains, both indicating aerobic metabolism levels[38]. The number and area proportion of type I muscle fibers were increased in the L group ($P < 0.05$), and accordingly, SDH activity was strengthened in this group ($P < 0.01$). Interestingly, An et al.[39] discovered a positive correlation between MDH and SDH and the expression of slow muscle fibers.

3.4 Metagenomic analysis

3.4.1 Core gene number and PCoA

In the raw data obtained by the Illumina sequencing platform, there is a certain proportion of low-quality data. To ensure the accuracy and reliability of subsequent analysis results, it is necessary to preprocess the raw sequencing data to obtain clean data for subsequent analysis (Tables S1 and S2). From the abundance table of genes in each sample, the gene number information can be obtained. The distribution of the number of genes in both groups is shown in Figure S1. From the point of view of location, the number of unique genes in feces samples of C and L groups were 518 788 and 520 582, respectively, while the number of coexisting genes was 1 582 767. It can be concluded that *L. reuteri* can change the number of genes in the samples. To explore whether the grouping of the samples was meaningful and to intuitively display the similarities and differences in the evolution of microorganisms in samples under different feeding conditions, UniFrac analysis was used to obtain the distance matrix. By extracting the most important elements and structures from the multidimensional data, the principal coordinates with the highest contribution rate were selected for the multivariate statistical method, PCoA. The circles in the figure represent confidence ellipses with a 95% confidence level, serving to compare the similarity of community structure composition between groups. After eliminating outliers, there were discernible distinctions in the composition of fecal genus-level microbiota between the two groups. The dispersion of samples at the species level was observed to be wider and less clustered compared to the PCoA map at the genus level, indicating a smaller between-group difference than within-group difference (Figure 4). The species-level distribution of microbial samples in the rectal feces of Sunit sheep fed with *L. reuteri* is more diverse compared to the C group, which is more conducive to the regulation of sheep quality and flavor in the later stage. Feeding *L. reuteri* has an impact on the genus level composition of Sunit sheep feces.

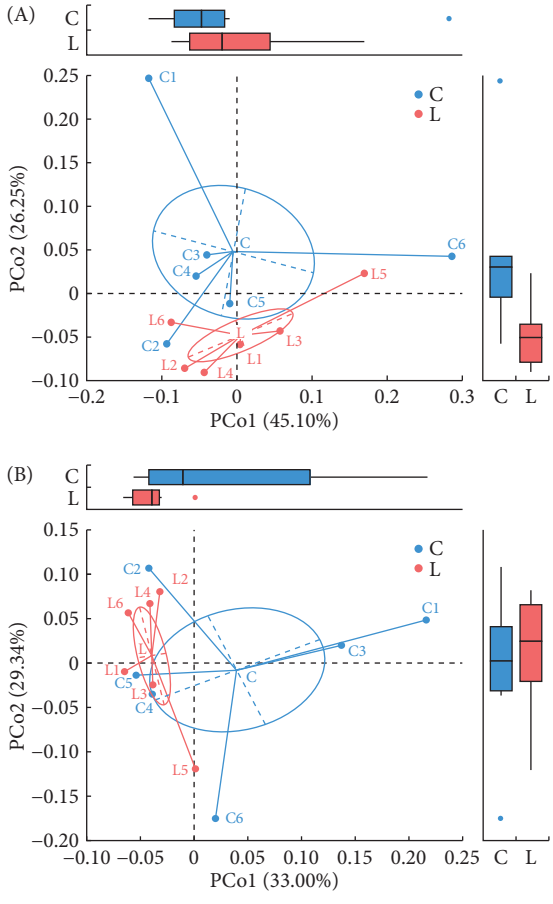


Figure 4 PCoA of feces microbial community structure of sheep fed basic diet (C group) or fed basic diet and *L. reuteri* (L group) at (A) genus and (B) species level. The edge box plot represents the distribution and skewness of the two sample data sets.

3.4.2 Effects of L. reuteri on gut microbiota

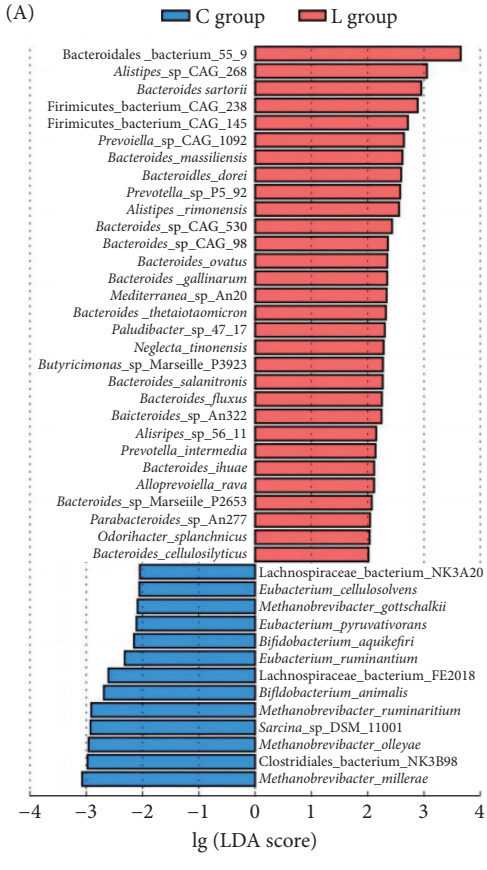
At the genus level (Table 2), there were 10 genus-level microorganisms with ≥ 1% relative abundance in feces. No significant differences were observed among the genus-level microorganisms except for *Alistipes*, *Succiniclasticum*, and *Methanobacterium*. Compared with the C group, the L group had a significantly higher abundance of *Alistipes* ($P < 0.05$). In the experiment conducted by Kong et al.[40], feeding probiotics could alleviate the imbalance of intestinal flora in obese mice with high fat and high glucose, and the abundance of *Alistipes* in the gut also increased. In the study on the effect of hawthorn polysaccharides on the regulation of intestinal microbiota and SCFAs metabolism, a significant enrichment of *Bacteroidobacteria* in the gut of mice was found after supplementation with hawthorn polysaccharides, which can produce acetic acid and propionic acid to inhibit enteritis[41]. In addition, sheep can promote nutrient absorption through the abundance of bacteria, such as *Alistipes*, which affect host metabolism by producing SCFAs[42]. The percentage of

Methanobacterium in the L group was significantly lower than that in the C group ($P < 0.05$). Methanogens are obligate anaerobic prokaryotes primarily found in the large intestine, responsible for methane production. Wichmann et al.[43] found that SCFAs produced by intestinal microbial metabolism can act as signaling molecules to regulate the secretion of glucagon-like peptide 1 (GLP-1) in the intestinal epithelium and promote energy absorption in the intestinal epithelium. Methanogenic bacteria use H_2 to produce methane, which may slow down intestinal motility and reduce the polysaccharides fermentation and SCFAs production. On the other hand, however, the emission of methane (CH_4) by ruminants has a negative impact on the environment by having a greenhouse effect. Jeyanathan et al.[44] employ direct feed microorganisms (DFM) to mitigate CH_4 emissions through competing biochemical pathways against methanogenesis. Therefore, feeding *L. reuteri* in the present study could reduce methane emissions to some extent and help alleviate environmental pressure.

Table 2 Composition of gut microbiota at genus and species levels.

Bacterium	Relative abundance (%)			*P* value
	C group	L group	Standard deviation	
Genus				
Prevotella	7.620	8.821	3.019	0.853
Bacteroides	6.900	7.965	1.610	0.759
Succinimonas	0.009	0.002	0.003	0.199
Roseburia	2.838	1.342	0.970	0.467
Alistipes	2.148	6.215	0.902	0.014
Clostridium	4.736	4.498	0.312	0.722
Selenomonas	0.040	0.036	0.003	0.489
Ruminococcus	1.684	1.860	0.209	0.696
Succiniclasticum	0.031	0.022	0.002	0.014
Bifidobacterium	0.811	0.093	0.274	0.203
Eubacterium	1.181	1.391	0.117	0.394
Lysinibacillus	0.456	0.026	0.222	0.357
Methanobrevibacter	1.229	0.152	0.271	0.040
Oscillibacter	1.358	1.606	0.178	0.514
Fibrobacter	0.129	0.570	0.191	0.269
Lachnoclostridium	1.273	1.086	0.139	0.528
Butyrivibrio	0.716	0.459	0.131	0.355
Succinatimonas	0.342	0.021	0.158	0.333
Niameybacter	0.295	0.015	0.141	0.346
Succinivibrio	0.183	0.013	0.083	0.330
Others	66.190	64.785	2.043	0.749
Species				
Succinimonas amylolytica	0.009	0.002	0.002	0.199
Roseburia sp. 499	1.986	0.219	0.926	0.365
Prevotella ruminicola	0.768	0.923	0.313	0.818
Firmicutes bacterium CAG:110	4.029	3.800	0.467	0.819
Prevotella sp. ne3005	0.084	0.077	0.031	0.909
Succiniclasticum ruminis	0.031	0.022	0.002	0.014
Bacteroides fragilis	0.611	0.121	0.268	0.388
Prevotella sp. MA2016	0.547	0.171	0.255	0.489
Prevotella bryantii	0.218	0.351	0.127	0.626
Selenomonas ruminantium	0.021	0.017	0.001	0.235
Ruminococcus flavefaciens	0.253	0.204	0.056	0.687
Clostridiales bacterium	0.345	0.345	0.050	0.999
Prevotella sp. tc2-28	0.136	0.083	0.055	0.653
Lysinibacillus odysseyi	0.339	0.015	0.167	0.359
Clostridium sp. CAG:413	0.286	0.563	0.162	0.420
Prevotella albensis	0.258	0.358	0.193	0.808
Succinatimonas sp. CAG:777	0.333	0.020	0.154	0.333
Niameybacter massiliensis	0.295	0.015	0.141	0.346
Succinivibrio dextrinosolvens	0.183	0.013	0.083	0.330
Bacteroides sp. 43_108	0.032	0.084	0.012	0.031
Others	89.236	92.607	1.087	0.126

Firmicutes bacterium CAG:110 and *Roseburia* sp. 499 were dominant with abundance ≥ 1% at the species level (Table 2). *Succiniclasticum ruminis* was more enriched in the C group, while *Bacteroides* sp. 43_108 was more enriched in the L group ($P < 0.05$). LEfSe analysis of the rectal microbial species level cluster heat map could be divided into two groups, among which the linear discriminant analysis (LDA) scores of *Bacteroides* species and *Prevotella* species were higher in the L group (Figure 5). In conclusion, *L. reuteri* can improve the level of rectal microbial species, *Bacteroides* and *Prevotella*, by promoting the production of volatile fatty acids. *Bacteroides* metabolizes polysaccharides and oligosaccharides, thereby providing nutrients to the host as well as other intestinal microbial inhabitants[45]. *Prevotella* metabolizes proteins and polysaccharides, producing mainly SCFAs such as propionate[46]. Since propionate serves as a hydrogen reservoir rather than acetate or butyrate, propionate-producing microbes could mitigate methane production. But the action of *L. reuteri* on the gut microbiota is still not clear, it probably acted differently such as producing bacteriocin, lactic acid, and growth substrates, and competing for dietary substrates or receptors on the intestinal mucosa[47].



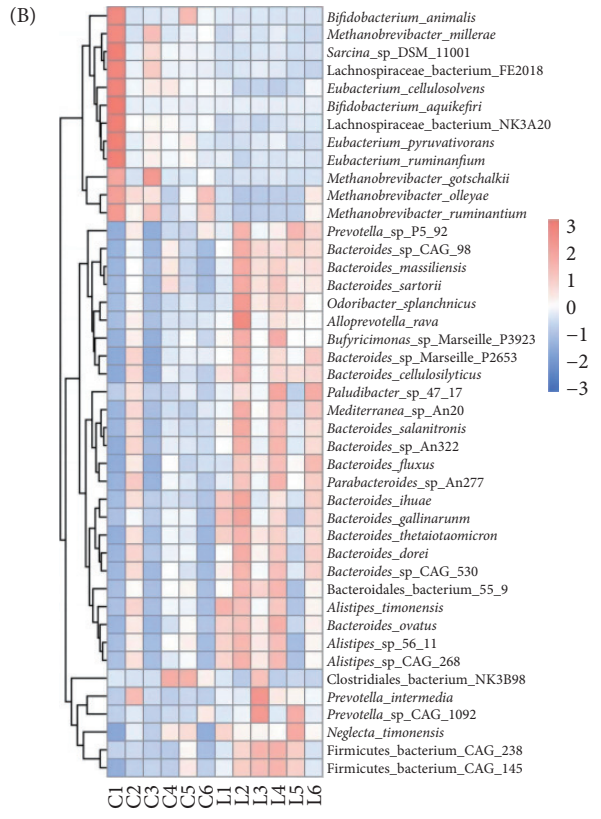


Figure 5 Feces microbial species level cluster heatmap and LEfSe analysis. (A) Gut microbes with significant differences at the species level. (B) Clusters of microorganisms at the species level in the two group samples.

3.4.3 *Effects of L. reuteri on differences in rectal microbial function*

The KEGG Orthology (KO) database serves as the foundation for constructing the pathway and module, with the KO pathway representing KEGG orthologs. According to the comparison with the KO database, the differentially expressed genes in the rectal microbial samples of L and C groups were clustered into two parts in the heat map (Figures 6A and B). In the L group, the main KO pathways were ko00603 (globular sphingolipid biosynthesis pathway), ko00513 (*N*-sugar biosynthesis), ko00604 (glycan biosynthesis and metabolism), and ko04216 (ferroptosis). Conversely, ko00350 (tyrosine metabolism), ko03410 (base excision repair), ko00440 (phosphonic acid and phosphonic acid metabolism), ko04122 (sulfur relay system), and ko00360 (phenylalanine metabolism) were most prominent in C group. Similarly, in the heat map classification of carbohydrate enzymes, a significant difference was found between C and L groups (Figures 6C and D). Carbohydrate enzymes with high abundance in the L group included PL1 (PL1 family polysaccharide lyase), CE8 (carbohydrate esterase family), PL22 (polysaccharide lyase family), and GH139 (glycoside hydrolase family). Carbohydrate enzymes with high abundance in the C group included GH75 (glycoside hydrolase family), CE5 (carbohydrate esterase family), GT81 (glycosyl transferase family), and CBM14 (carbohydrate binding module series). Therefore, in combination with the results of KEGG and CAZy analysis, it was found that the differential microorganisms in the samples after feeding with *L. reuteri* were mainly involved in carbohydrate metabolism and may affect the production of SCFAs. These results confirm the high proportion of *Bacteroides* and *Prevotella* found in the L group. In addition the functional annotation results (KEGG and CAZy analysis) showed that several coenzyme factors in the differential pathways of feces microorganisms were related to lipid and carbohydrate metabolism and energy metabolism, which was affected by the enzymes of metabolism. Therefore, *L. reuteri* regulated the relative abundance of microorganisms, further improving the fermentation function of the Sunit feces.

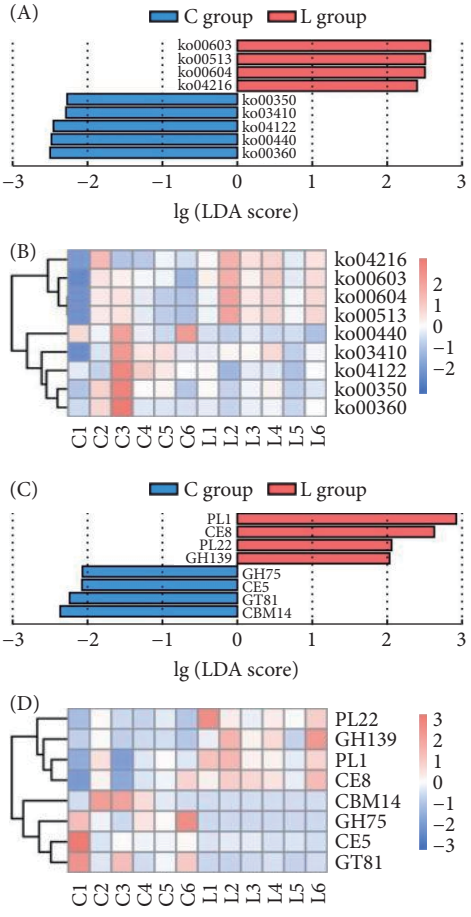


Figure 6 Composition of the microbial community at genus and species level in the feces of Sunit sheep. (A) Differential KO pathways between C and L groups. (B) Partition clustering of KO pathway. (C) Differential expression of carbohydrate-active enzymes between C and L groups. (D) Carbohydrate partition clustering of C and L groups.

3.5 Intestinal microbial metabolites and epithelial gene analysis

SCFAs are primarily metabolites generated via carbohydrate fermentation by microorganisms in the mammalian gastrointestinal tract. These SCFAs are synthesized through the carbon chain elongation pathway, involving metabolic routes such as Wood-Ljungdahl, and their levels can serve as indicators of the energy status within the intestinal microecology[48]. The butyric acid content in the L group was significantly higher than that in the C group ($P < 0.05$), as indicated by Table 3. This finding can be attributed to the elevated abundance of *Bacteroides*, known for their production of high levels of butyric acid, in the L group compared to the C group, consistent with previous reports[49]. It has been observed that the proportion of oxidative muscle fibers in sterile piglets is low, and their development may be influenced by the production of

butyrate by intestinal flora[50]. *Clostridium butyricum* has been shown to enhance muscle mass and fiber diameter, while reducing meat shear force in sheep. These effects are likely mediated through metabolic pathways involving butyric acid[51]. Chen et al.[52] demonstrated that intestinal flora can regulate skeletal muscle satellite cell homeostasis and muscle function through the butyric acid signaling pathway, with acetic acid, propionic acid, and butyric acid being the major constituents. Bai et al.[53] found that *Tolypocladium sinense* mycelium polysaccharide increased protein expression of ZO-1 and occludin in the colon, improving intestinal barrier homeostasis and reducing inflammatory responses. From a nutritional perspective, SCFAs are present in free form in the intestine and are regulated by H^+-coupled low-affinity monocarboxylate transporters in the colonic epithelium. The transport of proton-coupled mono-carboxylic acids, such as lactate and pyruvate, is facilitated by *MCT1* and Na^+-coupled low-affinity monocarboxylate transporters (*SMCT1*), which belong to the solute carrier 16 (*SLC16*) family. These transporters play a crucial role in mediating the transmembrane movement of various mono-carboxylic acids across the plasma membrane[54]. They enable the transfer of intracellular lactate to the extracellular space, thereby preventing glycolysis inhibition caused by intracellular lactate accumulation.

Table 3 Concentration of SCFAs in the intestinal tract.

SCFA	Concentration (mmol/L)			*P* value
	C group	L group	Standard deviation	
Acetic acid	27.36	27.20	1.43	0.940
Propionic acid	8.01	9.02	0.50	0.192
Butyric acid	5.18	7.52	0.55	0.023
Pentanoic acid	0.40	0.42	0.05	0.809
Isobutyric acid	0.82	0.88	0.12	0.726
Isovaleric acid	0.32	0.33	0.06	0.907

To quantify tight junctions (TJs) such as occludin and zonula occludens-1 (ZO-1) at the mRNA level, reverse transcription-polymerase chain reaction analysis was conducted. Gene expression analysis of nutrient absorption (*GPR43/120* and *MCT1*) and barrier integrity (*Occludins* and *Zonulin1*) revealed significantly elevated levels of *Zonulin1* and *GPR43* in the L group compared to the C group ($P < 0.05$) (Table 4). The production of SCFAs by *L. reuteri* may potentially contribute to maintaining the integrity of tight junctions, as elucidated by Pérez-Reytor et al.[55]. In summary, supplementation with *L. reuteri* may improve the functionality and integrity of the colonic epithelial barrier, maintain intestinal homeostasis and regulate butyric acid absorption.

Table 4 Expression levels (relative to *β-actin*) of genes involved in intestinal permeability in Sunit sheep.

Gene	C group	L group	Standard deviation	*P* value
Occludins	1.16	1.24	0.18	0.793
Zonulin1	1.09	1.99	0.17	0.007
GPR120	2.12	1.78	0.32	0.472
GPR43 (*FFAR2*)	1.08	2.60	0.36	0.020
MCT1 (*SLC16A1*)	1.06	1.17	0.16	0.659

4 Conclusion

Supplementation with *L. reuteri* led to an increase in the proportion and area ratio of type I muscle fibers and SDH activity, as well as a decrease in the proportion and area ratio of type IIB muscle fibers and LDH activity in muscle. This indicates that *L. reuteri* promoted the transition of muscle fiber type from glycolytic to oxidative and improved meat tenderness. In the analysis of sheep gut microbes, *L. reuteri* may reduce methane emissions, and the dominant genera and species were *Alistipes* and *Bacteroides* sp. 43 _108, respectively. Combined with the results of KEGG and CAZy analysis, it was found that the differential intestinal microbes of sheep fed with *L. reuteri* were mainly involved in carbohydrate metabolism. *L. reuteri* could regulate butyric acid absorption in the intestinal epithelium and colonic epithelial barrier homeostasis. In conclusion, the addition of 4 g/day of *L. reuteri* in the diet represents an efficacious strategy to improve the meat tenderness of Sunit sheep through modification of gut microbiota and muscle fiber type. Further studies should be conducted on diverse breeds and directly utilizing SCFAs, the main products of *L. reuteri*, in the diet of sheep to improve meat quality.

Conflict of interest

The authors declare no conflict of interest.

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Authors contribution

Ting Liu and Chenlei Wang: wrote the main text. **Mirco Corazzin, Taiwu Zhang, and Maoqin Zhai:** prepared all of figures. **Yue Zhang and Qiaoge Zhang:** prepared all of the tables. **Xin Zhao:** analyzed the data. **Ye Jin and Lina Sun:** modified the article.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://doi.org10.26599/FSAP.2024.9240096.

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