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Sex-specific microbiota associations with backfat thickness, eye muscle area, and rumen fermentation in Qinchuan cattle

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Abstract

Background Ruminant livestock are essential for global food production, and understanding sex-specific rumen fermentation and microbial differences is key to improving production efficiency and meat quality. This study explored sex-specific variations in backfat thickness, eye muscle area, rumen fermentation, and microbiota in Qinchuan cattle.

Results The results revealed that heifers exhibited higher backfat thickness, butyrate concentrations, and acetate/propionate ratio, whereas bulls had larger eye muscle areas and higher propionate concentrations. Volatile fatty acids (VFAs) transport-related genes (*CA4*, *DRA*, and *NHE1*) were more highly expressed in bulls. Heifers showed greater microbial diversity with distinct sex-specific community structures. Bulls had a higher abundance of *Prevotella*, while butyrate-producing bacteria like *Butyrivibrio* and *Pseudobutyrivibrio* were more abundant in heifers. Functional predictions revealed that bulls were enriched in glycan biosynthesis and amino acid metabolism pathways, whereas heifers showed enhanced lipid metabolism pathways. Correlation analyses showed that backfat thickness was positively correlated with acetate and butyrate production, and acetate/propionate ratio, but negatively correlated with *Veillonellaceae_UCG-001*. Eye muscle area was negatively correlated with isobutyrate production and the abundance of *Elusimicrobium* and *Anaeroplasma*, but positively correlated with *Lachnospiraceae_NK3A20_group*. Redundancy analysis (RDA) identified propionate and butyrate as key drivers of microbial community differences. The Random Forest model identified key predictors for backfat thickness, including rumen fermentation parameters, microbial taxa, and metabolic pathways, explaining 28% of the variation. However, eye muscle area was not well predicted by the current parameters.

Conclusion These findings enhance our understanding of sex-specific microbial and metabolic profiles, offering potential strategies for optimizing livestock management and breeding programs.

Keywords Qinchuan cattle, Backfat thickness, Eye muscle area, Rumen microbiota, VFA, Sex differences

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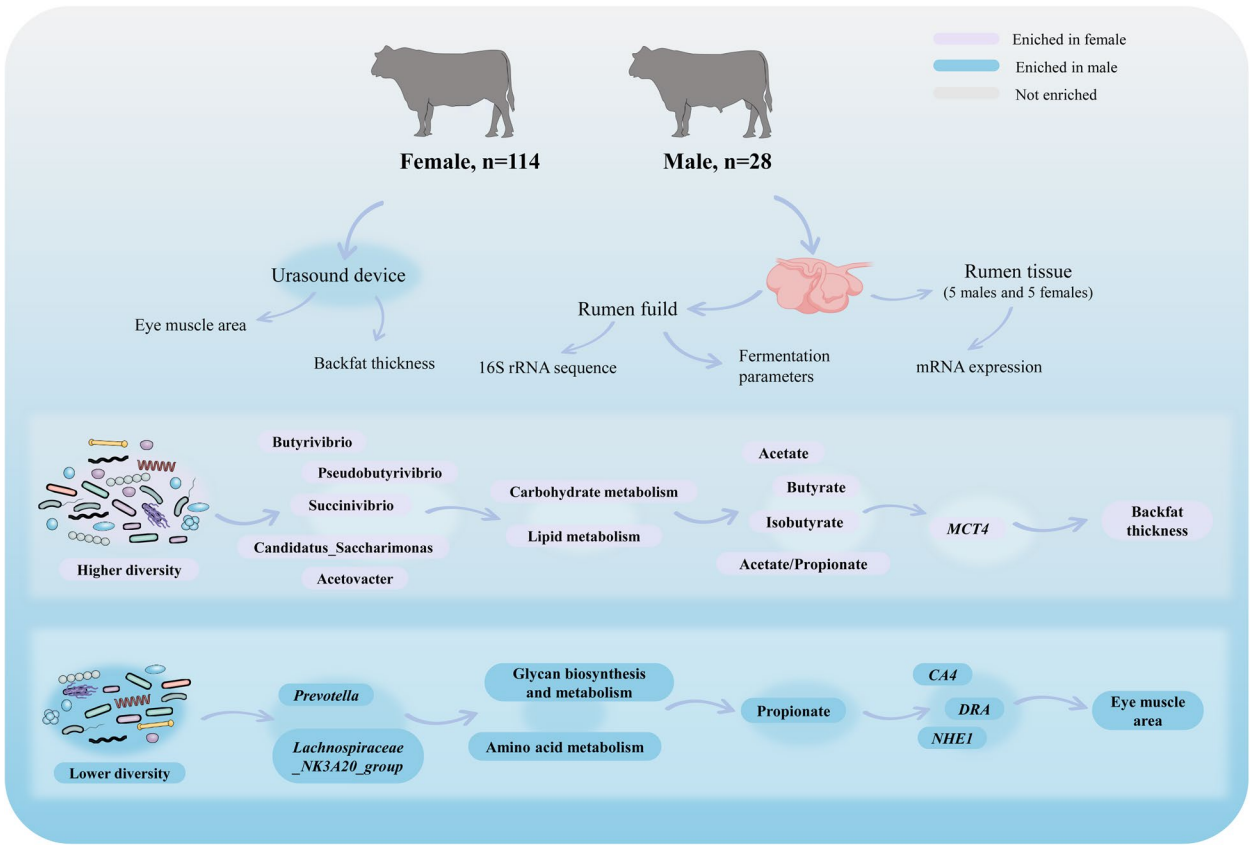
Graphical Abstract



Background

Optimizing livestock production efficiency and meat quality is a key objective in animal agriculture. Physiological traits such as fat deposition, muscle development, and rumen fermentation are key determinants of carcass composition, nutrient utilization efficiency, and economic value [1, 2]. Backfat thickness and eye muscle area serve as essential indicators, directly influencing meat quality traits such as marbling, tenderness, and consumer preference [3, 4]. Sex differences have been reported in Angus×Nellore cattle, Chikso, and Suffolk Down×Merino Precoz Aleman crosses lambs, with males generally exhibiting greater muscle development and large eye muscle area, while females accumulate more fat, resulting in higher backfat thickness and marbling scores and high levels of monounsaturated fatty acids (MUFAs) [5–7]. These sex-specific variations are primarily regulated by hormonal factors, with testosterone promoting muscle hypertrophy by regulating muscle protein synthesis and breakdown, while estrogen affecting adipogenesis [8, 9]. Moreover, these variations also affect carcass attributes and play a crucial role in determining beef flavor, texture, and nutritional properties, which are central to consumer preferences [10, 11]. Understanding these differences is vital for developing targeted breeding and management strategies to optimize production efficiency and quality.

Rumen microbiota plays a crucial role in fermentation processes and nutrient metabolism, influencing host energy utilization and production performance [9, 12–14]. Through the fermentation of carbohydrates, rumen microbes produce volatile fatty acids (VFAs), such as acetate, propionate, and butyrate, which serve as primary energy sources for cattle [15]. Acetate and butyrate are substrates for oxidation and as precursors of lipids, which predominantly utilized in lipogenesis [16], while propionate is the only glucogenic VFA, plays a key role in gluconeogenesis and supporting muscle protein synthesis [17–19]. Studies have identified significant sex differences in VFA production, correlating with observed disparities in fat distribution and muscle growth [2, 20, 21]. Studies have identified significant sex differences in

VFA production, correlating with variations in fat distribution and muscle growth. Females tend to product higher levels of acetate, favoring fat deposition, whereas males exhibit increased propionate production, which enhances muscle development [22]. These metabolic differences are largely shaped by rumen microbial composition and functional potential, which are influenced by host physiology, diet, and hormonal regulation [13, 23].

Despite advances in understanding rumen microbial roles, the mechanisms by which sex influences fat and muscle traits via rumen fermentation remain underexplored. Most studies have focused primarily on microbial taxonomy, leaving the functional roles of the microbiome and its metabolic contributions to host traits underexplored. This knowledge gap limits our ability to develop targeted interventions. This study hypothesizes that sex differences regulate rumen fermentation and microbial community composition, thereby affecting backfat thickness and eye muscle area. By integrating physiological measurements, fermentation parameter analysis, and 16S rRNA sequencing, this research aims to provide a comprehensive understanding of how sex-specific microbial and metabolic differences influence fat deposition and muscle development. The findings aim to provide novel insights into the interplay between host physiology and microbial functionality and expected to inform targeted strategies for optimizing beef cattle production and enhancing meat quality.

Materials and methods

Experiment design

A total of 142 healthy adult Qinchuan cattle from Qinchuan Cattle Breeding Farm within the National Beef Cattle Improvement Center at Northwest A&F University were selected and grouped by sex, including 114 heifers (4.12 ± 0.07 year-old) and 28 bulls (non-castrated males, 3.75 ± 0.25 year-old). No steers (castrated males) were included in this study to avoid hormonal confounding effects. All cattle have the same genetic backgrounds and management conditions. Backfat thickness and eye muscle area were measured, and rumen fluid samples were collected to assess rumen fermentation performance and for 16S rRNA sequencing of rumen microorganisms. After sampling, 10 individuals (5 bulls and 5 heifers) were selected for slaughter, and rumen tissue samples were collected. The contents of the tissue were quickly washed away with normal saline, then placed in cryogenic tubes, flash-frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction.

All experimental cattle were managed under a standardized feeding regimen, with feedings conducted at 06:00, 12:00, and 18:00 each day. Each cattle was fed 1.0 kg of concentrate and 10 kg of whole-plant corn silage daily, with free access to wheat straw and water. The concentrate formula was prepared according to the Feeding Standards of Beef Cattle (NY/T81-2004), and the composition and nutritional levels were shown in Table 1. Prior to sampling, all experimental cattle had undergone routine vaccinations and deworming.

Sample collection

Backfat thickness and eye muscle area were measured using an Aloka 500 V SSD ultrasound device (Aloka, Tokyo, Japan) with a 7.5 MHz probe. After removing the surface hair and applying coupling gel, the ultrasound probe was used to scan the cross-section of the 12th-13th rib, obtaining backfat thickness images. Each cow was measured three times consecutively.

Two hours after the morning feeding on the sampling day, rumen fluid was collected using an oral rumen tube. After discarding the initial 30 ml of saliva-contaminated fluid, 50 ml was collected in enzyme-free, sterile centrifuge tubes, flash-frozen in liquid nitrogen, and stored at -80°C for subsequent determination of rumen fermentation performance and microbial 16S rRNA sequencing. Following sample collection, anesthesia was induced in ten subjects (5 bulls and 5 heifers) via intravenous administration of sodium pentobarbital (50 mg/kg body

Table 1 Composition and nutrient levels of diet for cattle (dry matter basis)

Items	Contents
Ingredients, %	
Corn	53.0
Soybean meal	10.0
Cottonseed meal	15.0
Wheat bran	15.0
NaCl	1.00
$CaHPO_4$	1.00
Limestone	1.00
Premix[a]	4.00
Total	100
Nutrient levels[b]	
ME/(MJ/kg)	9.83
OM, %	96.2
CP, %	12.6
NDF, %	36.2
ADF, %	21.4
EE, %	3.96

[a] The premix provided the following per kg of diets: VA 2560 IU, VD 3 550 IU, VE 20 mg, Fe(as ferrous sulfate) 61 mg, Cu(as copper sulfate) 18 mg, Zn(as zinc sulfate) 49 mg, Mn(as manganese sulfate) 47 mg, Co 0.12 mg, I(as potassium iodide) 0.31 mg, Se(as sodium selenite) 0.36 mg. OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, Ether extract

[b] Metabolic energy (ME) was a calculated value [24], whereas the others were measured values

weight). After confirming deep anesthesia through absence of corneal reflex and withdrawal responses to nociceptive stimuli, euthanasia was performed by intravenous overdose of sodium pentobarbital (100 mg/kg). Subsequently, rumen tissue samples were collected for analysis.

Determination of rumen fermentation parameters

The concentrations of VFAs in the rumen fluid were measured using gas chromatography-flame ionization detection (GC-FID)(GC-6850, Agilent, USA). Ammonia nitrogen (NH_3-N) concentration was determined using an ultraviolet–visible spectrophotometer (colorimetric method; Cary 60, Agilent, USA).

Chemical analysis

The chemical composition of the feed sample was collected and dried in an oven at 65°C for 72 h to determine dry matter content. The dried samples were then ground to pass through a 1 mm sieve and stored in sealed containers at room temperature for nutritional analysis. Nitrogen content was measured using an automatic Kjeldahl nitrogen analyzer (UDK159, VELP, Italy), following the Chinese national standard GB/T 6432–2018 (semi-micro method). The crude protein (CP) content was calculated as N×6.25. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were analyzed with an automated fiber analyzer (F800, Hanon, Jinan, China), in accordance with the national standard GB/T 20806–2022 (filter bag method, ANKOM F57 filter bags) and industry standard NY/T 1459–2022 (filter bag method, ANKOM XT4 filter bags), respectively. Ash content was measured using a muffle furnace at 550°C for 5 h, with preliminary ashing conducted on an electric heating panel (F47910-33, Thermo Scientific, Waltham, MA, USA). Organic matter (OM) content was calculated by subtracting the ash content from 100%. The ether extract (EE) content was determined using an automatic Soxhlet extractor (SOX606, Hanon, Jinan, China).

DNA extraction and sequencing

Total bacterial DNA was extracted from rumen fluid samples using the TIANamp Stool DNA Kit (Tiangen Biotech Co., Ltd., China). The V3-V4 region of the 16S rRNA genes was amplified using specific primers (F: ACTCCTACGGGAGGCAGCA; R: GGACTACHVGGG TWTCTAAT). Sequencing adapters were added to the ends of the primers, followed by PCR amplification. The PCR products were then purified, quantified, and normalized to generate the sequencing library. After quality assessment, sequencing was performed on the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA). The sequencing process was carried out by Beijing Biomarker Technologies Co., Ltd.

Determination of mRNA expression in rumen tissue

Total RNA was extracted from the rumen epithelial tissues of Qinchuan cattle using *AG RNAex Pro* reagent (Accurate Biology, China). The RNA concentration and purity were assessed using the spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). A reverse transcription kit (*Evo M-MLV* RT Kit, AG Bio, China) was used to synthesize gDNA for qPCR, following the manufacturer's instructions. Specific primers for mRNA PCR were designed using Primer 5.0 software and listed in Table 2. Quantitative real-time PCR (qRT-PCR) was performed using the *PerfectStart*® Green qPCR SuperMix (TransGen Biotech, China). All reaction were performed with three biological replicates and three technical replicates to ensure reproducibility. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and GAPDH as the reference gene.

Bioinformatics analysis

The raw sequencing data were quality-filtered using Trimmomatic (version 0.33) [25], and primer sequences were identified and removed using Cutadapt (version 1.9.1) [26]. Paired-end reads were merged and chimeras were removed using USEARCH (version 10) [27] with the UCHIME algorithm (version 8.1) [28], resulting in high-quality sequences for further analysis. Denoising of the quality-controlled data was performed using the DADA2 algorithm in QIIME2 (version 2020.6) [29], applying a minimum read depth threshold of 5000 reads per sample to ensure high-quality sequence retention. Additionally, an amplicon sequence variant (ASV) filtering threshold of 0.005% of the total sequence count was implemented to remove low-abundance variants. SILVA (Release 138, http://www.arb-silva.de) was used as the reference database [30], and feature sequences were annotated using the Naïve Bayes classifier. α-diversity and β-diversity metrics were assessed in QIIME2 (version 2020.6) [29]. Microbial functional profiles were predicted using PICRUSt2 [31].

Statistics and analysis

To address concerns regarding the sample size imbalance between bull ($n=28$) and heifers ($n=114$), a post-hoc power analysis was performed using G*power (3.1.9.7) to evaluate the statistical power for detecting differences in backfat thickness and eye muscle area. Bootstrap resampling analysis (1000 iterations) was conducted in R (boot package) to further validate robustness. SPSS (version 26.0) was used to test for normality of distribution and homogeneity of variance. For data meeting these

Table 2 Primer sequences of fatty acid-related genes of cattle

Gene	Primer	Length	Accession number
GAPDH	F: AGTTCAACGGCACAGTCAAGG R: ACCACATACTCAGCACCAGCA	124 bp	NM_001034034.2
CA4	F: GACCCAATATGAACACCACAATGAAAG R: AGCGGAAGTAGTGCCTCAGAC	87 bp	NM_173897.1
NHE1	F: CCTTCGTTGCTTGTGGTGGTAG R: TCTGATGGTGCTGGCGGTAG	93 bp	NM_174833.2
NHE2	F: GGACCAGTTCATCATTGCCTACG R: GGAACACTGAGGACGGAAGGAG	83 bp	XM_002691185.7
SLC26A9	F: CTCGCTCATCTTCGCTCTTATCAG R: GCCACCACTACCACAATCATCTC	120 bp	XM_010813049.4
DRA	F: TCGTTGCCATCGGATTTCTCTTG R: CCTTTCGCCACAATCTTCGTATTTC	120 bp	NM_001083676.1
AE2	F: AGGAGGGCGAGGAAGAGGAG R: GTGTGGAGGCAGGCGAAGG	81 bp	NM_001205664.3
MCT1	F: CTGTTGGAGTCATTGGAGGTCTTG R: TGCCAGCGGTCGTCTCTTATAG	95 bp	NM_001037319.1
MCT4	F: TGGTGTCTGCGTCCTTCTGTG R: GTAGCGGTTGAGCATGATGAGTG	116 bp	NM_001109980.3

assumptions, *t*-tests were applied; otherwise, the Wilcoxon rank-sum test was used. Results were presented as mean ± standard error, with $P < 0.05$ considered statistically significant and $0.05 < P < 0.10$ indicating a trend. Microbial differences at the phylum and genus levels were analyzed using STAMP (version 2.1.3) [32], filtering out taxa with relative abundances below 0.01%. Microbial co-occurrence networks were constructed using Gephi software. Spearman correlation analysis was performed in SPSS 26.0, and correlation networks were visualized with Cytoscape 3.6.0. A redundancy analysis (RDA) was completed using the Wekemo Bioincloud (https://www.bioincloud.tech) [33]. To identify the best predictors for backfat thickness and eye muscle area, we used Random Forest regression models using Python (version 3.12.3) with the scikit-learn library (version 0.24.2). Feature importance scores from the Random Forest models were used to identify the key factors influencing backfat thickness and eye muscle area.

Results

Backfat thickness, eye muscle area, and rumen fermentation performance

Post-hoc power analysis demonstrated that the power of eye muscle area was 1.00 (Cohen's d = 1.85), confirming a robust statistical difference between bulls and heifers. For backfat thickness, the power was 0.76 (Cohen's d = -0.57), which was slightly below 0.8 but still provided moderate statistical confidence. Moreover, the bootstrap-estimated mean backfat thickness was 0.689 (95% CI: 0.641–0.745), and the mean eye muscle area was 65.30 (95% CI: 61.71–68.99). These confidence intervals confirm that the observed differences between bulls and heifers are statistically stable.

Backfat thickness and eye muscle area were analyzed in Qinchuan cattle of different sexes (Fig. 1). The results showed that backfat thickness was significantly higher of heifers than in bulls ($P = 0.006$), while eye muscle area was significantly larger in bulls ($P < 0.001$).

The concentration of rumen NH_3-N was significantly higher in bulls than in heifers ($P < 0.001$, Table 3). Moreover, propionate concentration was significantly higher in bulls, showing a 13.4% increase compared to heifers ($P = 0.021$). However, the concentrations of isobutyrate, butyrate, and the ratio of acetate/propionate were significantly higher in heifers ($P < 0.05$). Acetate concentration tended to be higher in heifers compared to bulls ($P = 0.086$). Nevertheless, no significant differences were observed in isovalerate, valerate, and total VFAs concentrations between bulls and heifers ($P > 0.05$).

VFA absorption-related gene expression

Significant differences were observed in the expression of VFA transport-related genes in rumen tissue between heifers and bulls (Fig. 2). The relative expression levels of *CA4, DRA*, and *NHE1* were significantly higher in bulls than in heifers ($P < 0.05$), while *MCT4* exhibited a trend toward higher expression in heifers ($P = 0.078$). However, no significant differences were detected for *MCT1*, *AE2*, and *NHE2* ($P > 0.05$).

Microbial diversity

A total of 74,266 ASVs were obtained from 142 samples using 16S rRNA gene sequencing. Alpha diversity of

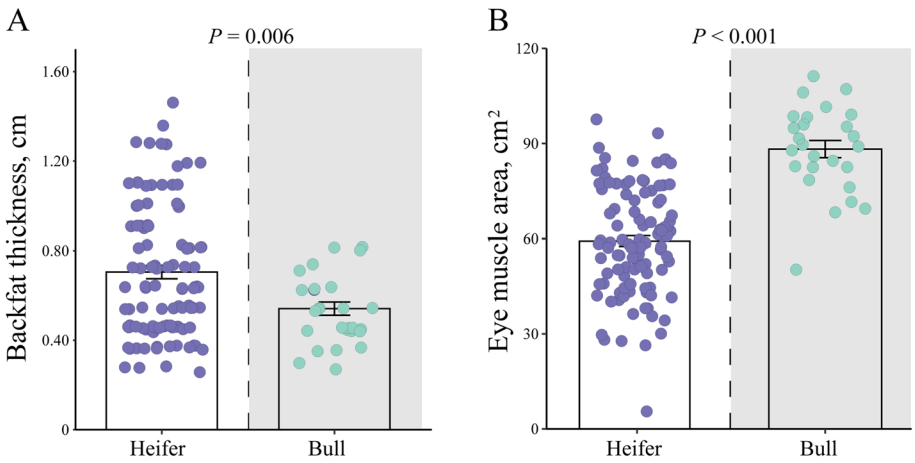


Fig. 1 Sex differences in backfat thickness (**A**) and eye muscle area (**B**). Each point represents a sample

Table 3 Analysis of sex differences in rumen fermentation performance

Items	Heifers	Bulls	SEM	*P*-value
NH_3-N, mmol/L	3.34	5.32	0.188	< 0.001
Concentration of VFAs, mmol/L				
Acetate	101.19	95.33	1.368	0.086
Propionate	23.12^{B}	26.57^{A}	0.538	0.010
Isobutyrate	1.37^{A}	1.07^{B}	0.026	< 0.001
Butyrate	11.36^{A}	9.66^{B}	0.266	0.010
Isovalerate	1.85	1.98	0.055	0.337
Valerate	1.367	1.53	0.052	0.199
Total VFAs	140.25	136.13	1.900	0.386
Acetate/Propionate	4.72^{A}	3.66^{B}	0.125	0.001

NH_3-N, ammonia nitrogen. VFAs, volatile fatty acid. SEM, standard error of the mean. Within the same row, distinct capital letter superscripts indicate significant differences between bulls and heifers ($P < 0.05$)

the rumen microbial communities indicated that Chao1 index, Shannon index, and PD whole-tree index was significantly increased in heifers than the bulls ($P < 0.001$, Fig. 3A-C), indicating an increase in microbial diversity and evenness in heifers. However, no significant difference was observed in Shannon index between bulls and heifers ($P > 0.05$, Fig. 3D). Non-metric multidimensional scaling (NMDS) analysis based on Bray–Curtis distance at the ASV level, combined with permutational multivariate analysis of variance (PERMANOVA), revealed differences in microbial community structure between bulls and heifer group ($F = 2.176$, $P = 0.001$). This suggests the higher spatial heterogeneity and lower community similarity in the rumen microbial communities among different sexes of Qinchuan cattle (Fig. 4).

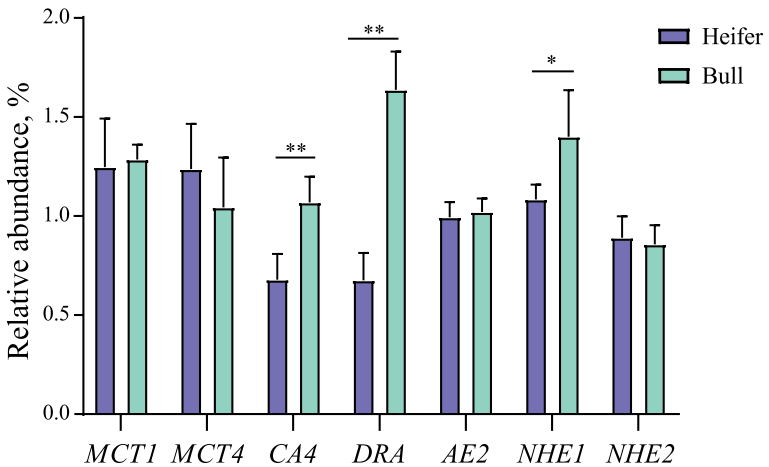


Fig. 2 Expression of VFA transport-related genes. * indicates $P < 0.05$; ** indicates $P < 0.01$

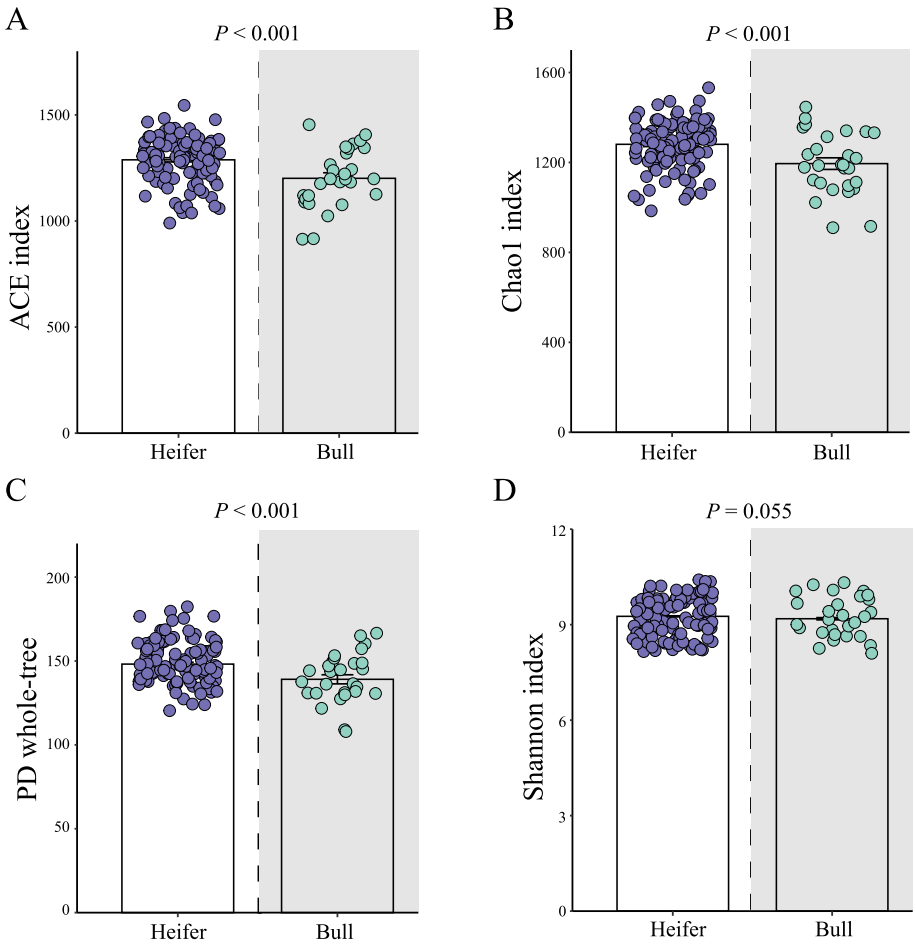


Fig. 3 Sex differences in rumen alpha diversity in Qinchuan cattle. Histogram show variations in α-diversity metrics, ACE index (**A**), Chao1 index (**B**), PD whole-tree (**C**) and Shannon index (**D**). Each point represents a sample

Microbial composition

In all samples, a total of 25 bacterial phyla and 486 bacterial genera were detected at the taxonomic level. Stacked histograms illustrate the microbial composition at the phylum and genus levels. Bacteroidota and Firmicutes were the dominant phyla, with *Prevotella* as the dominant genus (Fig. 5A, B). Microbial differences at the phylum and genus levels were analyzed using STAMP. At the phylum level, the relative abundance of Chloroflexi and Bacteroidota was significantly higher in bulls ($P < 0.05$), whereas the relative abundance of Proteobacteria, Patescibacteria, and Cyanobacteria were significantly higher in heifers ($P < 0.05$, Fig. 6A). At the genus level, *Prevotella*, *Lachnospiraceae_NK3A20_group*, and *Family_XIII_AD3011_group* were significantly more abundant in bulls ($P < 0.05$), while *Succinivibrio*, *Butyrivibrio*, *Pseudobutyrivibrio*, and *Candidatus_Saccharimonas* were more abundant in heifers ($P < 0.05$, Fig. 6B).

Linear discriminant analysis (LDA ≥ 4; $P < 0.05$) effect size (LEfSe) and cladograms were used to identify differentially abundant bacterial taxa in each group (Fig. 7). Specifically, 21 bacterial genera were identified in heifers, 9 bacterial genera in bulls (Fig. 7A). The cladogram illustrates the most significant taxonomic differences (Fig. 7B). The Patescibacteria phylum and Bacteroidales order were the most differentially abundant bacterial genera in heifers and bulls, respectively.

Microbial function prediction

To investigate how the functions of rumen bacteria vary with sex, we utilized PICRUSt2 to predict the functions of rumen bacteria. Four differential KEGG pathways at level 1 were identified (Fig. 8A). 'Metabolism' and 'Organismal system' pathways in bulls were significantly enriched ($P < 0.05$), while 'Environmental information processing' and 'Cellular processes' in heifers were significantly enriched ($P < 0.05$). A total of 32 significant sex-related pathways involved in 'metabolism' (Fig. 8B). Among them, 23 pathways related to 'Carbohydrate metabolism', 'Sulfur metabolism', 'Microbial

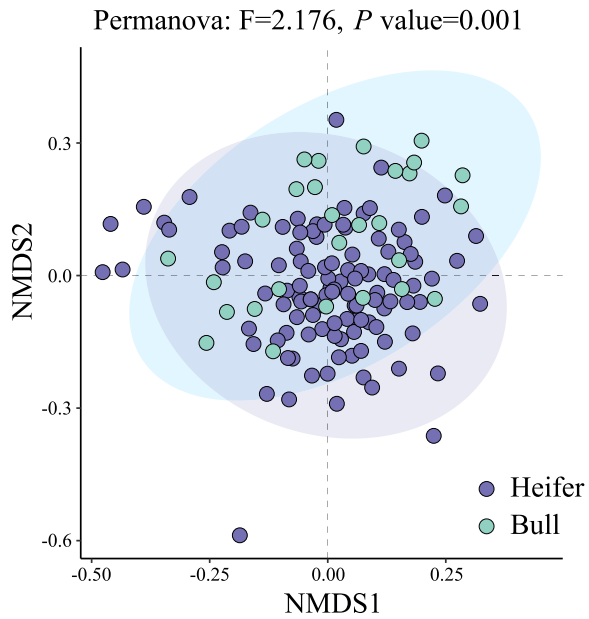


Fig. 4 Sex differences in rumen beta diversity in Qinchuan cattle. β-diversity, based on Bray–Curtis NMDS, illustrated the differences in microbial community composition between sexes

metabolism in diverse environments', 'Steroid hormone biosynthesis', 'Glutathione metabolism', 'Glycosphingolipid biosynthesis (globo and isoglobo series)' and 'Chloroalkane and chloroalkene degradation' were more abundant in heifers than in bulls ($P<0.05$). On the contrary, 9 pathways related to 'Carbon fixation in photosynthetic organisms', 'Alanine aspartate and glutamate metabolism', 'beta Alanine metabolism', 'Alanine aspartate and glutamate metabolism', 'Glycosaminoglycan degradation' and 'Glycosphingolipid biosynthesis (globo and isoglobo series)' were more abundant in bulls than in heifers ($P<0.05$).

Correlation analysis

Correlation analysis was conducted between backfat thickness, eye muscle area, rumen fermentation parameters, and rumen microbiota. The results showed that backfat thickness was significantly positively correlated with the concentrations of total VFAs, acetate, butyrate, and the acetate/propionate ratio (Fig. 9; $r=0.363$, 0.492, 0.223, and 0.464, respectively; $P<0.05$), while negatively correlated with *Veillonellaceae_UCG_001* ($r=$-0.205; $P<0.05$). Eye muscle area was negatively correlated with isobutyrate concentration, and the relative abundance of *Anaeroplasma* and *Elusimicrobium* ($r=$-0.217, -0.287, -0.205; $P<0.05$), while positively correlated with the relative abundance of *Lachnospiraceae_NK3A20_group* ($r=0.264$; $P<0.05$). Additionally, *Butyrivibrio*, *Lachnospiraceae_NK4A136_group*, *Ruminobacter* were positively correlated with butyrate concentrations (r = 0.219, 0.261, and 0.273, respectively; $P<0.05$). *Candidatus_Saccharimonas* was positively correlated with isobutyrate concentrations and acetate/propionate ratio ($r=0.241$, 0.204; $P<0.05$).

RDA was conducted to analyze the relationships between bacterial communities, rumen fermentation parameters, backfat thickness (BFT), and eye muscle area (EMA)(Fig. 10). RDA1 and RDA2 accounted for 28.17% and 24.08%, respectively, of the variation in bacterial community structure. Propionate and butyrate were the most significant factors associated with the bacterial community, explaining 32.91% and 17.32% of the variation, respectively, followed by NH_3-N concentrations (10.97%) and acetate (10.04%) ($P<0.05$). *Rikenellaceae*

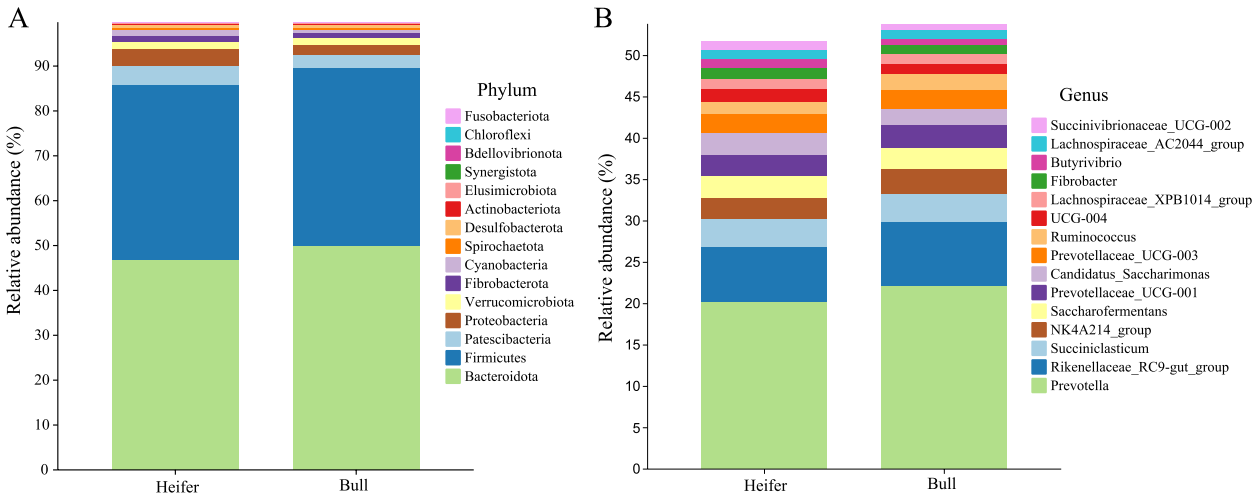


Fig. 5 Rumen microbial composition. The stacked histogram showed the microbial composition at the phylum (**A**) and genus (**B**) levels

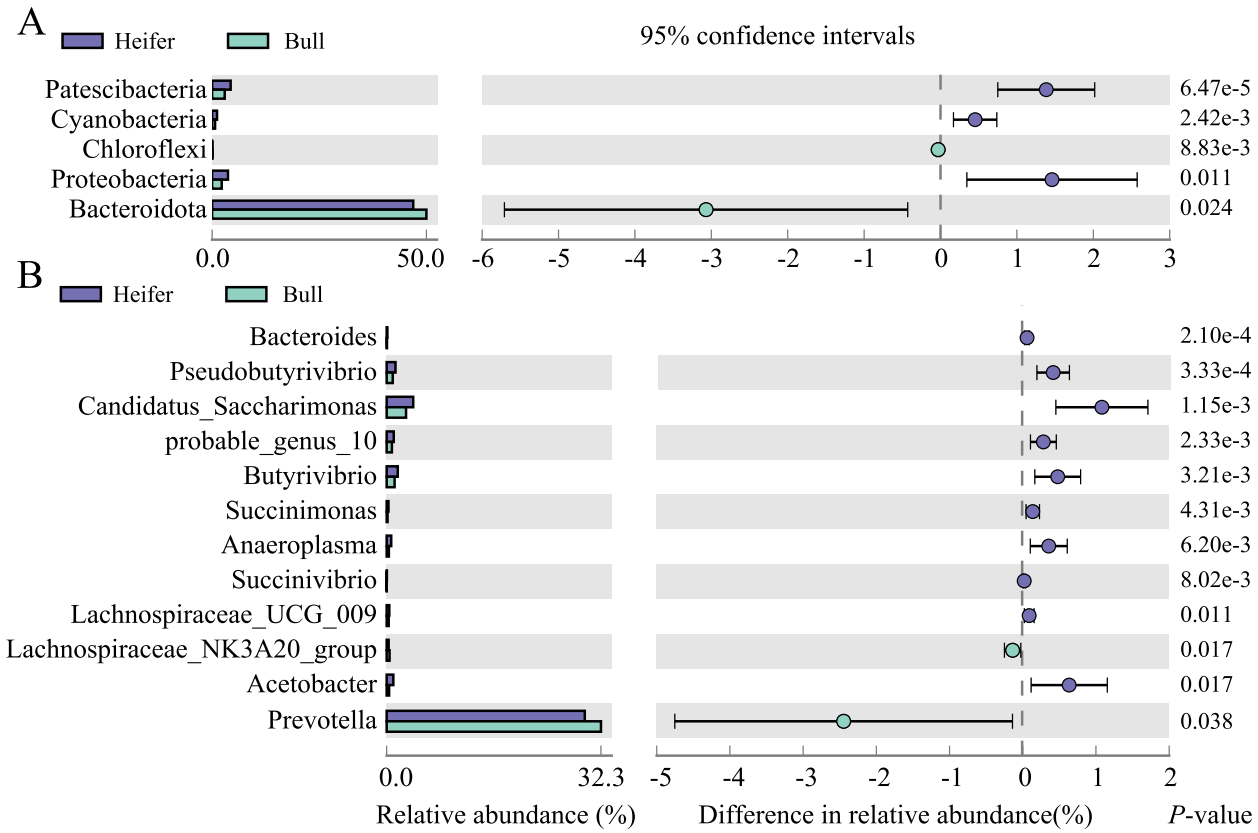


Fig. 6 Analysis of sex and age differences within the rumen community. The extended bar chart illustrates the differences between sexes at the phylum (**A**) and genus (**B**) levels. The bar chart on the left showed the average relative abundance (%) of microbiota, while the dots on the right shows the average proportion of change (%) in the relative abundance of microorganisms within the 95% confidence interval, exclusively displaying microbiota with $P < 0.05$

RC9-gut group and *Prevotellaece UCG-003* were positively correlated with backfat thickness, while *Saccharofermentans* was positively correlated with eye muscle area ($P < 0.05$).

Random forest prediction

The random forest models identified key predictors for both backfat thickness and eye muscle area. Rumen fermentation parameters, bacterial genera present in at least 90% of the samples, and level 2 KEGG metabolic pathways were included in the analysis. The feature importance results revealed that specific rumen fermentation parameters, microbial community composition, and metabolic pathways were highly influential in predicting these traits (Fig. 11). For BFT prediction, acetate, propionate, and butyrate concentrations, amino acid metabolism, *Prevotellaceae UCG-001*, Chao1 index, metabolism of cofactors and vitamins, *Lachnospiraceae NK3A20 group*, and *Rikenellaceae RC9-gut group* were identified as the most influential factors ($R^2 = 0.28$). In contrast, *Prevotella*, *Ruminococcus*, *U29-B03*, PD whole-tree index, *Lachnospiraceae XPB1014 group*, *Elusimicrobium*, *Ruminobacter* and NH_3-N were the most important predictors for EMA ($R^2 = -0.58$). However, the low R^2 value for EMA prediction suggests that EMA is a complex trait that cannot be accurately predicted using the current set of rumen fermentation parameters, microbial taxa, and metabolic pathways.

Discussion

The differences in backfat thickness, eye muscle area, and rumen fermentation performance between heifers and bulls of Qinchuan cattle offer significant insights into the physiological mechanisms underlying fat deposition and muscle development. These sex-based variations can be attributed to a complex interplay of hormonal regulation, microbial ecology, and metabolic pathways, all of which are influenced by sex-specific physiological demands and evolutionary adaptations.

The significantly higher backfat thickness in heifers can be explained by differences in energy allocation [34]. Heifers have larger subcutaneous adipose tissue for lipid storage and tend to prioritize fat deposition as a reserve for reproductive activities, which aligns with

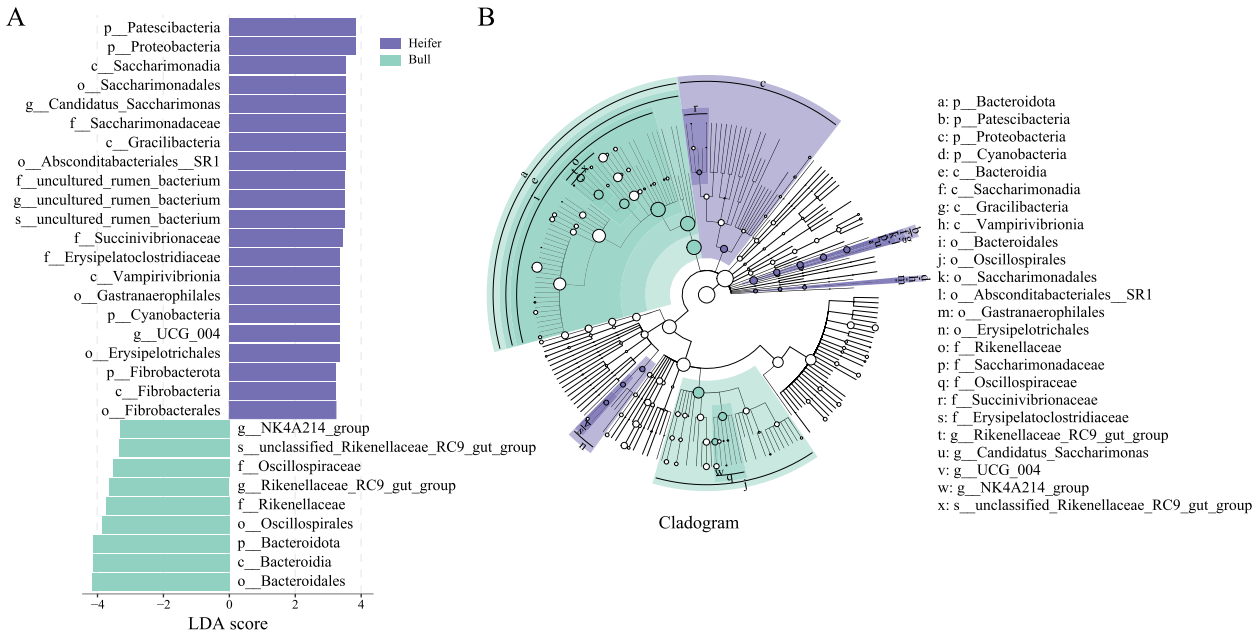


Fig. 7 Comparison of bacterial communities. **A**, **C** Linear discriminant analysis (|log10 LDA|≥ 4; $P < 0.05$; Wilcoxon rank-sun test) scores derived from LEfSe (Linear discriminant analysis effect size) analysis identified the genera of which abundances significantly differed in each group. Taxonomic levels: p, phylum; c, class; o, order; f, family; g, genus. **B**, **D** Cladogram generated from LEfSe analysis show taxonomic relationships (inner to outer rings, phylum, class, order, family, and genus). Colors denote the group where taxa were most abundant: lake green for bull, purple for heifer

their evolutionary role in gestation and lactation [35]. Studies have shown that estrogen promotes adipogenesis by upregulating lipogenic genes and enhancing adipocyte differentiation, thereby facilitating higher fat storage in heifers [36]. Moreover, heifers generally produce more acetate, a primary precursor for lipogenesis, contributing 70–80% of the substrates for synthesis in subcutaneous adipose tissue [22, 37, 38]. This acetate-driven fat deposition aligns with the higher backfat thickness observed in heifers, reflecting a metabolic tendency towards energy storage as adipose tissue. In contrast, the significantly larger eye muscle area in bulls is primarily driven by testosterone, which enhances muscle growth by stimulating protein synthesis and inhibiting protein degradation [39]. Testosterone has also been reported to influence rumen fermentation by promoting microbial communities that favor propionate production [40]. This is consistent with the higher propionate concentration observed in bulls. Propionate is a key gluconeogenic precursor in the liver, supporting muscle growth by providing energy for protein synthesis [41]. Moreover, propionate also promotes glucose uptake by muscle cells by influencing insulin secretion and sensitivity [42]. The increased propionate concentration in bulls suggests a metabolic shift toward greater energy availability for muscle development, contributing to the observed differences in eye muscle area. Previous studies have found that cows with high efficiency exhibit significantly higher propionate concentrations, as propionate competitively utilizes hydrogen to reduce methane production, thereby improving energy utilization [21, 43, 44]. This enhanced efficiency may further explain the faster growth rate and higher muscle yield observed in bulls.

The significantly higher concentrations of butyrate and the acetate/propionate ratio in heifers suggests a rumen environment that favors fat metabolism. Butyrate serves as a key energy source for rumen epithelial cells and can interconvert with acetate [45, 46]. It also has been associated with increased fat deposition in ruminants [47]. Additionally, butyrate promotes lipid synthesis through the β-hydroxy-β-methylglutaryl-CoA pathway by converting ketone bodies or acetyl-CoA into fatty acids, potentially contributing to adipose tissue accumulation [48]. However, further research is needed to clarify the specific mechanisms through which butyrate influences fat deposition in cattle. The difference in acetate/propionate ratio between bulls and heifers indicates distinct rumen fermentation patterns [49]. Under identical feeding and management conditions, this variation is primarily influenced by the differences in the rumen microbial community structure [50]. Moreover, heifers exhibit a higher acetate/propionate ratio, which aligns with their greater capacity for lipogenesis, as acetate is the primary substrate for fatty acid synthesis in ruminants [51].

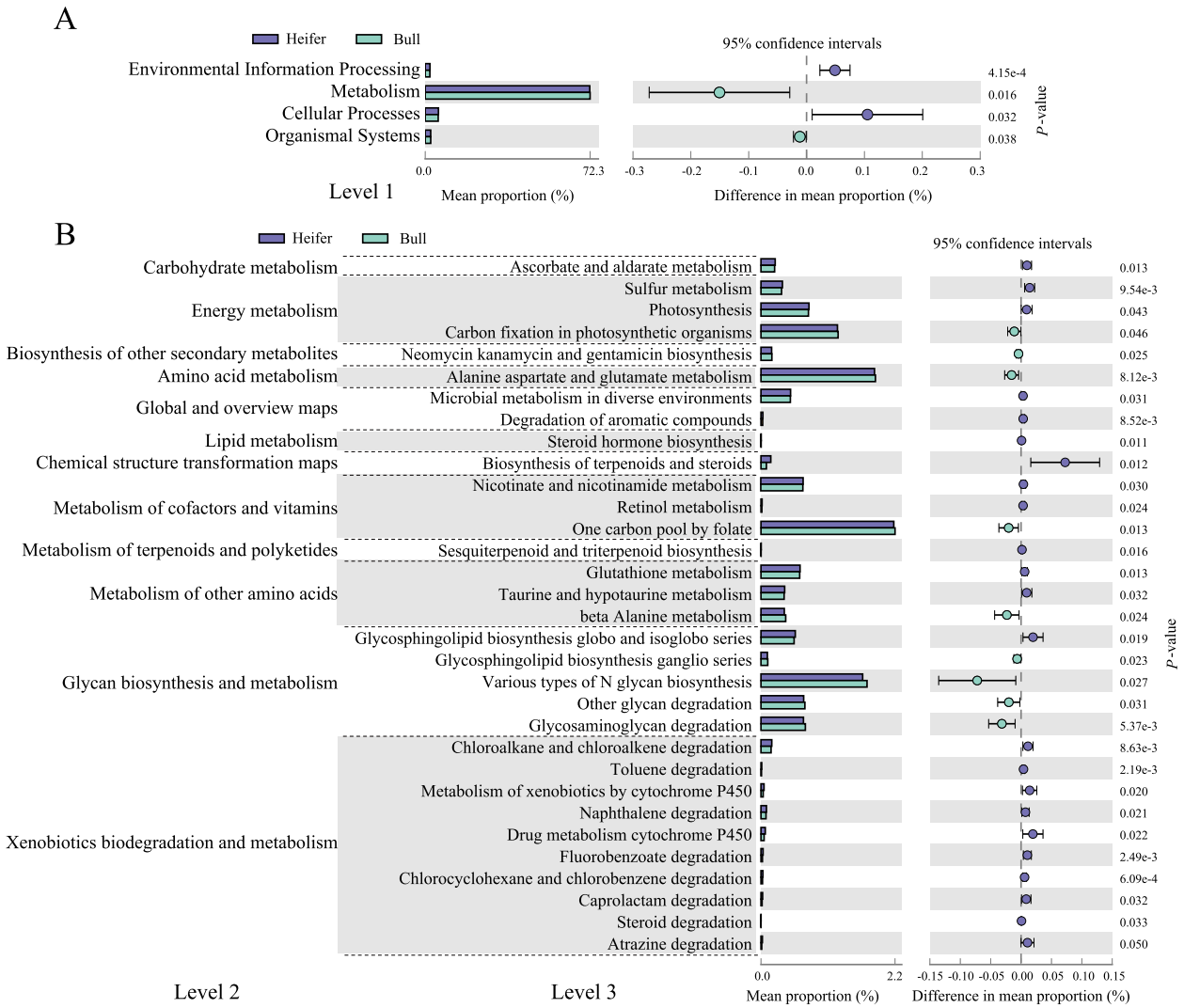


Fig. 8 The 8 most significant KEGG pathways of rumen bacteria between bulls and heifers at level 2 (**A**) and level 3 (**B**). The bar chart on the left showed the mean proportion (%) of KEGG pathways, while the dots on the right shows the difference in mean proportion (%) within the 95% confidence interval, exclusively displaying pathways with $P < 0.05$

NH_3-N in the rumen is a critical factor influencing microbial protein synthesis [52]. The significantly higher NH_3-N concentrations observed in bulls compared to heifers may reflect variations in protein metabolism and microbial activity. Elevated NH_3-N levels in bulls suggest increased protein degradation and deamination by rumen microbes, potentially due to higher intake of dietary protein or greater microbial turnover [53]. This aligns with the greater eye muscle area observed in bulls, as increased nitrogen availability enhances microbial protein synthesis, facilitates amino acid absorption in the small intestine, and ultimately contributes to muscle accretion [54].

Further analysis of the expression of genes involved in VFA absorption revealed that the relative expression levels of *CA4*, *DRA*, and *NHE1* were significantly higher in bulls compared to heifers, suggesting an enhanced capacity for VFA transport. *CA4* is involved in bicarbonate transport, facilitating the establishment of a favorable proton gradient essential for VFA uptake across the rumen epithelium, a process that optimizes rumen fermentation efficiency [55]. *DRA* is central to chloride-bicarbonate exchange, a mechanism crucial for maintaining ionic balance and enhancing VFA absorption. *NHE1* contributes to pH homeostasis by exchanging intracellular H^+ for extracellular Na^+, ensuring an

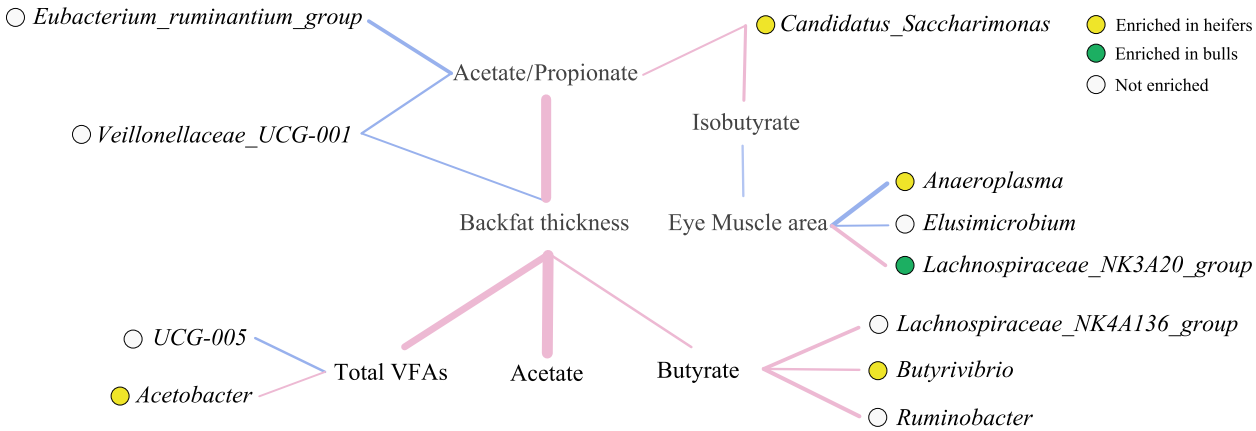


Fig. 9 Correlation network between backfat thickness, eye muscle area, rumen fermentation parameters, and rumen microbiota. Only nodes and edges with Spearman correlation coefficients |r|> 0.2 and *P* < 0.05 were displayed. Pink lines indicate positive correlations, while blue lines indicate negative correlations. The thickness of the lines represents the correlation value

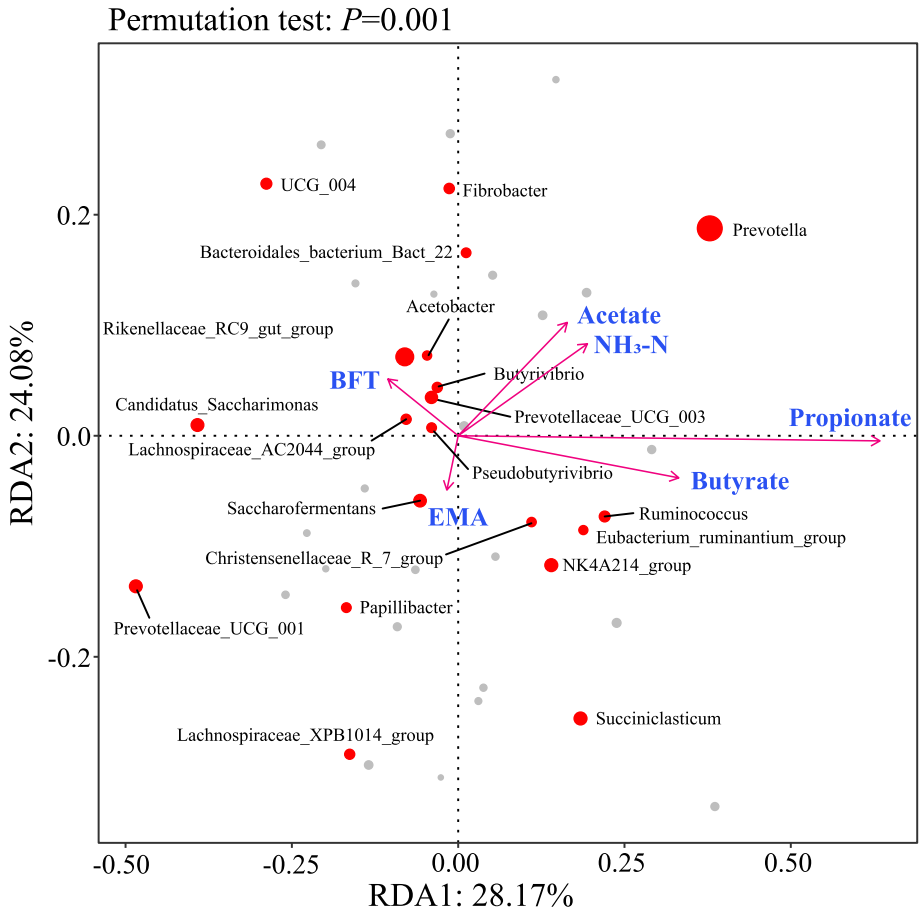


Fig. 10 Redundancy analysis (RDA) illustrating the correlations between bacterial communities and rumen fermentation, backfat thickness and eye muscle area. Each point represented a bacterial genus, with only genera presented in at least 90% of the samples included. The size of each point reflected the abundance of the corresponding genus, with the top 20 most abundant genera highlight in red. Arrow length indicated the correlation between explanatory variables and the distribution of rumen bacterial communities at the genus level. Longer arrows signify stronger correlations. BFT, backfat thickness; EMA, eye muscle area

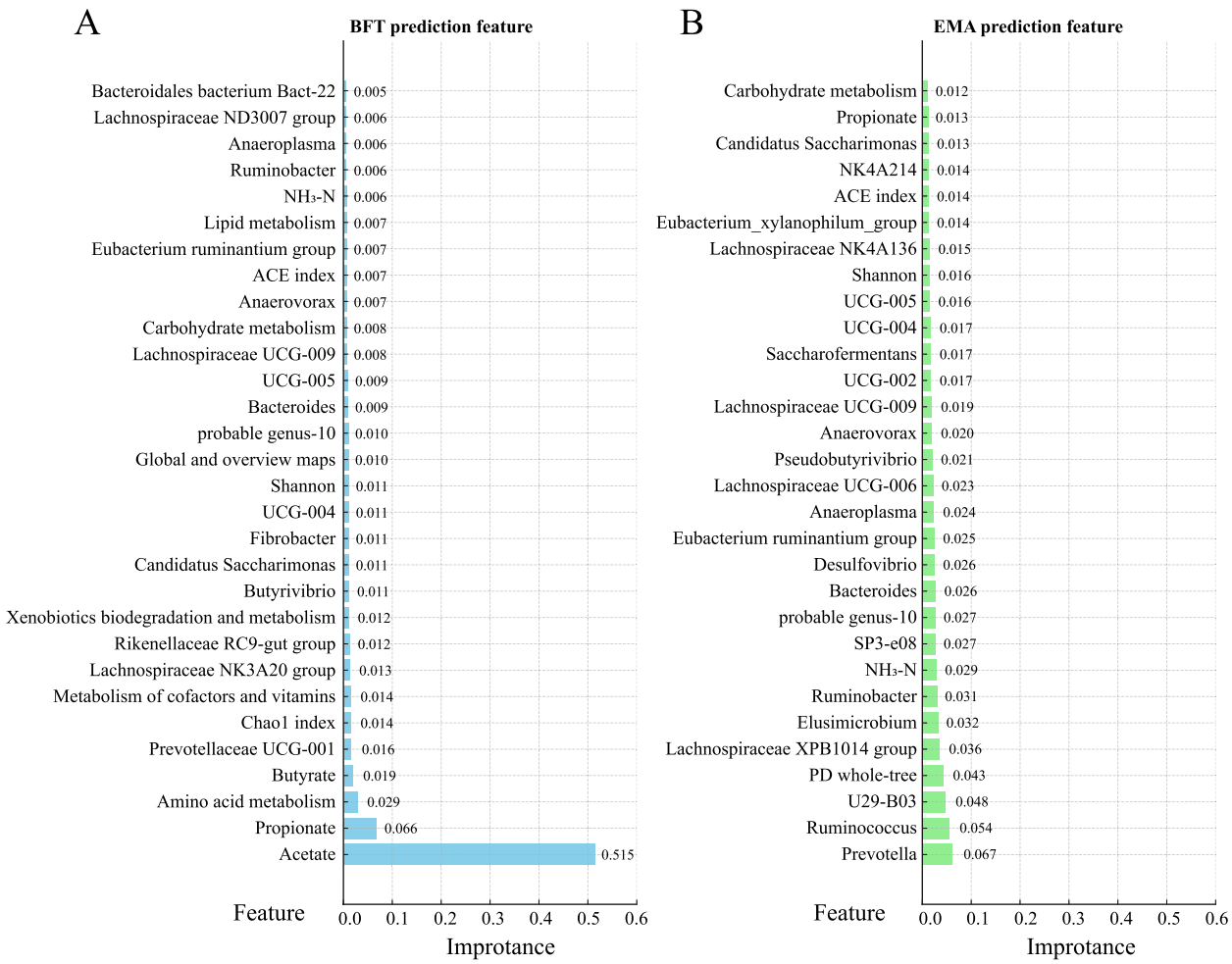


Fig. 11 Feature importance of predictors for backfat thickness in Qinchuan cattle, as determined by a random forest model. The horizontal bar plot shows the importance of various rumen fermentation parameters and microbial compositions in predicting backfat thickness. BFT, backfat thickness; EMA, eye muscle area

optimal intracellular environment for VFA transport [56, 57]. The increased expression of these genes in bulls indicates a greater capacity for VFA absorption, potentially linked to their higher energy demands for muscle development and growth. These funding suggest that sex-specific regulation of VFA transport genes may contribute to physiological differences in energy utilization and nutrient absorption between bulls and heifers. Interestingly, no significant differences were observed in the expression of *MCT1*, *MCT4*, *AE2*, and *NHE2*, suggesting that these transporters may play a less dominant role in sex-specific VFA absorption dynamics.

Microbial diversity analysis revealed that heifers exhibited significantly higher alpha diversity indices, including Chao1, Shannon, and PD whole-tree indices. This finding aligns with previous research on Tibetan sheep, which demonstrated greater bacterial diversity in adult heifers compared to bulls [58]. Higher microbial diversity is often associated with a more robust and resilient rumen ecosystem, which can efficiently metabolizing a wider variety of dietary substrates [59]. This enhanced diversity in heifers may contribute to their ability to process different feed types, particularly those that favor fat synthesis, as reflected in their higher backfat thickness. However, some studies have observed that individuals with higher feed efficiency tend to exhibit lower rumen microbial diversity but higher propionate concentrations, suggesting that a less diverse yet more specialized microbial community may better support the host's energy requirements [21]. In this study, the lower microbial diversity and higher propionate concentration observed in bulls are consistent with their potentially greater feed efficiency. Moreover, NMDS analysis showed significant differences in microbial community structure between bulls and heifers, indicating that sex influences not only the diversity but also the composition of the rumen

microbiota. These structural differences may underlie the observed variations in host traits [23, 60].

This study identified significant sex-specific differences in rumen microbial composition. The higher abundance of *Prevotella* in bulls suggests a greater capacity for carbohydrate fermentation, as *Prevotella* are known for their ability to degrade polysaccharides and produce propionate, a key glucogenic precursor [61]. Propionate is a key precursor for hepatic gluconeogenesis, providing glucose for muscle protein synthesis and maintenance of metabolic homeostasis [62, 63]. Increased propionate availability has been linked to enhanced feed efficiency and growth performance in cattle [62, 64], which may partially explain the greater eye muscle area observed in bulls. Furthermore, metabolic pathways related to amino acid and carbohydrate metabolism, including "Alanine, Aspartate and Glutamate metabolism", "Beta-Alanine metabolism", and "Glycosaminoglycan degradation" were significantly enriched in bulls, further supporting these findings. Conversely, *Butyrivibrio* and *Pseudobutyrivibrio* were more abundant in heifers, indicating an increased potential for fiber degradation and butyrate production. *Butyrivibrio* is well-known for its ability to ferment complex carbohydrates into butyrate, a key short-chain fatty acid associated with lipogenesis [65, 66]. The higher abundance of *Butyrivibrio* in heifers likely facilitates the conversion of dietary fiber into butyrate, increasing the supply of this fatty acid for lipogenesis and contributing to greater fat deposition in heifers [67]. Similarly, *Pseudobutyrivibrio*, a genus closely related to *Butyrivibrio*, is also a significant butyrate producer [68], aligning with the observed increase in backfat thickness in heifers. Additionally, *Butyrivibrio* is involved in biohydrogenation of unsaturated fatty acids [69], which may further influence lipid metabolism and fat composition in cattle. These findings suggest that microbial composition differences are not merely correlative but functionally linked to distinct metabolic strategies in bulls and heifers. *Prevotella* supporting muscle development through propionate-driven gluconeogenesis and *Butyrivibrio* supports lipid accumulation via butyrate-mediated pathways. Acetobacter, an important acetate-producing bacterium, generates acetate through the oxidation of sugars [70]. Previous studies have reported that a decline in *Acetobacter* is associated with reduced acetate levels in the rumen [70, 71]. In this study, *Acetobacter* was significantly more abundant in heifers, which may partially explain their higher acetate concentration. The positive correlation between *Acetobacter* and total VFAs suggests its contribution to acetate production. However, given the relatively low abundance of *Acetobacter*, further investigation is needed to verify the role of other bacteria in the elevated acetate concentrations observed.

The correlation and RDA revealed significant associations between rumen fermentation parameters, microbial taxa, and economically important production traits. The positive correlation between backfat thickness and total VFAs, acetate and butyrate concentrations, and the acetate/propionate ratio indicates that higher VFA availability, particularly acetate and butyrate, promotes lipogenesis and fat deposition in cattle. For eye muscle area, the positive correlation with *Lachnospiraceae NK3A20 group* is possibly due to its role in fiber degradation and energy metabolism [72]. The RDA results further highlighted the major role of propionate and butyrate in shaping the microbial community. Notably, *Rikenellaceae RC9-gut group* and *Prevotellaceae UCG-003* were positively associated with BFT, while *Saccharofermentans* correlated with EMA, suggesting distinct microbial contributions to fat and muscle deposition [73]. The Random Forest model identified acetate, propionate, and butyrate concentrations, amino acid metabolism, and key microbial taxa as influential predictors for BFT, whereas *Prevotella*, *Ruminococcus*, *U29-B03*, and NH_3-*N* were the most relevant for EMA. The relatively low R^2 for EMA prediction suggests that muscle development is regulated by additional genetic and environmental factors beyond microbial influence [74]. These findings highlight the potential of microbiome-driven interventions in fat regulation but indicate that EMA prediction requires a more integrative approach, including host genetic and metabolic data.

Overall, the observed sex-based differences in rumen fermentation, microbial diversity and composition in Qinchuan cattle highlight the intricate relationship between host physiology and microbiota. However, the study has several limitations. First, the sample distribution was uneven, which may affect the generalizability of the results. Although power analysis supports the reliability of the observed differences, future studies with a more balanced sample size are needed for validation. Furthermore, this study did not measure testosterone and estrogen levels, which play key roles in sex-specific metabolic differences. Future research should integrate hormonal data to better understand their influence on rumen microbiota and fermentation dynamics. Moreover, the study focused only on Qinchuan cattle, without considering breed-specific variations in microbial composition and metabolism. Expanding the study to multiple breeds would provide deeper insights into how genetic background influences host-microbiota interactions. By addressing these limitations, future studies can enhance our understanding of the complex relationships between sex, microbiota, and production traits in cattle.

Conclusion

This study highlights the significant sex-specific differences in backfat thickness, eye muscle area, rumen fermentation parameters, microbial composition, and metabolic pathways in Qinchuan cattle under the same feeding conditions. Heifers had higher backfat thickness, while bulls exhibited larger eye muscle areas. Rumen fermentation analysis showed that NH_3-N and propionate concentrations were higher in bulls, while butyrate concentration and acetate/propionate ratio were higher in heifers, and acetate showed a tendency to be higher in heifers. Gene expression related to VFA transport was also higher in bulls. Microbial diversity was greater in heifers than in bulls. The relative abundance of *Prevotella* and *Lachnospiraceae_NK3A20_group* was significantly increased in bulls, while *Butyrivibrio* and *Pseudobutyrivibrio* were significantly more abundant in heifers. Functional predictions revealed that pathways involved in "Lipid metabolism", "Carbohydrate metabolism" and "Xenobiotics biodegradation and metabolism" were significantly enriched in heifers, while "Amino acid metabolism" and "Glycan biosynthesis and metabolism" were enriched in bulls. Correlation analyses showed that backfat thickness was positively associated with acetate and butyrate production and the acetate/propionate ratio, while it was negatively associated with *Veillonellaceae_UCG-001*. RDA analysis identified propionate and butyrate as key drivers of microbial community differences. The Random Forest model identified key predictors for BFT, including rumen fermentation parameters, microbial taxa, and metabolic pathways, explaining 28% of its variation. However, EMA was not well predicted by the current parameters, suggesting that factors beyond the rumen microbiome play a more significant role. These findings provide a deeper understanding of how sex-specific microbial and metabolic profiles influence nutrient utilization and body composition, offering potential strategies for enhancing livestock management and breeding programs.

Abbreviations

VFA	Volatile fatty acids
BFT	Backfat thickness
NH_3-N	Ammonia nitrogen
OM	Organic matter
CP	Crude protein
NDF	Neutral detergent fiber
ADF	Acid detergent fiber
EE	Ether extract
ME	Metabolic energy
ASVs	Amplicon sequence variants

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Not applicable.

Authors' contributions

LZ: Conceptualization, Methodology, Supervision. WY: Conceptualization, Methodology. CM: Investigation, Resources. YP: Writing—original draft, Data curation, Formal analysis. GS: Investigation, Data curation. GL: Investigation, Data curation. SC: Investigation, Data curation. H-Liu: Investigation, Data curation. H-Li: Investigation, Data curation. All authors reviewed and approved the final manuscript.

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Data availability

All amplicon sequences are available in the Genome Sequence Archive of the National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences repository, under accession number CRA020672 (https://ngdc.cncb.ac.cn/gsa).

Declarations

Ethics approval and consent to participate

Approval of animal use and permission for specimen collection in this study was granted by the the Animal Welfare and Ethics Committee of Northwest A&F University under the laboratory animal operation guidelines (Protocol Number: NWAFUCAST2018-168). Written informed consent was obtained from the owners for the participation of their animals in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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