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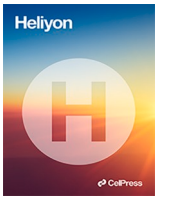

Alfalfa hay substitution for wheat straw improves beef quality via rumen microflora alteration

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ABSTRACT

The use of high-quality roughage to improve beef quality has become an important issue in China, as the country has become the world's largest beef consumer. This study aimed to evaluate the effects of different forage qualities (wheat straw vs alfalfa hay) on Simmental crossbreed cattle's meat quality, rumen fermentation and microbiota. AHG (Alfalfa hay group) improved the ADFI (Average daily feed intake) and ADG (Average daily gain) of the beef cattle, meat-to-bone ratio and EE (Ether extract). The C18:3n3 and C20:3n3 composition of LD in AHG was significantly higher than WSG. An increase in the relative abundance of *Firmicutes* and a decrease in *Bacteroidetes* was observed. AHG resulted in higher relative abundance of *Saccharomonospora*, *Streptomyces*. A negative correlation between *Treponema* and muscle PUFA was noticed. *Prevotella* was negatively correlated with starch and sucrose metabolism. In conclusion, current study demonstrates that feeding alfalfa hay can raise meat quality by altering the rumen microbiota, providing valuable information for the application of alfalfa hay in beef cattle breeding.

1. Introduction

With China's economy and population advancing, it has escalated into the largest beef importer worldwide [1]. The quality of beef has become a burgeoning apprehension for consumers, with their mindful discernment towards the diet-heart theory, demonstrating that an unbalance in cholesterol and fat consumption might drive the onset of cardiovascular disease (CVD). In support of sound dietary practices, health practitioners worldwide advocate a decline in the uptake of SFAs and an upsurge in unsaturated fatty acids [2]. Market research has substantiated that customers are prepared to pay more for beef selections that are healthier [3].

Several factors have a significant impact on the quality of beef, including genetics, region, gender, and primarily diet composition. Inclusion of roughage in beef cattle's diet is vital for maintaining rumen health and preventing subacute acidosis. Historically, low-quality and inexpensive wheat straw has been used as roughage in the majority of beef cattle rations in China, however, its low digestibility and inadequate nutrient provision for the rumen and animal absorption render it an unsuitable source of roughage. The production of high-quality beef necessitates the utilization of high-quality roughage, alfalfa, a high-quality forage grass with high

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List of abbreviations

OTU	Operational Taxonomic Units
NDF	Neutral Detergent Fiber
ADF	Acid Detergent Fiber
DMI	Dry Matter Intake
SFAs	Saturated Fatty Acids
ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
VFAs	Volatile Fatty Acids
MUFA	Monounsaturated Fatty Acid
PUFA	Polyunsaturated Fatty Acid
EE	Ether extract
LD	Longissimus dorsi

protein content and abundant unsaturated fatty acids has been shown to improve growth performance and meat quality when fed to ruminants [4]. The digestion of roughage in ruminants is dependent on the unique digestive organ known as the rumen. The ability of ruminants to digest and absorb structural carbohydrates found in plants is facilitated by the presence of rumen microorganisms. The VFAs (Volatile fatty acids) produced by these microorganisms through fermentation are employed by the host and eventually transformed into microbial proteins [5]. The composition of the diet is the primary factor that induces changes in the rumen microorganisms. A shift towards a diet containing higher levels of concentrate has been shown to reduce the richness and diversity of the rumen bacteria, as evidenced by a decline in the relative abundance of *Bacteroides* and *Firmicutes* [6]. Conversely, feeding dairy cows with alfalfa hay instead of corn stalks can increase the rumen microbial richness, this leads to alterations in the prevalence of bacterial genera like *Prevotella* [7]. Additionally, high levels of dietary starch have been associated with a decrease in rumen microbial α diversity and the relative abundance of *Fibrobacteres* and *Spirochaetes* [8]. Existing studies predominantly concentrate on specific aspects of alfalfa hay's impact, such as growth performance, beef quality, or rumen microflora [9–11]. Comprehensive investigations evaluating the overall effect of alfalfa hay on beef cattle are relatively scarce Therefore, this study hypothesized that different qualities of forage (wheat straw vs. alfalfa hay) in the diet would selectively impact the colonization of rumen bacteria, high quality roughage leads to improved meat characteristics and lipid profile. The objective of this study was to assess the impact of high-quality forage (alfalfa hay) and low-quality forage (wheat straw) in diets on growth performance, meat characteristics, muscle fatty acids, rumen fermentation, and rumen microorganisms. The study also aimed to investigate the associations among rumen microorganisms and meat characteristics, FAs, and rumen fermentation parameters, and provided reference for the application of alfalfa hay in beef cattle breeding.

2. Materials and methods

2.1. Animals, diet, and experimental design

The Institutional Animal Ethics Committee of Henan Agricultural University approved the research (Number: HENAU-2020-036). This research was conducted at Shuangmiao farm (Henan province, China). Two dietary treatments, AHG and WSG, were applied to 40 Simmental hybrid cattle (4 replicates in each treatment and 5 cattle in each replicate, both AHG and WSG treatments consisted of 20 cattle each, Supplementary Material Fig. 1 shows the experimental design). (Supplementary Table S1 displays the feed's constituents and chemical composition.), each weight 415 kg, they were all 18 months old, with an entirely random design during a 7 d adaptation phase followed by a 90 d feeding phase (Total 97 d). At 7 a.m. and 2 p.m., TMR feed was delivered, while manure disposal and barn spray disinfection were done regularly, and feeding management and vaccinations were carried out using standard procedures. All the animals were managed jointly. The starting and ending weights were measured as initial weight and final weight.

2.2. Sample collection

For each treatment, 50 ml of rumen fluid from one ccattle chosen randomly from each replication was taken days before slaughter (97 d). The rumen fluid was promptly submerged in liquid nitrogen and kept cold until bacterial diversity testing. One day before slaughter (97 d), the 8 cattle from which rumen fluid was collected were subsequently transported to Hengdu Commercial Abattoir in Henan Province, China. All the animals were stunned and exsanguinated by skilled workers following the animal welfare protocols. Body weight and $pH45_{min}$ (A portable pH meter, SFK-Technology, Copenhagen, Denmark) were collected after the slaughter, animal corpses were divided in half and aged at 4 °C. Steaks measuring 2.5 cm in thickness were obtained from the left posterior LD before aging, following aging and pH48 was noted.

2.3. Meat quality measurements

Ash, crude protein, moisture, and EE were determined using samples of aged muscle, according to Zhong et al. [12]. To calculate drip loss, aged muscle samples were utilized, and the samples were reweighted 24 h later at 4 °C.

2.4. Fatty acid analysis

To identify fatty acids, 1 g muscle tissue was subjected to methylation, a Gas Chromatography (8890-7000D GC/MSD; Agilent, Santa Clara, CA, USA) outfitted with a Flame ionization detector and a 30-m fused silica capillary column with 0.18 mm inner diameter and 0.2 m film thickness (Agilent J&W Scientific, Folsom, CA, USA) was used to separate and quantify methyl esters. The GC procedure was initiated at 80 °C and maintained for 0.5 min before being ramped up to 175 °C at a rate of 70 °C per minute. This temperature was then sustained for 1 min before being increased to 230 °C at a rate of 8 °C per minute, and finally held at 80 °C for 2 min. The carrier gas, helium, flowed through the system at a rate of 1.0 ml per minute.

2.5. Determination of rumen fermentation parameters

The pH level of the rumen fluid was determined during fluid collection with a pH meter (Sartorius PB-10, Sartorius, Göttingen, Germany). The level of SCFAs in the fluids from the rumen was measured using GC. The HP-88 column was used to analyze the samples (100 m length, 0.25-mm diameter, and 0.2-μm film thickness), separation was achieved via the use of Trace 1310 GC equipped with FID. The initial temperature was set to 70 °C for 1 min, followed by a 25 °C/min increase to 180 °C for 1 min, then maintained at 200 °C for 11 min, then a 10 °C/min increase to 220 °C and a 10 min hold time, and finally increased to 240 °C at 20 °C/min for a 6 min hold time. The sample was run at a column flow rate of 1.3 ml/min and a split ratio of 20:1. Hydrogen was used as the carrier gas. The injector temperature was set at 270 °C, and the detector temperature was set at 290 °C.

2.6. Rumen bacterial diversity analysis

Microbial DNA was extracted from rumen liquid samples using an E.Z.N.A. soil DNA kit (Omega Bio-Tek, Norcross, GA). The concentration and purity of the DNA were evaluated using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, DE), and the purity was additionally assessed by 1 % agarose gel electrophoresis. The hypervariable regions V3–V4 of the bacterial 16S rRNA gene were amplified by PCR (GeneAmp 9700; ABI) with primers 338F and 806R (5′-ACTCCTACGGGAGGCAGCAG-3′ and 5′-GGACTACHVGGGTWTCTAAT-3′), using the following conditions: 3 min of denaturation at 95 °C, 27 cycles of 30 s at 95 °C, 30 s of annealing at 55 °C, and 45 s of elongation at 72 °C, and a final extension for 10 min at 72 °C. Each PCR experiment used a 20-μl reaction mixture containing 4 μl of 5 × FastPfu buffer, 2 μl of 2.5 mM deoxynucleoside triphosphates, 0.8 μl of each primer (5 M), 0.4 μl of FastPfu polymerase, and 10 ng of template DNA. The PCR products were purified using the AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA) following extraction from 2 % agarose gels, and their concentration was measured with a QuantiFluor-ST device (Promega), according to the manufacturer's guidelines. The purified amplicons were combined in equal proportions and sequenced using paired-end sequencing (2 × 300 bp) on an Illumina MiSeq platform (Illumina, San Diego, CA) using standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The study's data collections are available on NCBI under accession number PRJNA992952.

2.7. Bioinformatics analysis of sequencing data

In order to maintain quality control, the original fastq files underwent quality filtering with Trimmomatic and were subsequently merged using FLASH based on certain criteria. These criteria involved the truncation of reads that had an average quality score below 20 within a 50 bp sliding window, as well as the combination of sequences with an overlap length greater than 10 bp, taking into account the degree of overlap while allowing for no more than 2 bp mismatches; Barcodes and primers were used to isolate each sample's sequences. The operational taxonomic units (OTUs) were categorized using a 97 % similarity threshold with UPARSE, and any chimeric sequences were detected and removed using UCHIME. The biodiversity of the samples was assessed using the ACE, Chao1, and Shannon indices. Graphs and colony histograms were generated using R tools. Column charts are used to display changes in bacteria's relative abundance. The composition of the rumen microbiota was analyzed using weighted Unifrac principal coordinate analysis and hierarchical cluster analysis of Bray-Curtis data depending on OTU level. To investigate the relationship between the posterior segment microbiota and apparent performance, redundancy analysis (RDA) was conducted at the genus level using the vegan package in the R programming language. Majorbio Co., Ltd., a Chinese company, used PICRUSt2 for Pathway to acquire information on three levels of metabolic pathways, the relative abundance tables for each level were acquired and analyzed subsequently.

2.7. Statistics and analysis

One-way analysis of variance (ANOVA) was used to analyze the data using the SPSS 23.0 program (IBM, New York, NY, USA). Statistical significance was defined as a P value of 0.05.

3. Results

3.1. Growth performance and carcass characteristics

Table 1 shows the impact of various diets on the growth performance and carcass characteristics. The AHG group exhibited a significantly higher ADFI, ADG and meat mass/bone mass compared to the WSG group ($P < 0.05$). No significant differences were observed between the two separate dietary groups in F/G, cold carcass weight, F/G, cold carcass weight, dressing percentage, net meat mass, net meat percentage, and loin-eye area ($P > 0.05$).

3.1.1. Meat quality

Table 1 displays the results of meat quality analysis, where it was observed that the EE content in beef was significantly higher in the AHG group ($P < 0.05$), but different diets did not affect moisture, ash, crude protein contents of meat, cooking yield, drip loss at 24 h, pH45min, and pH48.

3.2. Fatty Acids Composition

Different muscle FA compositions of the LD muscle were found in the WSG and AHG (Table 2), the AHG group exhibited a trend towards an increase in C18:2n6 (P = 0.09) and PUFA (P = 0.07) in comparison to WSG. Meanwhile, the alfalfa group diet significantly increased the content of C18:3n3, C20:3n3 and n−3 PUFA in the longissimus dorsi muscle ($P < 0.05$).

3.3. Rumen fermentation

Table 3 displays that no significant differences were observed in pH values between the two groups, in general, compared to the WSG group, The consumption of alfalfa resulted in a significant decrease in rumen propionate content, while significantly increasing rumen acetic acid content and the A/P($P < 0.05$), no significant differences in the content of NH3–N, butyrate, isobutyric, valeric, and isovaleric were observed ($P > 0.05$).

3.4. Rumen microbes

The Illumina MiSeq sequencing equipment generated a total of 1165785 high-quality 16S rRNA gene sequences. However, during the analysis, it was observed that over 5 % of these sequences (58893 out of 1165785) were identified as chimeric and were subsequently excluded from further analysis. After filtering, the average length of the remaining sequence reads was found to be 414 bp. Alpha diversity analysis at the OTU level was performed, and it was observed that the Chao, Sobs, and Shannon indices did not exhibit significant shifts between the groups (Fig 1 A, B, and C, $P > 0.05$). Subsequently, beta diversity analyses (Fig 1 D) were conducted, which revealed similarities in microbial diversity between the WSG and AHG groups. Fig 2 displays the microbial composition at both

Table 1
Performance, carcass characteristics, chemical compositions, and meat quality of longissimus dorsi muscle in Simmental crossbred steers (DM%). crossbred cattle muscles.

Items	WSG	AHG	SEM	*P*-Value
Growth performance				
Initial Weight/kg	403.25	414.00	6.04	0.59
Final Weight/kg	546.58	585.92	11.95	0.06
ADFI/kg/d	9.08[b]	10.99[a]	0.84	0.05
ADG/kg/d	1.48[b]	2.02[a]	0.09	0.01
F/G	6.02	5.51	0.68	0.60
Carcass characteristics				
Cold carcass weight, kg	295.50	320.00	11.76	0.11
Dressing percentage, %	0.57	0.56	0.02	0.24
Net meat mass, kg	262.69	287.96	10.62	0.14
Net meat percentage, %	0.51	0.50	0.02	0.24
Meat mass/Bone mass	8.01[b]	9.00[a]	0.20	0.03
Loin-eye area, cm	130.55	138.25	9.89	0.67
Chemical compositions				
Moisture %	0.26	0.26	0.01	0.36
Crude protein %	22.04	21.79	0.78	0.42
EE %	3.11[b]	4.04[a]	0.32	0.02
Ash %	0.83	1.02	0.25	0.80
Meat quality				
Cooking yield, %	0.60	0.60	0.02	0.96
Shear force (N)	104.02[a]	83.44[b]	7.01	0.03
Drip loss at 24 h, %	0.03	0.04	0.01	0.81
pH45min	5.43	6.06	0.33	0.67
pH48	6.57	6.89	0.09	0.25

Table 2
The Effects of Different Dietary Forage Combinations on fatty acids in Simmental crossbred cattle muscle.

Items (% of total fatty acid)	WSG	AHG	SEM	*P*-Value
C14:0	2.63	3.13	0.27	0.24
C14:1	0.54	0.77	0.20	0.44
C15:0	0.36	0.39	0.03	0.49
C16:0	28.48	30.02	0.56	0.10
C16:1	3.47	4.00	0.50	0.48
C17:0	0.79	0.75	0.05	0.64
C17:1	0.65	0.56	0.04	0.19
C18:0	17.23	15.77	1.24	0.35
C18:1	39.91	40.34	1.02	0.78
C18:2n6	2.78	4.25	0.50	0.09
C18:3n6	0.09	0.08	0.01	0.54
C18:3n3	0.20[b]	0.67[a]	0.03	0.06
C20:0	0.10	0.09	0.01	0.60
C20:1	0.15	0.15	0.01	0.87
C20:3n3	0.41[b]	1.04[a]	0.13	0.03
C24:1	0.12	0.06	0.02	0.19
SFA	50.66	49.08	1.19	0.55
MUFA	45.83	44.72	1.46	0.62
PUFA	3.45	6.08	0.65	0.07
n−6 PUFA	2.86	4.34	0.50	0.09
n−3 PUFA	0.59[b]	1.74[a]	0.16	0.05
n−6 PUFA/n−3 PUFA (%)	4.91	3.67	0.44	0.10

SFA, saturated fatty acids (14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0).
MUFA, monounsaturated fatty acids (14:1 + 16:1 + 17:1 + 18:1 + 20:1).
PUFA, polyunsaturated fatty acids (18:2n−6 + 18:3n−3 + 18:3n−6 + 20:3n−3).
n−6 PUFA (C18:2n6+18:3n−6).
n−3 PUFA (18:3n−3 + 20:3n−3).
a,b Means within rows with different superscript letters differ ($P < 0.05$).

Table 3
Rumen fermentation parameters in Simmental crossbred cattle.

Items	WSG	AHG	SEM	*P*-Value
pH	6.53	6.43	0.69	0.235
NH_3–N/mg/dl	40.66	36.20	7.87	0.187
Acetic acid/mmol/L	40.02[a]	34.48[b]	1.92	0.028
Propionic acid/mmol/L	11.06[b]	15.85[a]	1.74	0.033
Butyrate acid/mmol/L	9.74	12.02	2.74	0.371
Isobutyric acid/mmol/L	0.29	0.41	0.11	0.631
Valeric acid/mmol/L	1.15	1.11	0.04	0.276
Isovaleric acid/mmol/L	0.50	0.62	0.12	0.318
A/P	3.79[a]	2.21[b]	0.54	0.027

the phylum and genus levels. At the phylum level, the most abundant microorganisms in both groups were *Firmicutes* (WSG 52 %, AHG 59 %, Fig 2A) and *Bacteroidetes* (WSG 31 %, AHG 44 %, Fig 2A). At the genus level, *Prevotella* (WSG 21 %, AHG 8.7 %, Fig 2 B), *Rikenellaceae_RC9_gut_group* (WSG 7.7 %, AHG 7.7 %, Fig 2 B), and *NK4A214_group* (WSG 6.2 %, AHG 7.1 %, Fig 2 B) were found to be the dominant genera. At the phylum level, no significant differences in microbial composition were observed between the two groups (Fig 3 A, $P > 0.05$). Additionally, the relative abundance of *unclassified_f_Oscillospiraceae* in the AHG group was observed to be lower compared to the WSG group (Fig 3 B, $P < 0.05$). Furthermore, the AHG group exhibited a significantly higher relative abundance of *Saccharomonospora*, *Streptomyces*, *norank_f_Actinomycetaceae*, and *Oceanobacillus* in comparison to the WSG group (Fig 3 B, $P < 0.05$).

3.5. The correlation of bacterial genera between meat quality and VFA concentrations

For each breed, Spearman correlations were independently obtained to connect the bacterial genera to the rumen fermentation parameters and muscle fatty acid deposition. Only top 15 relative abundant genera were considered which showed significant connections. The groups of *norank_f_muribaculaceae* exhibited a significantly negatively relationship with butyric acid content in the rumen, and the groups of *Treponema* were significantly negatively correlated with the muscle PUFA (Fig 4 A, B). At the same time, our investigation indicated a meaningful direct correlation between *norank_f_Actinomycetaceae* and the level of propionic acid in the rumen, we also observed a significant positive correlation between *Oceanobacillus* and the concentration of acetic acid in the rumen (Fig 4 C, D).

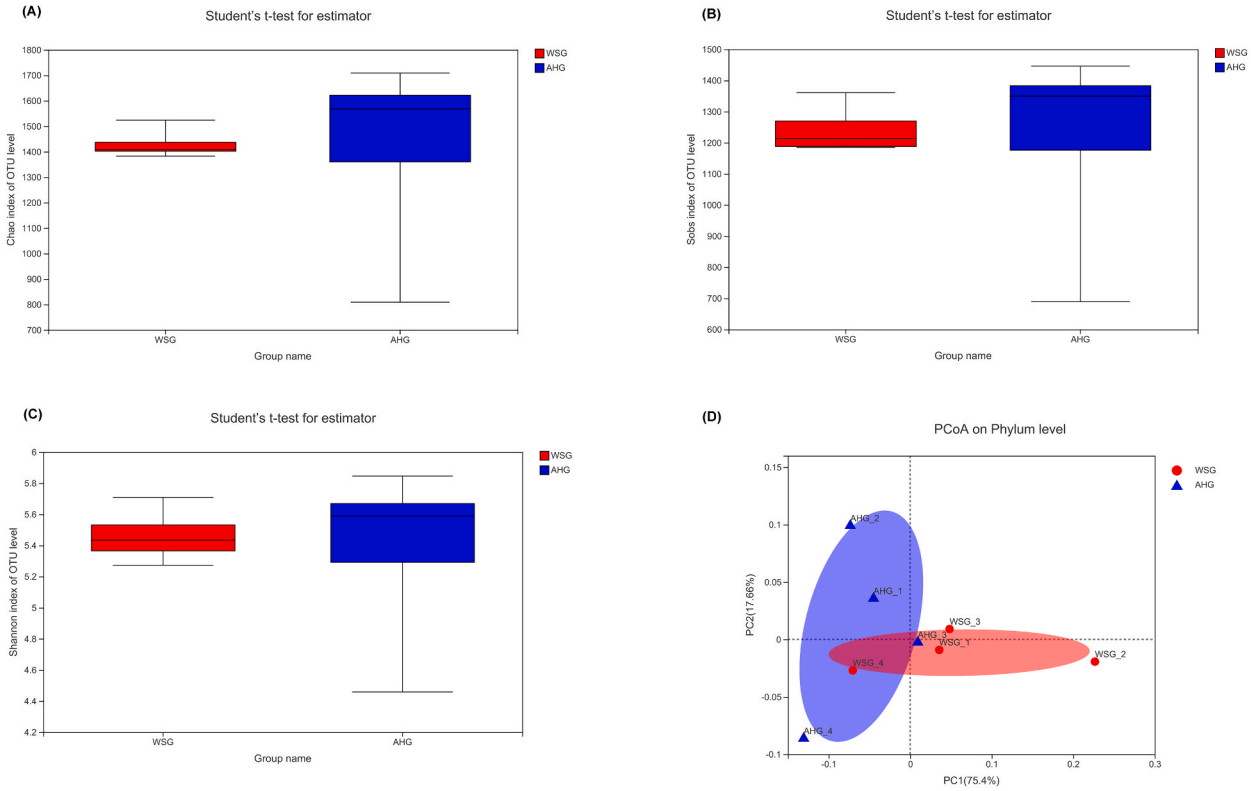

Fig. 1. | Alpha and Beta diversity statistics comparison. **(A)** Chao index of OTU level. **(B)** Sobs index of OTU level. **(C)** Shannon index of OTU level. **(D)** Principal coordinate analysis (PCoA) of rumen bacterial community structures of steers in the three groups. PCoA plots based on weighted UniFrac distance. Steers were fed a wheat straw diet (WSG), n = 5; Steers were fed an alfalfa hay diet (AHG), n = 5. Error bars represent standard deviations and their lengths are adjusted at a 95 % confidence interval.

3.6. The effects on the metabolic pathway

The results of the PICRUSt2 function prediction are shown in the Fig 5 A, Only the top 50 metabolic pathways out of 205 retrieved were considered. The top three pathways include Metabolism, Genetic information processing, and Cellular processes. Meanwhile, Fig 5 B shows the differential pathways. The consumption of alfalfa diet resulted in a significant increase in the Staurosporine biosynthesis, Renin secretion, Biosynthesis of type II polyketide backbone, Tetracycline biosynthesis pathways ($P < 0.05$), and greatly improved the Calcium signaling pathway, Hypertrophic cardiomyopathy (HCM) ($P < 0.01$). Fig. 6 displays the associations among rumen bacteria and metabolic pathways, the relative abundance of *Prevotella* strongly inversely linked Starch and sucrose metabolism. The relative abundances of *Ruminococcus* were significantly negatively correlated between the Pentose phosphate pathway, conversely, the relative abundances of *Ruminococcus* were significantly positive association with Alanine, aspartate, and glutamate metabolism pathways. The relative abundances of *Rikenellaceae_RC9_gut_group* were significantly negatively correlated with 2-Oxocarboxylic acid metabolism. The relative abundances of *NK4A214_group* were significantly positively correlated with Glycolysis/Gluconeogenesis, Phenylalanine, tyrosine and tryptophan biosynthesis, Glyoxylate and dicarboxylate metabolism, Biosynthesis of secondary metabolites, Purine metabolism, Methane metabolism, Metabolic pathways and Glycine, serine and threonine metabolism.

4. Discussion

Growth performance and slaughter performance are key indicators in measuring the profitability of beef cattle farming. In this study, there was a tendency for the AHG diet to increase growth performance in beef cattle compared to the WSG diet, which was probably due to the alfalfa hay's higher CP and lower NDF levels. Higher levels of NDF in the diet can reduce feed intake by increasing ruminant time and reducing digestibility. Madruga [13] examined the impact on beef cattle growth performance of switching from 10 % wheat straw to 19 % alfalfa hay, with the alfalfa diet providing lower levels of NDF than the wheat straw diet and the same reduction in intake observed with the wheat straw diet. Swanson [14] used different sources of forage to feed beef cattle and also mentions that wheat straw diets reduce intake compared to alfalfa and corn silage diets. The cold carcass weight of steers fed alfalfa hay increased, along with net meat mass, meat mass/bone mass, and loin-eye area, despite no significant effect of the two roughage diets on slaughter performance. These findings further substantiate alfalfa hay's superiority as a roughage source, enhancing the fattening effect and slaughter performance of Simmental crossbred steers.In beef production, the fat content of muscles is a critical factor. Higher fat

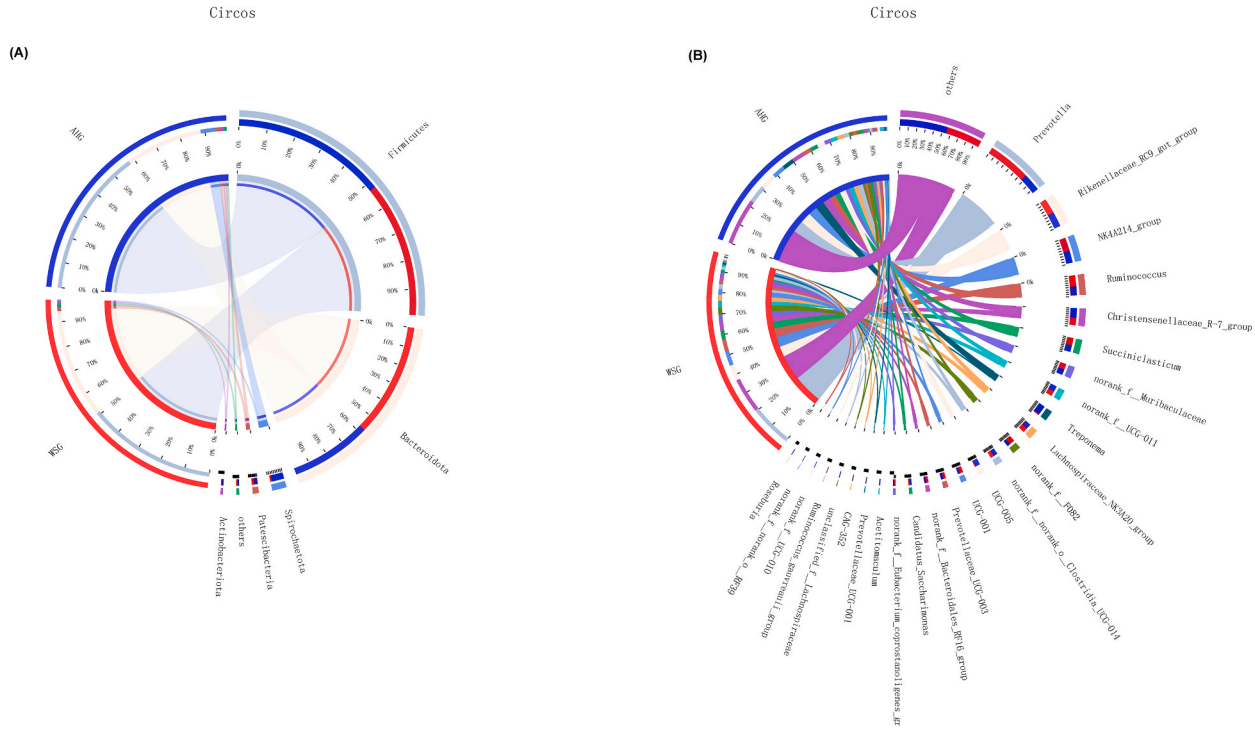

Fig. 2. | The relative abundance of phyla and genera in the rumen bacteria community. **(A)** Community abundance on phylum level in two treatment groups. **(B)** Community abundance on genus level in two treatment groups. Steers were fed a wheat straw diet (WSG), n = 5; Steers were fed an alfalfa hay diet (AHG), n = 5.

content directly influences meat shear force, making it easier to chew and promoting fragmentation during chewing. In the present study, the AHG group exhibited superior meat quality compared to the WSG group, with significantly higher fat deposition percentage in muscles and improved muscle shear force. Both treatment groups' pH48 values fell within the typical range, reflecting protein hydrolysis activity and muscle glycolysis rate after slaughter, ensuring product reliability.Rumen fermentation and the nutritional makeup of alfalfa hay may be connected to the improvement in fat deposition for the AG group. As a result, feeding Simmental crossbred steers alfalfa hay throughout the fattening stage has the potential to provide excellent-grade meat. On the other hand, The FA content of fat is thought to be significant for maintaining human health. It is well known that a high consumption of dietary cholesterol and fat has been linked to the development of atherosclerosis and cardiovascular disease (CVD) [2]. SFAs, such as C14:0 and C16:0, stimulate the creation of cholesterol and the accumulation of low-density lipoprotein, each of these are important hazards for human cardiovascular disease. As a result, researchers have proposed that reducing SFA intake while increasing PUFA intake may help minimize these risks [15,16], unsurprisingly, beef products with better fatty acid profiles have grown in popularity among consumer. The fatty acid content of beef is able to be altered by dietary interventions, feeding beef cattle PUFA-rich alfalfa hay has been shown to considerably boost levels of C18:2n6 and C20:3n3, both of which have been linked to neural development, anti-inflammatory, and cardiovascular benefits.

Improving the composition of FAs in beef necessitates protecting the dietary PUFAs against ruminal biohydrogenation. Microbial lipases in the rumen hydrolyze dietary lipids, primarily releasing PUFAs that are toxic to ruminal microbes. To mitigate the toxic effects, ruminal microbes undergo biohydrogenation of PUFAs, this detoxification mechanism has been confirmed by multiple studies [17–19], meanwhile, n-3 fatty acid levels in beef are typically low, inhibition of rumen hydrogenation is critical to enhance unsaturated fatty acid deposition in beef. By blocking ruminal hydrogenation, alfalfa hay tannins have been discovered to decrease the formation of C18:0 [20], this effect on hydrogenation may be due to tannin influence on bacterial species involved in fatty acid biohydrogenation, which may occur through selective inhibition of cell wall synthesis. Studies suggest that tannins change hydrogen ion production, leading to disintegration of cell walls and liposomes, disruption of oxidative phosphorylation metabolic pathways, and decreased substrates necessary for bacterial growth. These alterations have a direct impact on ruminal fatty acid metabolism [21]. Nonetheless, tannin presence in alfalfa did not significantly affect pH and total VFAs [22], this is in line with earlier findings. The concentration of rumen NH3–N, a diet's protein consumption is favorably associated with a protein degradation product. Interestingly, we observed that compared to WSG, the increase in dietary protein content of AHG did not result in higher rumen NH3–N concentration, but rather in its reduction. The reduction in rumen NH3–N concentration can be attributed to the protective effect of tannins in alfalfa on ingested protein. By generating protein-tannin complexes in the pH 6–7 mildly acidic rumen environment, tannins increase the quantity of rumen-bypass protein, these complexes are protected from degradation by rumen microorganisms and dissociate in the abomasum (pH < 3.5), increasing the amount of protein that can be absorbed in the small intestine.

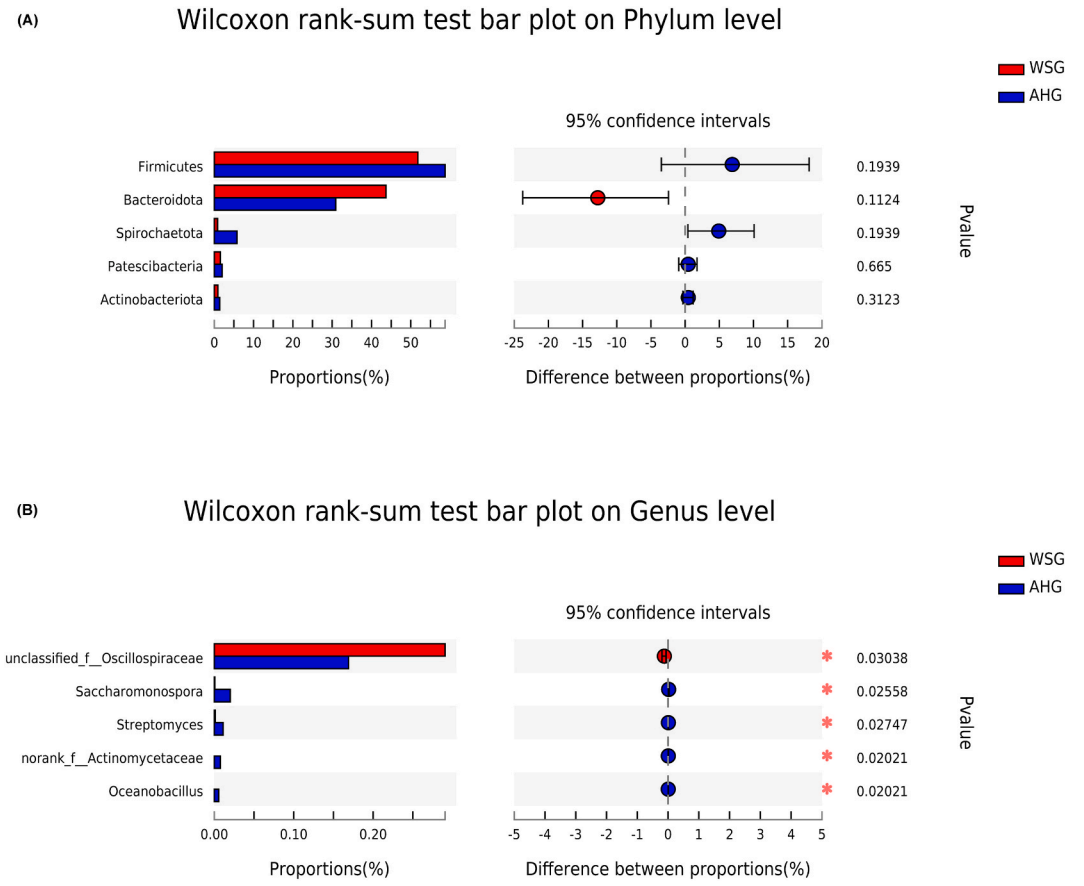

Fig. 3. | Difference of rumen bacteria at the phylum and genus level. **(A)** Difference of rumen bacteria at the phylum level (B) Difference of rumen bacteria at the genus level. WSG, wheat straw group, n = 5; AHG, alfalfa hay group, n = 5. $^{*}P < 0.05$, $^{**}P < 0.01$.

An analysis of the ruminal bacterial composition was also performed, consistent with previous studies [23], the rumen-dominant microbiomes of the two treatments were composed primarily of *Bacteroidetes* and *Firmicutes* at the phylum level, which together accounted for approximately 90 % of bacterial species. Interestingly, the AHG group demonstrated a comparatively higher level of *Firmicutes*, with *Bacteroides* following in relative abundance, while the WSG group exhibited the highest relative abundance of *Bacteroides*, with *Firmicutes* being the second most abundant group. Zhu et al. [24]. also made a similar observation and put forth the hypothesis that the reduction in ruminal pH caused a reduction in the relative abundance of *Bacteroidetes* and an accompanying rise in the relative abundance of *Firmicutes*. Unfortunately, our experimental findings did not disclose a significant variance in ruminal pH. It has been reported in prior investigations that polysaccharides procured from flora-rooted fibers, such as cellulose, xylan, arabinogalactan, and pectin, along with plant-based starches, encompassing amylose and amylopectin, have the potential to act as the cardinal energy reservoir for the *Bacteroides* species [25,26]. Adequate fibrous substrates in WSG diets enhance *Bacteroides*' relative abundance, which plays a crucial role in utilizing low-nutritional feeds in the rumen [27]. According to some research *Firmicutes* can generate enzymes capable of degrading proteins, lipids, and cellulose. In contrast. Krajmalnik et al. [28]. argued that *Firmicutes* utilize energy from nutrient-rich meals more effectively than *Bacteroides*. Correlation with the accumulation of intramuscular fat and the ratio of *Firmicutes* to *Bacteroidetes* in animals has been extensively studied. Guo et al. [29]. investigated the gut microbiome of pigs being fattened and discovered that fat deposition was accompanied by a corresponding increased *Firmicute* relative abundance and *Firmicute* to *Bacteroidetes* ratio in the gut. Jami et al. [30]. made similar observations in cows. Vijay et al. [31]. replicated this phenomenon in obese mice and showed that the fat deposition phenotype could be transferred by fecal microbiota transplantation in germ-free mice. The ratio of *Firmicutes* to *Bacteroidetes* over the past decade as a marker of fat deposition. In this research, the ratio of *Bacteroidetes* to *Firmicutes* was observed to be indicative of a greater level of fat deposition in AHG beef cattle, which concurs with the observed superiority in ADG of AHG beef cattle as compared to WSG. At the genus level, *Prevotella* and *Rikenellaceae_RC9_gut_group* were the predominant microorganisms in both dietary treatments, consistent with previous studies [32]. *Prevotella* is crucial to the rumen microbiome of ruminants, playing a central role in carbohydrate and hydrogen metabolism. It secretes diverse digestive enzymes for carbohydrate utilization, leading to the production of propionic acid, which is essential for hepatic gluconeogenesis in ruminants due to their limited glucose intake [33]. The high prevalence of Prevotella in the rumen is commonly observed in healthy ruminants [34]. Our research showed that using wheat straw as a low-quality feed source increased the relative abundance of *Prevotella*, attributed to

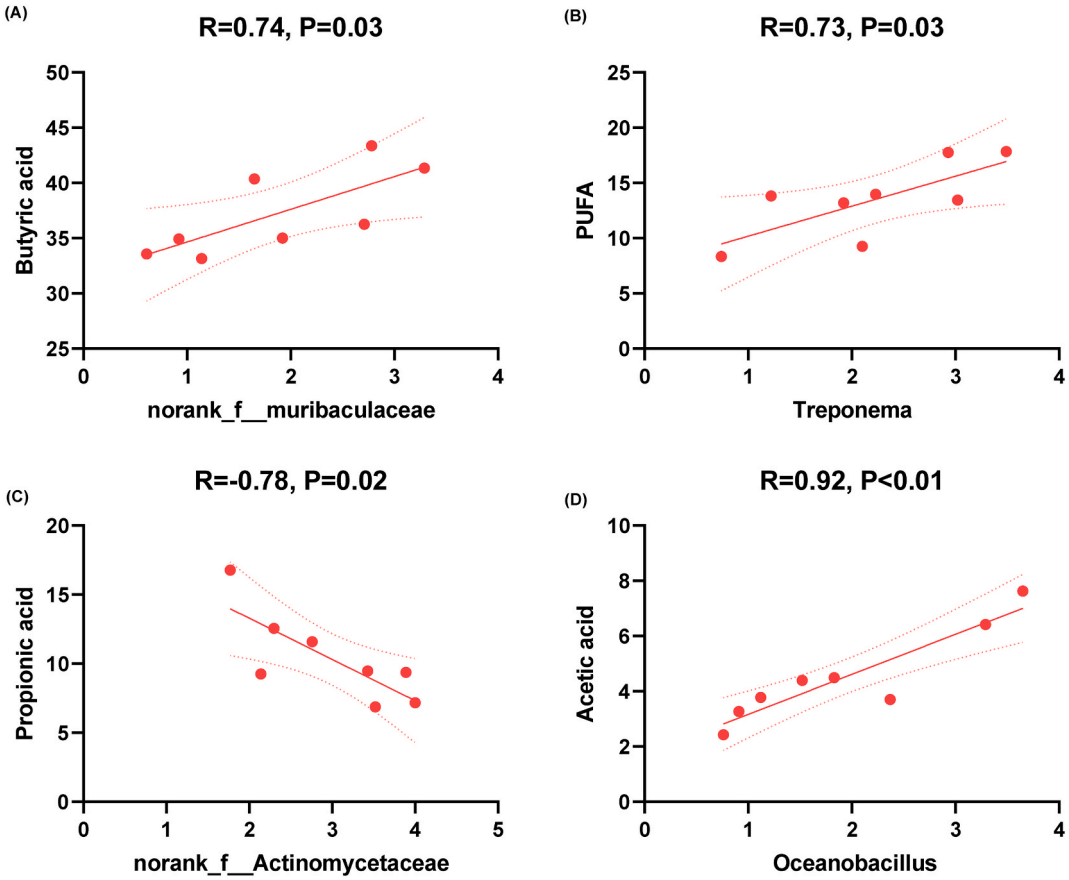

Fig. 4. | Correlation between rumen microorganism and environmental factors (LD Fatty Acids Composition and Rumen fermentation). **(A)** The correlation between the relative abundance of *norank_f_muribaculaceae* and the concentration of butyric acid in the rumen. **(B)** The correlation between the relative abundance of *Treponema* and the concentration of PUFA in LD. **(C)** The correlation between the relative abundance of *norank_f_Actinomycetaceae* and the concentration of Propionic acidin the rumen. **(D)** The correlation between the relative abundance of *Oceanobacillus* and the concentration of Acetic acid in the rumen.

the abundant availability of lignin as a fermentation substrate. Consequently, there was an increase in the concentration of propionic acid, which is the primary metabolite produced by the Prevotella genus. *unclassified_f_Oscillospiraceae* belongs to *Firmicutes*. Fu et al. [35]. discovered that the level of *unclassified_f_Oscillospiraceae* was significantly elevated in the gut microflora of grazing yaks and goats, in comparison to that of ruminants that were barn-fed. Thus, *unclassified_f_Oscillospiraceae* was established as a hallmark microbiota indicative of ruminants that are grazed. More research results also support the conclusion of Fu et al. After in-depth research on African herbivores, Kartzinel et al. [36] found that *Oscillospiraceae* is the most important family of ruminants such as giraffes, cape buffalo, antelopes, and zebra. The diet of wild animals is composed mainly of high-fiber but low-energy plants, which are more difficult to digest. In this context, *Oscillospiraceae* play a crucial part in the breakdown of cellulose, and the primary metabolite produced is butyric acid [37]. In our study, *Treponema* relative abundance and the level of PUFA in the LD muscle were shown to be significantly negatively correlated. *Treponema* in the rumen can be divided into two functional groups, one specialized in the degradation of pectin and the other involved in fiber digestion. Bekele et al. [38]. found that *Treponema* relative abundance was higher in the rumens of ruminants fed an alfalfa diet. Previous studies have demonstrated that the anaerobic degradation of pectin by *Treponema*, represented by *T. saccharophilum*, occurs through the Entner-Doudoroff pathway, and the final fermentation product is acetate, with no other VFAs produced. *T. bryantii*, another representative of *Treponema*, is unable to utilize pectin, but it has been shown to interact effectively with cellulolytic bacteria like *F. succinogenes* [39]. The link between *Treponema* and PUFA in the LD muscle remains to be explored and further research is necessary to gain a deeper understanding.

Upon further correlation analysis between the rumen microbiota and the KEGG pathways, significantly positively correlated were shown between the *Ruminococcus* and *NK4A214_group* and multiple pathways involved in amino acid metabolism. The *Ruminococcus* and *NK4A214_group* belong to the *Firmicute*, and the primary sources of amino acids involve the breakdown of proteins in the diet and the synthesis of rumen microorganisms by *Firmicutes*, which are known to secrete enzymes that can break down proteins. Additionally, ruminants excrete urea into the rumen, and the nitrogen derived from the degradation of urea is indispensable for the synthesis of bacterial protein by ruminal microorganisms. As observed by Jin et al. [40]., *Firmicutes* have the capability for urea metabolism. A

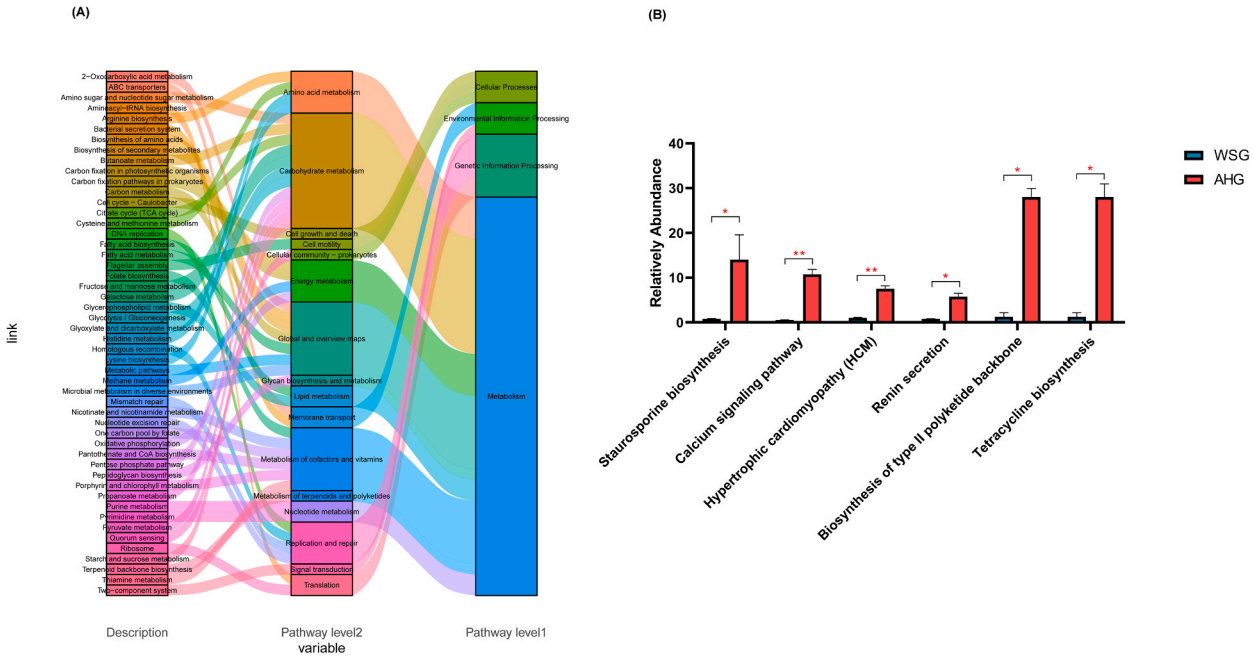

Fig. 5. | Prediction of KEGG pathways by PICRUSt2. **(A)** Top 50 metabolic pathways out of 205 retrieved **(B)** Analysis of differences in rumen microbial functions. $*P < 0.05$, $**P < 0.01$.

further investigation was carried out by Mann et al. [41]. demonstrated that the *Firmicutes* modulate urea exchange through the expression of the urease gene, thereby altering the ruminal ecosystem. This could account for the significant positive correlation between the *Ruminococcus* and *NK4A214_group* with both amino acid metabolism and purine metabolism. Meanwhile, an important finding of our study was a significant negative relationship detected with the relative abundance of *Prevotella* and starch metabolism pathways. The breakdown of starch in the rumen is likely facilitated by the presence of rumen wall bacteria, as evidenced by the expression of glycogen phosphorylase and alpha amylase. Although the specific bacterial species involved in this process has yet to be identified, we speculate that *Prevotella* may engage in a competitive relationship with these bacteria, hindering harmonious coexistence in the rumen. The correlation between the metabolic functions of rumen microorganisms and their impact on host cells remains an area in need of further investigation.

5. Conclusions

In conclusion, our research demonstrates that the inclusion of alfalfa hay as a high-quality forage in the diets of Simmental crossbreed cattle resulted in significant improvements in ADFI and ADG, as well as improved Meat mass/Bone mass and increased crude fat deposition in the LD muscle. The incorporation of alfalfa hay in the diet has been demonstrated to improve the weight gain of beef cattle and facilitate the accumulation of polyunsaturated fatty acids (PUFA). Furthermore, this nutritional intervention elicited modifications in the microbial community residing in the rumen and ameliorated the fermentation process, ultimately resulting in an improvement in the meat quality.

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Author contribution statement

Zimin Gao: Performed the experiments; Wrote the paper. Boshuai Liu; Shaokai La: Performed the experiments. Defeng Li; Xiaoyan Zhu: Analyzed and interpreted the data. Hao Sun; Sen Ma: Contributed reagents, materials, analysis tools or data. Yalei Cui; Yinghua Shi: Conceived and designed the experiments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

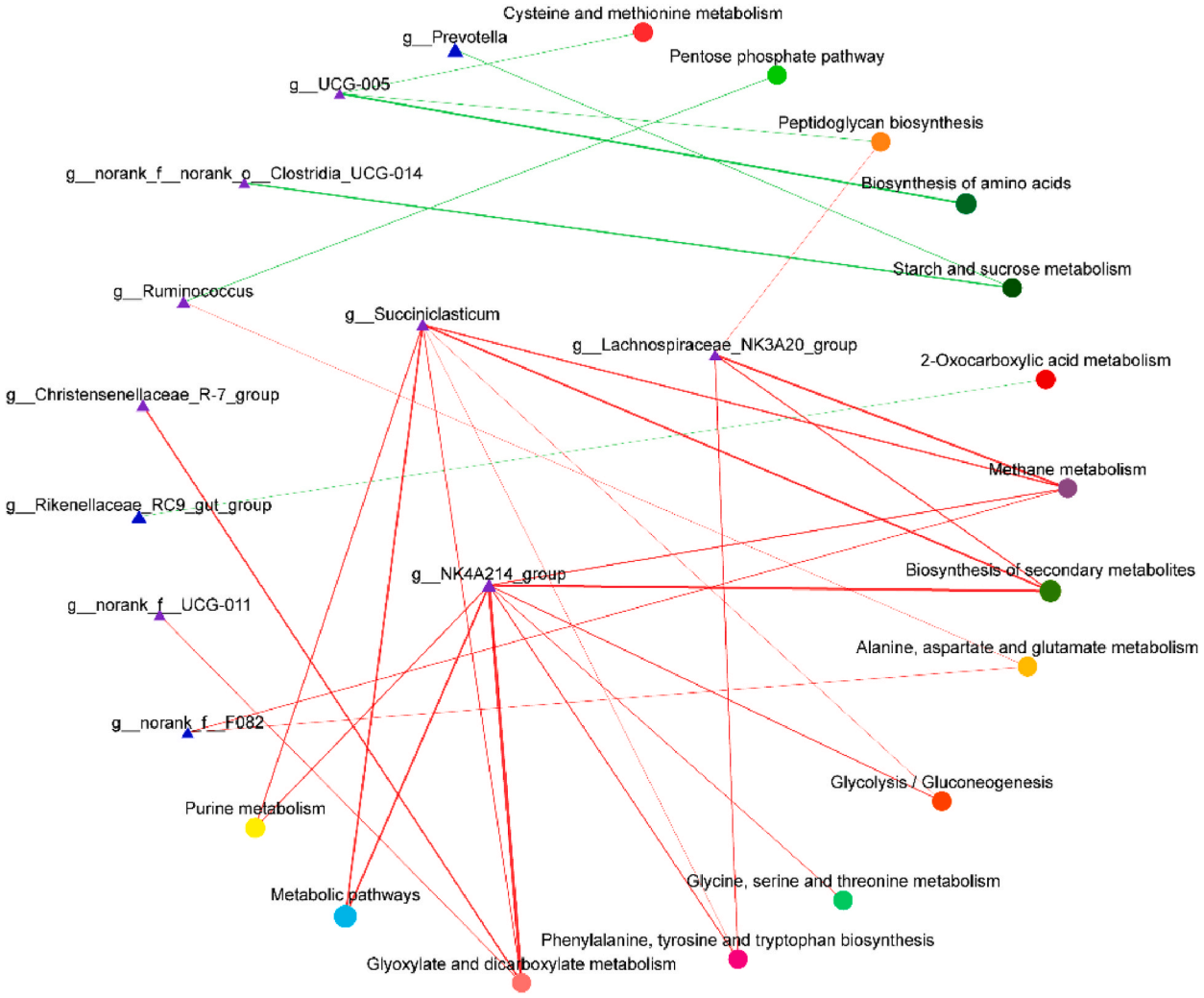

Fig. 6. | Correlation between rumen microorganism and KEGG pathways. Only display significant correlations. The red line indicates a positive correlation, and the green line indicates a negative correlation. The thickness of the line describes the magnitude of the correlation coefficient, The thicker line means a higher correlation between rumen microorganisms and KEGG pathways. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20803.

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