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Original Research Article

Integrated multi-omics reveals the relationship between growth performance, rumen microbes and metabolic status of Hu sheep with different residual feed intakes

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ABSTRACT

Residual feed intake (RFI) is a metric that provides a more accurate measure of feed efficiency. The lower the RFI, the higher the feed efficiency. The changes in the host microbiome and metabolome contribute to the greater feed efficiency of low RFI (LRFI) animals. The aim of this study was to explore the differences in rumen microorganisms, rumen metabolites and plasma metabolites of Hu sheep with differing RFI through the microbiome and metabolome. A total of 80 Hu sheep were used. The experiment consisted of a 15-d pretrial period and a 128-d experimental period. The RFI in the experimental period was calculated for all sheep, and the sheep were screened into high RFI (HRFI, $n = 8$) and LRFI ($n = 8$) groups. The HRFI and LRFI sheep did not differ in their initial and final body weights, average daily gain and body measurements, but the dry matter intake of LRFI sheep was significantly decreased (28.4%, $P < 0.001$). The sheep with LRFI had higher digestibility of crude protein ($P = 0.010$) and ether extract ($P = 0.010$) compared to HRFI group. The concentrations of acetate ($P = 0.036$), propionate ($P = 0.010$), valerate ($P = 0.027$) and total volatile fatty acids ($P = 0.048$) in rumen of LRFI group were higher compared to HRFI group. The results of 16S rDNA sequencing indicated that the sheep with LRFI had higher proportions of *Prevotella* genus in rumen liquid ($P = 0.031$). The rumen metabolome and plasma metabolome results showed that the citrate cycle, pyruvate metabolism and alanine, aspartate and glutamate metabolism processes were more active for sheep in LRFI group, which provided more energy substrate such as malic acid, oxoglutaric acid and citric acid. In conclusion, sheep with LRFI can utilize feed more efficiently, and the more active energy metabolism pathway and the production of energy substances may account for the higher feed efficiency.

1. Introduction

Feed cost is an important factor constraining the development of the livestock industry, which can account for 40% to 60% of the total cost of farming (Shalloo et al., 2004). With the rising cost of feed ingredients worldwide, improving feed efficiency and reducing production costs have become a major concern for the livestock industry (Kenny et al., 2018). Improving feed efficiency not only reduces production costs (Connor et al., 2012), but also improves milk quality (Xie et al., 2021) and reduces greenhouse gas emissions (Hegarty et al., 2007). Scholars have reported a variety of

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methods to evaluate feed efficiency, among which residual feed intake (RFI) proposed by Koch is more commonly used and accurate (Koch et al., 1963). The RFI is the difference between the actual dry matter intake (DMI) of the animal and the predicted DMI based on the energy required to sustain the animal's life activities. The RFI is independent of traits such as body weight (BW), average daily gain (ADG) and DMI, and is not affected by changes in specific growth performance (Hoque et al., 2007; Crowley et al., 2010). Therefore, RFI can more accurately evaluate the feed efficiency of animals.

As the animal's "biodigester", the rumen is rich in a complex community of microorganisms that play an important role in the digestion, metabolism, immunity and even behaviour of the host (Hanning and Diaz-Sanchez, 2015). Feed consumed by ruminants can be degraded by rumen microorganisms to produce products such as volatile fatty acids (VFA) and microbial proteins (MCP) (Liu et al., 2021). The feed efficiency can be affected by many factors including gene expression (Zhang et al., 2019), microbial activity (Liu et al., 2022), and host metabolism (Jorge-Smeding et al., 2022). It has been shown that rumen fermentation variables and nutrient uptake can cause changes in the RFI of cattle (Kong et al., 2016; McDonnell et al., 2016). Ruminal macrogenomics and metabolomics studies in cattle with different RFIs showed differentially expressed ruminal microorganisms and differential metabolites, and there were also significant differences in blood metabolites (Xue et al., 2020). This suggested that differences in host microbiota may cause differences in metabolism, which in turn affect feed efficiency.

Hu sheep is a local protected breed in China. They have good characteristics such as rough feeding tolerance and seasonal estrus. Studies on the liver transcriptome of Hu sheep with different RFI showed that immune-related genes were highly expressed in the high residual feed intake (HRFI) group, leading to an enhanced immune response, increased energy consumption and decreased feed efficiency (Zhang et al., 2019). Additional studies also had reported differences in apparent digestibility (Zeng et al., 2023), rumen microbiota (Zhang et al., 2021a) and the liver transcriptome (Zhang et al., 2021b) in Hu sheep with different RFI. However, few studies have reported differences and associations between rumen microorganisms, rumen metabolism, and plasma metabolism in Hu sheep with different RFI. Therefore, the current study aims to evaluate the differences between rumen microorganisms, rumen metabolism and plasma metabolism of Hu sheep with different RFI and further explore the associations, which can provide a theoretical reference for improving the feed efficiency of Hu sheep.

2. Materials and methods

2.1. Animal ethics statement

All experimental procedures were approved by the Animal Protection and Utilization Committee of Zhejiang Agriculture and Forestry University, Hangzhou, China (ZAFUAC2016005) and were in accordance with the university's animal research rules. The experiment was performed in accordance with the ARRIVE guidelines.

2.2. Animals and experimental design

A total of 80 Hu sheep (postnatal days = 97 ± 6.1 d, BW = 23.33 ± 3.58 kg; mean ± standard deviation [SD]) were used in this study, all of which were from Zhejiang Provincial Animal Husbandry Technology Promotion and Monitoring Station of Breeding Livestock and Poultry, a main breeding sheep farm in the Zhejiang Province. The 80 sheep were evenly distributed into 10 pens, each pen was equipped with electronic devices for radio frequency identification (WL-CD-Y03, Beijing Huiqi Tongda IOT Technology Co., Ltd., China) to record the daily feeding data of each sheep. The experiment included a 15-d pretrial period, which allowed the sheep to adapt the environment, and a 128-d experimental period. The diet was fed at 08:00 and 20:00 daily during the whole experiment, ensuring 24 h free access to feed and water (Table 1). The BW of each sheep was measured at the beginning and end of the experimental period, and the ADG was calculated according to the change in BW.

2.3. RFI calculation

The RFI was calculated for all sheep using individual data from d 16 to 143. The individual DMI, middle body weight (MBW) and ADG were calculated at the end of the experimental period and analyzed by linear regression according to the following formula: $DMI_i = \beta_0 + \beta_1 (ADG_i) + \beta_2 (MBW_i) + e_i$, where β_0 represents the regression intercept; ADG_i is the average daily gain; β_1 represents the extent to which ADG affects feed intake; β_2 represents the extent to which average interim metabolic weight affects feed intake; and e_i represents RFI. The MBW is given by the formula: $MBW_i = [1/2 \times (FBW_i + IBW_i)]^{0.75}$, where FBW is the final BW, and IBW is the initial BW. The sheep were divided into HRFI and low residual feed intake (LRFI) groups according to mean ± 0.5 SD. Eight sheep per group were selected for subsequent analysis (Table S1).

2.4. Measurement of body measurements

Body measurements of sheep were determined according to Zhang (2001). Body height: vertical distance from the highest point of the dorsal fins to the ground; body oblique length: straight line distance from the end of the shoulder to the posterior end of the

Table 1
Ingredients and chemical composition of the experimental diet (%, DM basis).

Item	Content
Ingredients	
Corn grain	27.3
Corn germ meal	32.0
Wheat bran	8.0
Soybean straw	5.0
Soybean meal	2.0
Barley malt root	8.0
Bacterial residue[1]	2.0
Rice husk powder	2.0
Chinese wildrye hay	8.0
Molasses	2.0
Limestone	1.5
$NaHCO_3$	0.2
Premix (fattening sheep)[2]	2.0
Chemical composition	
CP	14.7
EE	2.7
Crude ash	10.0
ADF	11.4
NDF	36.7
Ca	1.4
P	0.4

DM = dry matter; CP = crude protein; EE = ether extract; ADF = acid detergent fiber; NDF = neutral detergent fiber; Ca = calcium; P = phosphorus.
[1] This is the culture substrate left after harvesting of edible mushroom. It mainly included wood chips, cottonseed shells, rice bran, etc.
[2] Provided per kilogram of DM total mixed rations: Cu ≥ 15 mg, Fe ≥ 65 mg, Mn ≥ 60 mg, Zn ≥ 140 mg, I ≥ 1.5 mg, Se ≥ 0.85 mg, Co ≥ 0.05 mg, vitamin A ≥ 13,000 IU, vitamin D ≥ 4000 IU, vitamin E ≥ 40 mg.

sciatic tuberosity; thoracic circumference: circumference around the thorax from the posterior edge of the scapulae to the vertical body axis; thoracic breadth: straight line distance between the posterior edges of the scapulae on both sides; thoracic depth: straight line distance from the highest point of the dorsal fins to the bony surface of the sternum; tail length: distance from the anterior edge of the first caudal vertebrae to the end of the tail; tail breadth: straight line distance from the widest point of the tail in the lateral direction; tail circumference: horizontal circumference around the tail from the widest transverse axis of the tail; testis circumference: the longest circumference of the testes of the rams when they are standing.

2.5. Determination of nutrient apparent digestibility

Feces and diet were collected from 2 groups of sheep at 09:00, 15:00, 21:00 and 03:00 on d 140 to 143 of the trial. The feces and diet were mixed at 4 time points individually and dried at 65 °C for 72 h for nutrient digestibility analysis. The nutrient digestibility was determined using the acid insoluble ash (AIA) method. Briefly, the samples were pulverized using a shredder and passed through a 40-mesh sieve. The AIA in the diets and feces were analyzed according to the method as described by Van Keulen and Young (1977). The following equation was used to calculate the apparent digestibility of the nutrient in the gastrointestinal tract (Zhong et al., 2008): Digestibility (%) = [1 - (AIA concentration in diet/AIA concentration in feces) × (nutrient concentration in feces/nutrient concentration in diet)] × 100.

Ether extract (EE; method 983.15; AOAC, 1990), crude protein (CP; method 988.05; AOAC, 1990), crude ash (method 942.05; AOAC, 1990), dry matter (DM; method 934.01; AOAC, 1990), calcium and phosphorus (Ca and P; method 945.46; AOAC, 1990) in the feces and diet were analyzed. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to the method described by Van Soest (1991), and α-amylase and sodium sulfide were used in NDF analysis.

2.6. Sample collection and determination of rumen fermentation parameters

Rumen fluid samples were collected before morning feeding on d 140 by an oral gastric tube. The collected samples were filtered through 4 layers of gauze and the pH value was determined by using a pH meter (Ph818M, Hong Kong Smart Sensor Ltd., China). After that, the samples were stored at −80 °C for determination of rumen fermentation parameters, microorganisms, and metabolomes. Blood samples were collected from the jugular vein into a heparin tube on d 140 of the trial, and immediately centrifuged at 4000 × *g* at 4 °C for 15 min to obtain plasma. The samples were stored at −80 °C for analysis of metabolomes. The VFA content in the rumen was determined by gas chromatography (Agilent 7890B, CA, USA) by adding 1 mL of 25% metaphosphoric acid to 5 mL of rumen fluid. Ammonia-nitrogen (NH_3-N) concentration was determined according to the method described by Broderick et al. (1980). The concentration of MCP was determined according to the method described by Makkar et al. (1982).

2.7. 16S rDNA amplification and sequencing

Bacterial DNA was extracted from rumen fluid using a PF Mag-Bind Stool DNA kit (Omega Bio-Tek, Norcross, GA, U.S.) and tested using a NanoDrop 2,000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA) for DNA purity and concentration. The DNA integrity was detected by 1% agarose gel electrophoresis set at a voltage of 5 V/cm for 20 min. The V3–V4 region of bacterial 16S rRNA was amplified using the upstream primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and the downstream primer 806R (5′-GGACTACHVGGGGTWTCTAAT-3′). The polymerase chain reaction (PCR) reaction system consisted of 338F 0.8 μL, 806R 0.8 μL, template DNA 10 ng, 5 × FastPfu Buffer 4 μL, 2.5 mmol/L dNTPs 2 μL, FastPfu DNA polymerase, 0.4 μL, BSA 0.2 μL, and made up with ddH_2O to 20 μL. The PCR amplification conditions were: 95 °C for 3 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s (30 cycles), and 72 °C for 10 min. The PCR products were further purified using PCR clean-up Kit and quantified by Quantus Fluorometer assay. Sequencing was performed on an Illumina PE300 platform (Maiorbio Bio-Pharm Technology Co., Ltd., Shanghai, China).

2.8. Ruminal 16S rDNA sequencing data processing and analysis

The raw sequenced sequences were quality controlled using fastp (Chen et al., 2018) software (https://github.com/OpenGene/fastp, version 0.20.0) and spliced using FLASH (Magoč and Salzberg, 2011) software (http://www.cbcb.umd.edu/software/flash, version 1.2.11). The annotated chloroplast and mitochondrial sequences were removed from all samples using UPARSE software (Edgar, 2013; Stackebrandt and Goebel, 1994) (http://drive5.com/uparse/, version 11) and non-repetitive sequences were extracted for the optimised sequences, removing single sequences without repeats. Operational taxonomic units (OTU) clustering of non-repetitive sequences (excluding single sequences) was performed according to 97% similarity, and chimeras were removed during clustering to obtain OTU representative sequences. All the optimised sequences were mapped to the OTU representative sequences and the sequences with 97% or more similarity to the OTU representative sequences were selected to generate the OTU table. Alpha diversity indices, including Chao1, Shannon, Simpson, Ace, Coverage, and Sobs, were calculated using publicly available software (Mothur v. 1.30.2, http://www.mothur.org/wiki/Calculators) (Schloss et al., 2009), and the Wilcoxon rank sum test was used for the analysis of intergroup differences in alpha diversity. Similarity in microbial community structure among samples was tested using principal coordinates analysis (PCoA) based on the Bray–Curtis distance algorithm and partial least squares discriminant analysis (PLS-DA). The Wilcoxon rank sum test was used to analyse between-group community differences.

2.9. Determination of rumen fluid and plasma metabolomics

Rumen fluid or a plasma sample (100 μL) was added to a 1.5 mL centrifuge tube with 400 μL of solution (acetonitrile to methanol ratio = 1:1, vol:vol) containing 0.02 mg/mL internal standard (L-2-chlorophenylalanine) to extract metabolites. The samples were mixed by vortex for 30 s and sonicated at low-temperature for 30 min (5 °C, 40 kHz), and were then placed at −20 °C for 30 min to precipitate the proteins. After that samples were centrifuged for 15 min (4 °C, 13,000 × *g*). The supernatant was removed and blown dry under nitrogen. The sample was then re-solubilized with 100 μL solution (acetonitrile to water ratio = 1:1) and extracted by low-temperature ultrasonication for 5 min (5 °C, 40 kHz), followed by centrifugation at 13,000 × *g* and 4 °C for 10 min. The supernatant was transferred to sample vials for LC-MS/MS analysis. As a part of the system conditioning and quality control process, a pooled quality control (QC) sample was prepared by mixing equal volumes of all samples. The QC samples were disposed and tested in the same manner as the analytic samples.

The LC-MS/MS analysis of samples was conducted on a Thermo UHPLC-Q Exactive HF-X system equipped with an ACQUITY HSS T3 column (100 mm × 2.1 mm, 1.8 μm; Waters, USA) at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The mobile phases consisted of 0.1% formic acid in water: acetonitrile (95:5, vol:vol) (solvent A) and 0.1% formic acid in acetonitrile:isopropanol:water (47.5:47.5:5, vol:vol:vol) (solvent B). Separation gradient in positive ion mode: 0 to 3 min, mobile phase B was increased from 0% to 20%; 3 to 4.5 min, mobile phase B was increased from 20% to 35%; 4.5 to 5 min, mobile phase B was increased from 35% to 100%; 5 to 6.3 min, mobile phase B was maintained at 100%; 6.3 to 6.4 min, mobile phase B was decreased from 100% to 0%; 6.4 to 8 min, mobile phase B was maintained at 0%. Separation gradient in negative ion mode: 0 to 1.5 min, mobile phase B rises from 0% to 5%; 1.5 to 2 min, mobile phase B rises from 5% to 10%; 2 to 4.5 min, mobile phase B rises from 10% to 30%; 4.5 to 5 min, mobile phase B rises from 30% to 100%; 5 to 6.3 min, mobile phase B linearly maintains 100%; 6.3 to 6.4 min, the mobile phase B decreased from 100% to 0%; 6.4 to 8 min, the mobile phase B was linearly maintained at 0%. The flow rate was 0.40 mL/min and the column temperature was 40 °C. The mass spectrometric data were collected using a Thermo UHPLC-Q Exactive HF-X Mass Spectrometer equipped with an electrospray ionization (ESI) source operating in positive mode and negative mode. The optimal conditions were set as followed: source temperature at 425 °C; sheath gas flow rate at 50 arb; auxiliary gas flow rate at 13 arb; ion-spray voltage floating (ISVF) at −3500 V in negative mode and 3,500 V in positive mode, respectively; normalized collision energy, 20–40–60 V rolling for MS/MS. Full MS resolution was 60,000, and MS/MS resolution was 7500. Data acquisition was performed with the data dependent acquisition (DDA) mode. The detection was carried out over a mass range of 70 to 1050 *m*/*z*.

2.10. Metabolome sequencing data processing and analysis

Progenesis QI software (Waters Corporation, Milford, USA) was used to pre-process the LC/MS raw data and export the 3D data matrix in CSV format. The information in the 3D data matrix included: sample information, metabolite name and mass spectral response intensity. The data matrix obtained by searching the database was uploaded to the Majorbio cloud platform (https://cloud.majorbio.com) for data analysis. Firstly, the data matrix was pre-processed, as follows: at least 80% of the metabolic features detected in any set of samples were retained. After filtering, for specific samples with metabolite levels below the lower limit of quantification, the minimum metabolite value was estimated, and each metabolic signature was normalized to the sum. Meanwhile, the variables of QC samples with relative standard deviation (RSD) > 30% were excluded and $\log_{10}$ logarithmized, to obtain the final data matrix for subsequent analysis.

The R package "ropls" (Version 1.6.2) was used to perform principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), and 7-cycle interactive validation evaluating the stability of the model. The metabolites with variable importance in the projection (VIP) > 1, $P < 0.05$ were determined as significantly different metabolites based on the VIP obtained by the OPLS-DA model and the *P*-value generated by *t*-test. Differential metabolites among two groups were mapped into their biochemical pathways through metabolic enrichment and pathway analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/). Enrichment analysis was used to analyze a group of metabolites in a function node for whether they appear or not. Python packages "scipy.stats" (https://docs.scipy.org/doc/scipy/) was used to perform enrichment analysis to obtain the most relevant biological pathways for experimental treatments.

2.11. Statistical analysis

Independent variance *t*-tests were performed to compare the growth performance, plasma biochemical indices, nutrient digestibility and rumen VFA concentration between the two groups using SPSS 22.0 software (IBM Corp., Armonk, New York, USA). The correlation between metabolites and growth performance was analyzed using Bary and Euclidean in the R "vegan, ggcor" software package (https://www.r-project.org). Data are reported as least square means, and declared significant if $P < 0.05$ and declared as trends at $0.05 \leq P < 0.10$.

3. Results

3.1. Growth performance and body measurements

The differences of growth performance and body measurements between HRFI and LRFI groups are shown in Table 2 and Table 3. There were no significant differences in IBW ($P = 0.288$) and FBW ($P = 0.406$) between HRFI and LRFI groups. The DMI in the HRFI group was greater than that in the LRFI group ($P < 0.001$). However, the ADG was not different between the two groups ($P = 0.297$). The feed to gain ratio (F/G) in the HRFI group was significantly higher compared to the LRFI group ($P < 0.001$). In addition, the RFI was also higher in the HRFI group ($P < 0.001$) (Table 2).

There were no significant differences in body measurements including body height ($P = 0.644$), body slanting length ($P = 0.968$), chest circumference ($P = 0.429$), tail length ($P = 0.535$), tail width ($P = 0.764$), tail circumference ($P = 0.728$), chest depth ($P = 0.922$), chest width ($P = 0.750$), scrotal circumference ($P = 0.592$), backfat thickness ($P = 0.638$) and loin-eye area ($P = 0.846$) between the HRFI and LRFI groups (Table 3).

3.2. Apparent digestibility

The differences in apparent digestibility between the HRFI and LRFI groups are shown in Table 4. The digestibility of CP ($P = 0.010$) and EE ($P = 0.010$) was significantly higher in the LRFI group compared to the HRFI group. There were no significant differences in the digestibility of NDF ($P = 0.341$), ADF ($P = 0.800$), Ca ($P = 0.812$), and P ($P = 0.602$) between the HRFI and LRFI groups.

3.3. Rumen fermentation parameters

The rumen fermentation parameters of the HRFI and LRFI groups are shown in Table 5. There was no difference in pH between the HRFI and LRFI groups ($P = 0.860$). There was a tendency for NH_3-N concentration in the LRFI group to be higher than that in the HRFI group ($P = 0.051$). The concentration of MCP was higher in the

Table 2
Characterization of feed intake and growth performance of Hu sheep with HRFI and LRFI ($n = 8$).

Item	HRFI	LRFI	SEM	*P*-value
DMI, kg/d	2.24	1.60	0.086	<0.001
ADG, kg/d	0.29	0.31	0.010	0.297
IBW, kg	25.13	24.19	0.427	0.288
FBW, kg	64.50	62.56	1.121	0.406
F/G, kg feed DM/kg gain	8.14	6.18	0.272	<0.001
RFI	0.29	−0.28	0.076	<0.001

HRFI = high residual feed intake; LRFI = low residual feed intake; SEM = standard error of the mean; DMI = dry matter intake; ADG = average daily gain; IBW = initial body weight; FBW = final body weight; F/G = feed to gain ratio; RFI = residual feed intake.

Table 3
Body measurements (cm) of Hu sheep with HRFI and LRFI ($n = 8$).

Item	HRFI	LRFI	SEM	*P*-value
Body height	70.45	71.90	1.509	0.644
Body slanting length	76.28	76.22	0.725	0.968
Chest circumference	91.84	90.46	0.845	0.429
Tail length	15.32	15.86	0.420	0.535
Tail width	18.68	18.20	0.767	0.764
Tail circumference	41.27	40.02	1.725	0.728
Chest depth	32.28	32.34	0.295	0.922
Chest width	20.06	20.28	0.333	0.750
Backfat thickness, mm	6.05	5.66	0.395	0.638
Scrotal circumference	26.26	27.36	0.989	0.592
Loin-eye area, cm^2	14.99	14.91	0.204	0.846

HRFI = high residual feed intake; LRFI = low residual feed intake; SEM = standard error of the mean.

Table 4
Apparent digestibility (%) of Hu sheep with HRFI and LRFI ($n = 8$).

Item	HRFI	LRFI	SEM	*P*-value
CP	68.58	77.06	1.927	0.010
EE	27.34	44.73	3.934	0.010
NDF	67.51	70.68	1.540	0.341
ADF	55.46	54.20	2.217	0.800
Ca	54.31	55.31	1.816	0.812
P	35.45	38.62	2.728	0.602

HRFI = high residual feed intake; LRFI = low residual feed intake; SEM = standard error of the mean; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; Ca = calcium; P = phosphorus.

LRFI group compared to the HRFI group ($P = 0.002$). Acetate ($P = 0.036$), propionate ($P = 0.010$), valerate ($P = 0.027$) and total VFA ($P = 0.048$) were significantly higher in the LRFI group compared to the HRFI group. There were no differences in isobutyrate ($P = 0.660$), butyrate ($P = 0.658$), isovalerate ($P = 0.472$), and the ratio of acetate to propionate (A/P) ($P = 0.681$) between the HRFI and LRFI groups.

3.4. Ruminal microbial community characteristics

A total of 388,288 clean data from 1,526 OTUs were observed, with an average of 24,268 clean data per sample. As shown in Table 6, there were no significant differences in Sobs ($P = 0.607$), Shannon ($P = 0.200$), Simpson ($P = 0.103$), Ace (0.882), Chao1 ($P = 0.839$) and Coverage ($P = 0.983$) alpha diversity indices of rumen microbial community between HRFI and LRFI groups. No significant difference was observed according to the PCA (Fig. 1A), but the results of the PLS-DA (Fig. 1B) showed that the bacterial community was clearly separated between the HRFI and LRFI groups. The sequences were taxonomically analyzed using the ribosomal database project (RDP) classifier Bayesian algorithm, and a total of 16 phyla and 332 genera were identified. In terms of microbial composition, Firmicutes and Bacteroidetes are the main phyla (Fig. 1C) with the major difference at the phyla level being the bacterium Fibrobacterota, having a higher abundance in the HRFI group compared to the LRFI group ($P = 0.040$) (Fig. 1E). *Prevotella* and *ruminococcus* are the main genera (Fig. 1D), and the differential bacteria at the genus level were *Prevotella*, *Fibrobacter*, *Colidextribacter*, *Mitsuokella* and *Anaerofustis*. Among them, *Fibrobacter* ($P = 0.040$), *Colidextribacter* ($P = 0.048$) and *Anaerofustis* ($P = 0.039$) were higher in the HRFI group, but *Prevotella* ($P = 0.031$) and *Mitsuokella* ($P = 0.039$) were higher in the LRFI group (Fig. 1F), where the relative abundance of *Prevotella* was 20.71% higher in the LRFI group than that of the HRFI group.

Table 5
Ruminal fermentation parameters (mmol/L) of Hu sheep with HRFI and LRFI ($n = 8$).

Item	HRFI	LRFI	SEM	*P*-value
pH	6.47	6.47	0.020	0.860
NH_3–N, mg/dL	10.30	10.88	0.152	0.051
MCP, mg/100 dL	77.96	81.48	0.632	0.002
Acetate	26.29	32.70	1.566	0.036
Propionate	10.45	12.64	0.454	0.010
Isobutyrate	0.27	0.28	0.020	0.660
Butyrate	5.97	6.52	0.595	0.658
Isovalerate	0.25	0.22	0.018	0.472
Valerate	0.29	0.40	0.028	0.027
Total VFA[1]	43.48	52.77	2.400	0.048
A/P[2]	2.52	2.60	0.091	0.681

HRFI = high residual feed intake; LRFI = low residual feed intake; SEM = standard error of the mean; NH_3–N = ammonia-nitrogen; MCP = microbial crude protein; Total VFA = total volatile fatty acids.
[1] Total VFA = acetate + propionate + isobutyrate + butyrate + isovalerate + valerate.
[2] A/P = acetate to propionate ratio.

3.5. Rumen and plasma metabolome

For rumen metabolome, a total of 4837 negative ion peaks and 5211 positive ion peaks were detected, and 984 positive ion metabolites and 1142 negative ion metabolites were identified by searching the library. The rumen metabolites were significantly different between the two groups (Fig. 2A). Ruminal differential metabolites were screened by *P*-value ($P < 0.05$), VIP (VIP ≥ 1) and fold change (FC, FC ≥ 1 and FC ≤ 0.5, Fig. 2B). The results indicated there were 16 differential metabolites between the HRFI and LRFI groups, including galactonic acid, lysoPC (18:3(6Z,9Z,12Z)/0:0), S-lactoylglutathione, D-galactose, PC (16:0/0:0), PC(18:0/0:0), PE (15:0/0:0), PE (17:0/0:0), PC (18:1(6Z)/0:0), succinic acid, lysoPC (18:1(11Z)/0:0), lysoPC (20:5(5Z,8Z,11Z,14Z,17Z)/0:0), lysoPC (0:0/18:1(9Z)), lysoPE (18:0/0:0), lysoPC (14:0/0:0) and maltotriose. The results of KEGG pathway enrichment analysis for 16 differential metabolites showed that the major differential metabolic pathways were galactose metabolism, the citrate cycle (TCA cycle), sulfur metabolism, butanoate metabolism, propanoate metabolism, carbon fixation pathways in prokaryotes, pyruvate metabolism, alanine, aspartate and glutamate metabolism, phenylalanine metabolism and lysine degradation (Fig. 2C).

For the plasma metabolome, a total of 2467 negative ion peaks and 2480 positive ion peaks were monitored, and 185 negative ion metabolites and 328 positive ion metabolites were finally identified. The metabolites of the two groups were clearly separated in the OPLS-DA score plots of the positive and negative ion patterns, there was a significant difference between the HRFI and LRFI groups (Fig. 3A). Plasma differential metabolites were screened by *P*-value ($P < 0.05$), VIP (VIP ≥ 1) and FC (FC ≥ 1 and FC ≤ 0.5). There was a total of 7 differential metabolites between the HRFI and LRFI

Table 6
Analysis of rumen microbial diversity by 16S rRNA sequencing of Hu sheep with HRFI and LRFI ($n = 8$).

Item	HRFI	LRFI	SEM	*P*-value
Observed species	473.5	441.4	29.76	0.607
Shannon index	3.56	3.13	0.164	0.200
Simpson index	0.10	0.18	0.024	0.103
Ace index	609.98	597.91	37.782	0.882
Chao1 index	606.12	589.41	38.995	0.839
Coverage	0.99	0.99	0.001	0.983

HRFI = high residual feed intake; LRFI = low residual feed intake; SEM = standard error of the mean.

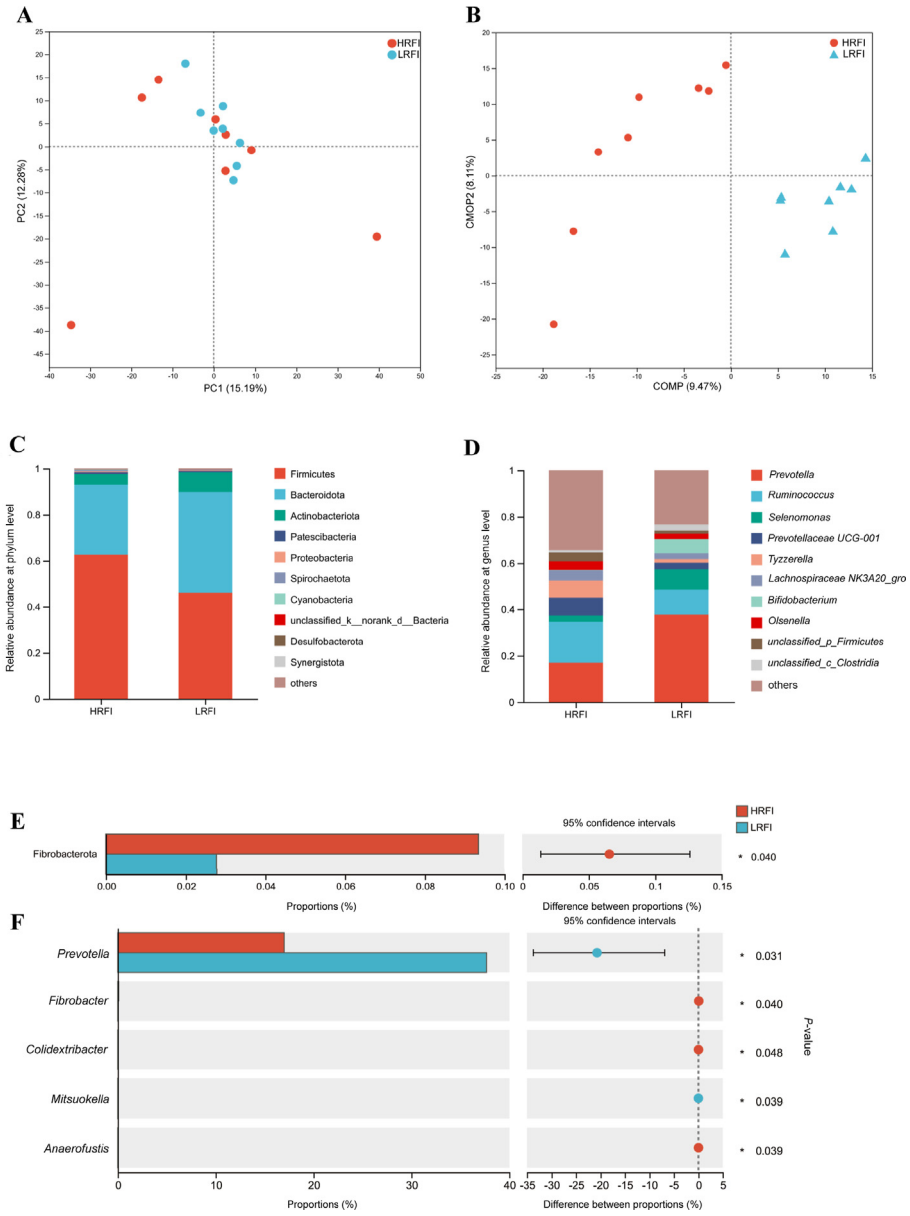

Fig. 1. Comparative analysis of rumen microbial species diversity in high and low residual feed intake Hu sheep (HRFI and LRFI, $n = 8$). (A) Principal component analysis (PCA). (B) Partial least squares discriminant analysis (PLS-DA). (C) The relative abundance of bacteria at the phylum level. (D) The relative abundance of bacteria at the genus level. (E) Differential bacteria between HRFI and LRFI groups at the phylum level. (F) Differential bacteria between HRFI and LRFI groups at the genus level. HRFI = high residual feed intake; LRFI = low residual feed intake.

groups, of which 4 metabolites were down-regulated and 3 were up-regulated in the LRFI group. Down-regulated metabolites included L-leucine, pantothenic acid, decanoylcarnitine, N-acetylserotonin. Up-regulated metabolites included citric acid, oxoglutaric acid and malic acid (Fig. 3B). The results of KEGG enrichment analysis showed that 7 different metabolites were enriched in 12 metabolic pathways, including the TCA cycle, tryptophan metabolism, pantothenate and CoA biosynthesis, pyruvate metabolism, glyoxylate and dicarboxylate metabolism, alanine, aspartate and glutamate metabolism, taurine and hypotaurine metabolism, biosynthesis of cofactors, aminoacyl-tRNA biosynthesis, valine, leucine and isoleucine biosynthesis, beta-alanine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis (Fig. 3C).

3.6. Relationships among rumen microorganisms, the rumen metabolome and the plasma metabolome and their interpretation of RFI phenotypes

To understand the effects of 16 rumen metabolites, 7 plasma metabolites, and 2 microorganisms screened on the growth performance of sheep with differing RFIs, Pearson's analysis was carried out. The results of correlation analysis between rumen microorganisms and growth performance showed that *Prevotella* had a significant negative correlation with RFI and F/G (Fig. 4). The results of correlation analysis between rumen metabolites and growth performance showed that 15 differential metabolites screened were significantly correlated with RFI. In addition, galactonic acid, lysoPC (18:3(6Z,9Z,12Z)/0:0), S-lactoylglutathione, D-

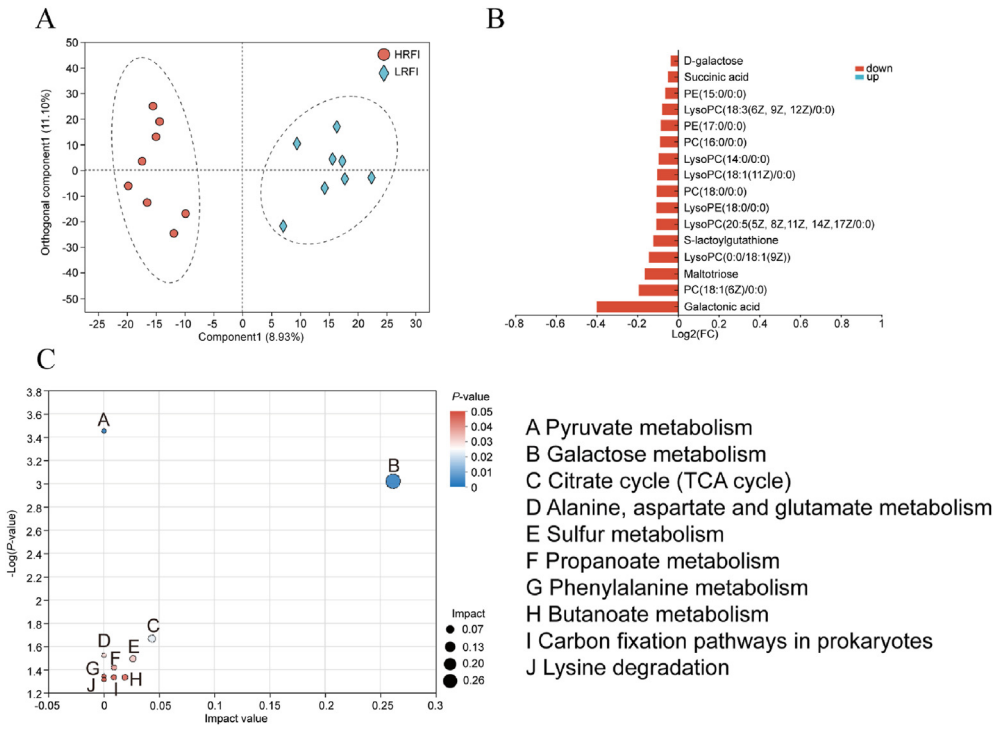

Fig. 2. Ruminal metabolome profiles of Hu sheep with high and low residual feed intake groups (HRFI and LRFI, $n = 8$). (A) Metabolic profiles of rumen anions and cations based on orthogonal partial least squares discrimination analysis. (B) Ruminal differential metabolites between HRFI and LRFI groups using *t*-test. (C) Pathway enrichment analysis performed using the significantly different ruminal metabolites between HRFI and LRFI groups. HRFI = high residual feed intake; LRFI = low residual feed intake, FC = fold change.

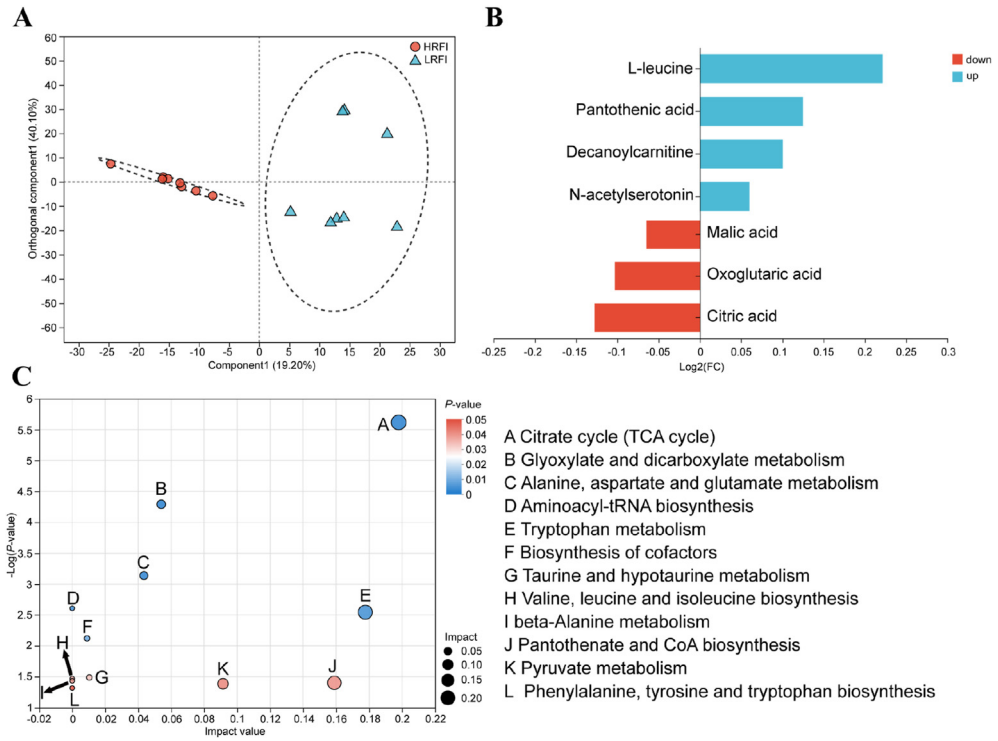

Fig. 3. Plasma metabolome profiles of Hu sheep with high and low residual feed intake groups (HRFI and LRFI, $n = 8$). (A) Metabolic profiles of plasma anions and cations based on orthogonal partial least squares discrimination analysis. (B) Plasma differential metabolites between Hu sheep with HRFI and LRFI groups using *t*-test. (C) Pathway enrichment analysis performed using the significantly different plasma metabolites between HRFI and LRFI groups. HRFI = high residual feed intake; LRFI = low residual feed intake, FC = fold change.

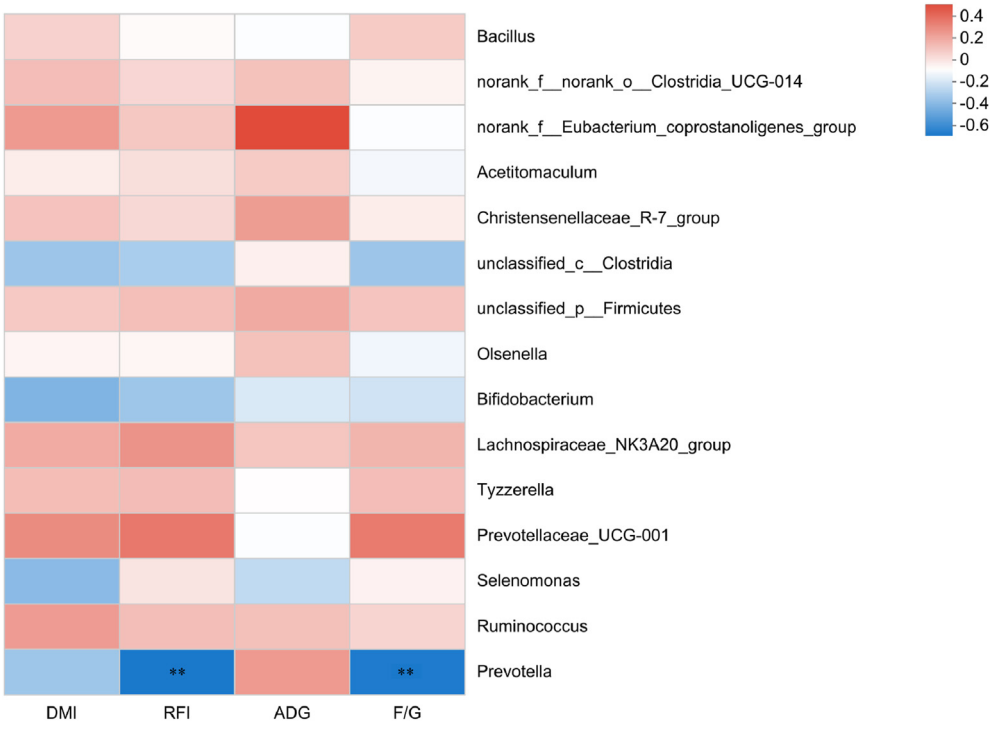

Fig. 4. Correlation analysis between rumen microorganisms and growth performance. DMI = dry matter intake; RFI = residual feed intake; ADG = average daily weight gain; F/G = feed to gain ratio. **Correlation was significant at $P < 0.01$.

galactose, lysoPE (18:0/0:0) and lysoPC (14:0/0:0) were significantly negatively correlated with DMI (Fig. 5A). The results of correlation analysis between plasma metabolites and growth performance showed that RFI was significantly negatively correlated with malic acid, citric acid, and oxoglutaric acid, but positively correlated with O-acetylcarnitine, L-tryptophan, N-acetylserotonin, L-leucine, pantothenic acid, and decanoylcarnitine. Malic acid, citric acid and oxoglutaric acid in plasma were significantly negatively correlated with DMI (Fig. 5B). The results of Venn diagrams for rumen and plasma differential metabolic pathways showed that the TCA cycle, Pyruvate metabolism, and Alanine, aspartate and glutamate metabolism pathways were shared in rumen and plasma metabolism (Fig. 6).

4. Discussion

Zeng et al. (2023) reported that the LRFI sheep had a significantly lower DMI compared to HRFI sheep during the 56-d trial period, however, in the absence of a difference in IBW, there was no difference in ADG as well as FBW between the two groups. This was in agreement with our findings that DMI of sheep in the LRFI group was 28.4% less than that in the HRFI group. In addition, there were no significant differences between the HRFI and LRFI groups in terms of 11 body measurements including body height, backfat thickness and eye muscle area, etc. This suggested that sheep with LRFI were able to improve feed efficiency without affecting growth performance. The apparent digestibility of CP and EE in LRFI group was higher than that in HRFI group in the current study. The results were similar to Johnson et al. (2019) who reported that heifers with low RFI had higher CP digestibility compared to heifers with high RFI. Fischer et al. (2018) reported that cows with high feed efficiency ruminated 44 min more daily than inefficient cows; the higher comminution efficiency may contribute to higher digestibility. In addition, Kong et al. (2016) reported rumen epithelial cells of LRFI steers may have a stronger capacity for tissue morphogenesis, which may increase paracellular permeability to facilitate nutrient absorption and increase energy production.

The rumen NH_3—N concentration reflects the degradation of protein in the diet, and NH_3—N is also the main nitrogen source for rumen microorganism to synthesize MCP (Rodríguez et al., 2007). In the current study, the concentrations of NH_3—N and MCP in rumen were higher in the LRFI group, which was similar to the findings of Zeng et al. (2023) who also observed higher NH_3—N and MCP concentration in sheep with LRFI. The higher digestibility of CP in LRFI group might contribute to the higher NH_3—N, which can be utilized by rumen microorganism to generate more MCP. Acetate, propionate, and butyrate are the most productive VFAs in rumen fermentation and are main energy sources for ruminants (Bergman, 1990). In the current study, acetate content in rumen liquid was higher in the LRFI group than that of the HRFI group. This was similar to the findings of Lage et al. (2020) who reported that preweaned claves in the LRFI group had higher ruminal acetate content. Studies have shown that rumen epithelial cells can absorb 29% to 59% of the acetate content by passive diffusion, and the extent and speed of passive diffusion absorption is affected by the osmotic pressure of VFA concentration, which is directly dependent on the concentration difference between rumen fluid and blood; the larger the difference, the faster the diffusion into the blood (Gregory B. et al., 2009). Acetate is absorbed through the portal vein and transported to peripheral tissues for oxidative energy supply by the TCA cycle or for use in fatty acid synthesis (Annison et al., 1963). Several studies had shown that rumen propionate content was significantly higher in ruminants with a LRFI (Liang et al., 2017; Zeng et al., 2023), which is in line with our results. Propionate is the main precursor for hepatic gluconeogenesis utilization in ruminants, accounting for 60% of glucose consumption in animals (Harfoot, 1978). Zhang et al. (2015) reported that propionate concentration raised as the proportion of dietary carbohydrates increases. Therefore, the higher propionate concentration in sheep with a LRFI may indicate that they can utilize carbohydrates more

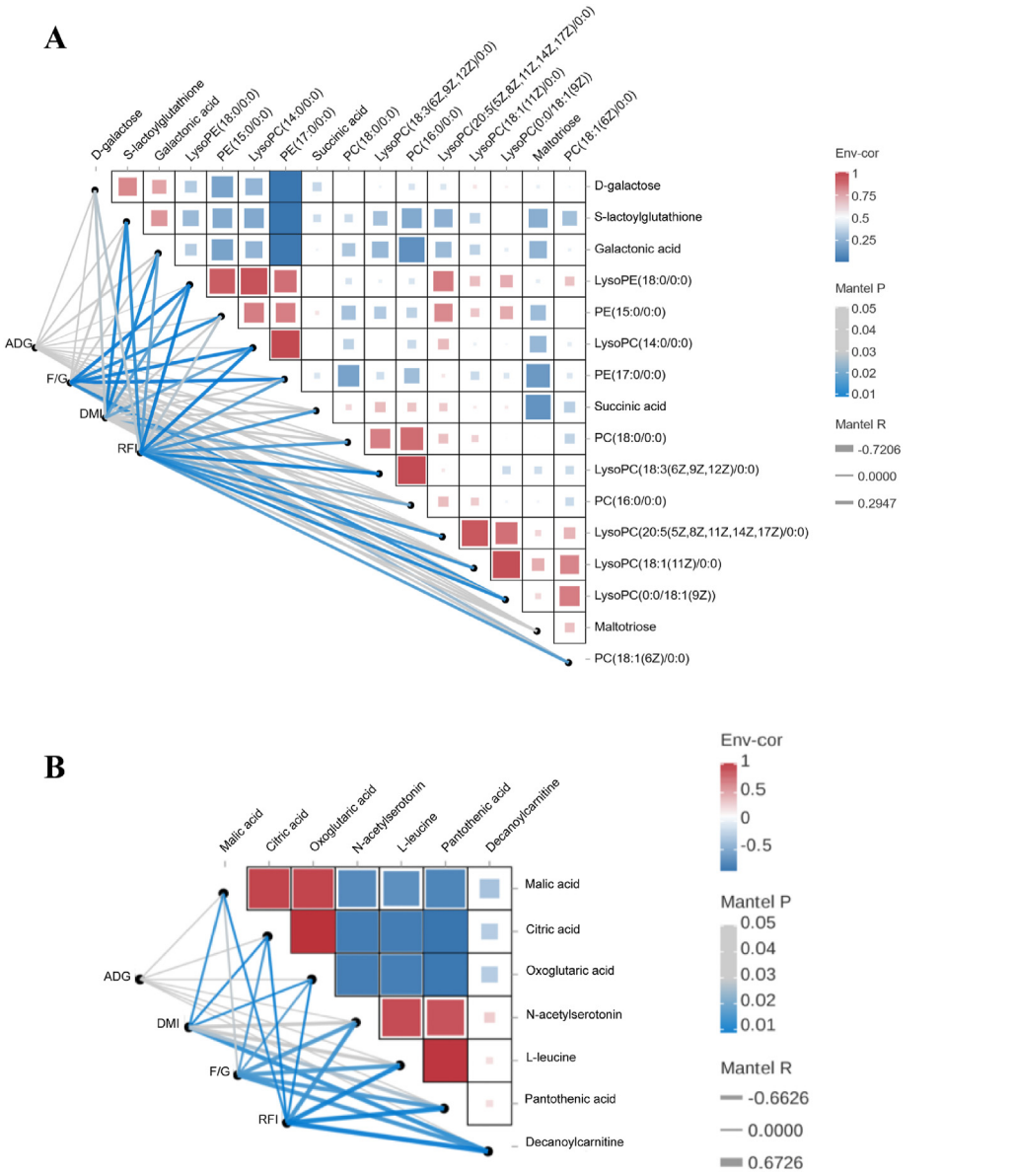

Fig. 5. Correlation analysis between metabolites and growth performance. (A) correlation analysis between ruminal metabolites and growth performance. (B) correlation analysis between plasma metabolites and growth performance. DMI = dry matter intake; ADG = average daily weight gain; F/G = feed to gain ratio; RFI = residual feed intake; Env-cor = environments-correlation.

efficiently to produce more propionate. In addition, valerate and total VFA content were higher in the LRFI group, which was similar to the results reported by Guan et al. (2008) who observed higher valerate and total VFA concentrations in steers with a LRFI when fed 64.5% barley grain and 20% oats. Changes in VFA may indicate that LRFI sheep can break down feed more efficiently to produce more VFA, thus providing more energy. However, NDF and ADF digestibility were not different between the two groups in the current study. During digestion, fibre is digested to pyruvate which is then converted to acetate, propionate and CH_4, similarly to starch (Fleming et al., 2021). Therefore, the higher concentration of acetate in rumen of LRFI sheep may be due to a more efficient conversion of carbohydrates to VFA when fed the high-concentrate diet in the current study. Although the rumen concentration of VFA was higher in the LRFI group, there was no significant difference in pH between the two groups. When the rumen epithelium takes up VFA by passive diffusion, a H^+ will be removed from the ruminal contents, however, once the H^+ is present in the cytosol, the VFA will dissociate and the released H^+ will be expelled from the cell to maintain intracellular pH. Monocarboxylate transporter and Na^+/H^+ exchangers involved in intracellular pH regulation return H^+ to the rumen or to extracellular space (Kirat et al., 2006; Fleming et al., 2021). As a result, ruminal pH can remain relatively stable. However, this result for rumen pH should be treated with caution as it was not taken as a ruminal dynamic pH profile.

Bacteria are key players in most feed biopolymer degradation and fermentation processes (Shabat et al., 2016; Xue et al., 2020). At the genus level, the relative abundance of *Prevotella* in the LRFI group was significantly higher than that in the HRFI group. This was similar to the finding of Xue et al. (2020) who reported that *Prevotella* accounted for about 40% of the total rumen genera in LRFI group. *Prevotella* plays an important role in the biosynthesis of VFA, and can utilize protein and starch fermentation to produce succinic acid. This may be one of the reasons for the higher acetate content.

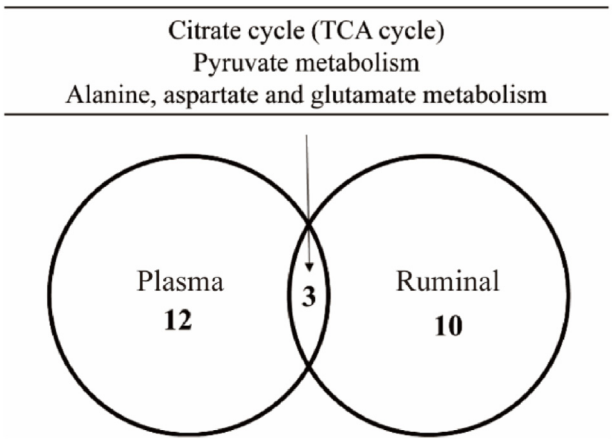

Fig. 6. Comparison of residual feed intake associated metabolites enriched pathways between rumen and plasma.

In addition, acetate- and succinate-producing *Mitsuokella* genus was also higher in the LRFI group, which may also account for the higher ruminal acetate in the sheep with a LRFI.

The current study showed that rumen and plasma metabolite compositions were different in sheep with differing RFI. Maltotriose is a product of starch digestion, which promotes energy production (Chen et al., 2022). Higher levels of maltotriose in the rumen in the LRFI group may be due to the rumen microorganisms of sheep in the LRFI group being able to break down feeds more efficiently, producing more energy precursors, lowering the necessary DMI to some extent. The contents of galacturonic acid and D-galactose in rumen were also higher in the LRFI group. D-galactose is commonly found in plants and animals. After being absorbed, galactose undergoes a series of reactions in hepatocytes to generate glucose 1-phosphate, which eventually enters the glucose metabolic pathway to provide energy for the organism (Coelho et al., 2015). S-lactoylglutathione can be oxidized to pyruvate by D-lactate dehydrogenase, and pyruvate can be further reacted to provide energy for the organism (de Bari et al., 2002). S-lactoylglutathione is also an intermediate in the glyoxalase system, which provides energy for milk protein synthesis (Atlante et al., 2005). In addition, succinic acid was higher in LRFI group, which was consistent with the findings of Wang et al. (2019) who reported that higher levels of succinic acid in plasma differential metabolites in LRFI Jersey and Holstein cattle. As an important intermediate compound in energy metabolism, succinic acid is involved in both TCA cycle regulation of energy metabolism and fatty acid synthesis (Tretter et al., 2016). Notably, succinic acid was enriched in 9 out of 10 differential metabolic pathways of rumen metabolism, suggesting that it may play an important role in feed efficiency in sheep. It has been shown that acetate absorbed by ruminants can go directly into the cytosol to be converted into acetyl CoA. Therefore, more acetate in the rumen of sheep in LRFI group may be absorbed by the organism and then converted to succinic acid via acetyl CoA. Succinic acid is involved in a variety of metabolic pathways to provide energy for the organism. In plasma metabolism, citric acid, oxoglutaric acid and malic acid were significantly higher in the LRFI group, similar to the results reported by Elolimy et al. (2020) and Wang et al. (2019). These three substances are intermediates in the TCA cycle. The results suggested that the sheep with a LRFI were not only highly productive, but also highly efficient in energy conversion. In fatty acid metabolism, short-chain acylcarnitines are intermediate metabolites of fatty acids, amino acids, and glucose, whereas long- and medium-chain acylcarnitines are intermediates in fatty acid metabolism (Makrecka-Kuka et al., 2017). The lower intracellular levels of these intermediates may indicate a greater ability of the organism to accomplish oxidative energy supply through the TCA cycle (Martin et al., 2021). Interestingly, a higher apparent digestibility of EE in LRFI sheep means that the animal absorbed more fatty acids and participated in more metabolic processes, while lower levels of acylcarnitine, which acts as a key transporter during fatty acid oxidation, may indicate that the organism is more metabolically active and uses the absorbed nutrients more efficiently (Hoppel, 2003). In addition, L-leucine is a branched-chain amino acid, and short-chain acylcarnitines are breakdown products of branched-chain fatty acids, which inhibit the initial step of β-oxidation if the degradation pathway of branched-chain amino acids is saturated (Kirchberg et al., 2015). It had also been shown that accumulation of long-chain acylcarnitines may interfere with insulin sensitivity (Jorge-Smeding et al., 2022, 2021). Pantothenic acid is a precursor substance for the formation of CoA, which plays an important role in metabolic processes such as the TCA cycle and fatty acid synthesis and catabolism (Branca et al., 1984). In the current study, the lower pantothenic acid content in the LRFI group may be due to the higher metabolic efficiency that allows a large amount of pantothenic acid to be involved in metabolism in the form of CoA, which generates energy for the organism. It had been shown that tryptophan stimulates feed intake by enhancing ghrelin, 5-hydroxytryptamine (5-HT), neuropeptide Y, and pituitary growth hormone-insulin-like growth factor signaling pathways (Miao et al., 2019; Zhao et al., 2019). Tryptophan, a precursor of 5-HT, dominates the rate of 5-HT synthesis both in the gut and centrally (Capello and Markus, 2014; Wu et al., 2012). In addition, N-acetylserotonin is a reaction intermediate in the endogenous synthesis of serotonin to melatonin. Therefore, higher concentrations of tryptophan and N-acetylserotonin in the plasma of sheep in the HRFI group in the current study may be due to the higher feed intake, similar to the results reported by Han et al. (2021).

Recent studies had reported that host metabolism and rumen microbiota can influence host feed efficiency (Elolimy et al., 2020; Sasson et al., 2017; Xue et al., 2020). The results about the effects of rumen microbes, the rumen metabolome and the plasma metabolome on host feed efficiency in the current study further confirm the above. In our study, rumen microorganisms had a certain correlation with RFI, among which *Prevotella* was negatively correlated with RFI; the higher the relative abundance of *Prevotella*, the lower the RFI and the feed efficiency. In addition, correlation results showed that 9 plasma metabolites and 16 rumen metabolites played an important role in sheep with high feed efficiency. The TCA cycle, pyruvate metabolism, and alanine, aspartate, and glutamate metabolism are common differential metabolic pathways in rumen and plasma. As the common home of catabolism of the three major nutrients, sugar, fat, and protein, TCA cycle not only plays an important role in energy production and energy conversion, but also connects the inter-transformation of various nutrients (Fernie et al., 2004). Pyruvate is an intermediate product with a key role in glucose metabolism, which can interconvert sugars, fats and amino acids through the acetyl CoA and TCA cycle (Chaudhry and Varacallo, 2023). In turn, pyruvate, α-ketoglutarate, and other substances resulting from the metabolism of alanine, aspartate, and glutamate can be further reacted to provide a large amount of energy for the organism (Brosnan, 2000; Brosnan and Brosnan, 2013; Jubouri et al., 2021). Thus, higher levels of energy metabolism in sheep with a LRFI may account for higher feed efficiency. Synthesizing the colony microbiome and metabolome, we found some characteristics of rumen microorganisms and metabolites that may be related to feed efficiency in sheep.

5. Conclusion

In summary, we studied the changes of rumen microorganisms, rumen metabolism and plasma metabolism of Hu sheep with different RFIs. The sheep with a LRFI had a lower DMI but similar ADG compared to sheep with a HRFI. The high relative abundance of *Prevotella* in the rumen of sheep with a LRFI may enhance the metabolism of acetate in the rumen, which in turn improves the TCA cycle, pyruvic acid metabolism, alanine, aspartic acid metabolism, and glutamic acid metabolism in the rumen and plasma, generating a large amount of energy for the host. The differences in microorganisms and metabolites significantly affects the feed efficiency of Hu sheep, and the specific mechanism needs to be further explored.

Author contributions

Yanzhen Zhang: Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft, Visualization. **Xiaowei Zhang:** Conceptualization, Investigation, Methodology. **Dingren Cao:** Investigation, Methodology, Formal analysis. **Jinyong Yang:** Conceptualization, Supervision, Project administration. **Huiling Mao:** Conceptualization, Writing – review & editing. **Lingling Sun:** Conceptualization, Methodology, Formal analysis, Supervision, Writing – review & editing. **Chong Wang:** Conceptualization, Supervision, Funding acquisition, Project administration, Resources.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.04.021.

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