Microbiome

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No rumen fermentation profiles and associated microbial diversities difference were found between Hu sheep and Karakul sheep fed a cottonseed hull diet

Jishu Wang[1,2], Guangxu E[1,2], Meng Zhang[4], Yanlong Zhang[1,2], Tiantian Bai[4], XuanXuan Pu[1,2,3], Junfeng Liu[1,2,3], Xuefeng Guo[1,2,3*], Subir Sarker[5,6] and Long Cheng[7]

Abstract

Background This research aimed to investigate differences in rumen fermentation characteristics between Karakul sheep and Hu sheep reared under identical conditions. The test subjects included newborn Hu and Karakul sheep, which were monitored across three stages: stage I (Weaning period: 15 ~ 30 days), stage II (Supplementary feeding period: 31 ~ 90 days), and stage III (Complete feeding period: 91 ~ 150 days). During the supplementary feeding period, cottonseed hulls were the main roughage source. To analyze the dynamics of rumen fermentation, 16S rRNA sequencing and metabolomics methods were employed, alongside measurements of rumen fermentation parameters and cellulase activity. This comprehensive approach aimed to investigate the potential impact of breed on rumen fermentation indicators, microbial community structure, and metabolites in Hu and Karakul sheep.

Results The 16S rRNA sequencing analysis revealed no significant differences in the relative abundance or dominant bacterial communities in the rumen across all stages. In stage II, rumen bacteria in both Hu and Karakul sheep were relatively stable. However, the Simpson index of Hu sheep in stage II was substantially greater than that of Karakul sheep, demonstrating similarities in the rumen microbial structure between stages II and III. Dynamic variations in fermentation parameters and cellulase activity in the rumen revealed that the indicators in both sheep breeds stabilized at 150 days. Metabolomic results revealed that the metabolic pathways in stage I were mainly concentrated in purine metabolism and lipid metabolism, while stage II was dominated by amino acid metabolism. Stage III involved mainly in pyrimidine and purine metabolism. An exploration of the relationships among rumen microbial biomarkers, key differentially abundant metabolites and rumen characteristics indicated that Karakul sheep exhibited superior lipid metabolism compared to Hu sheep.

Conclusion These findings reveal that there were no interbreed differences in the rumen characteristics of Hu and Karakul sheep when fed the same cottonseed hull diet, despite differences in their metabolic pathways. The findings also indicate that the first 20 days represent the initial stage of rumen bacteria in Hu sheep, followed by a transition phase between 20 and 90 days, and a relatively stable stage from 90 to 150 days. These results provide a scientific basis for further understanding the rumen function of sheep and for optimizing their feeding strategies.

Keywords Hu sheep, Karakul sheep, Rumen bacteria, Dynamic changes, Rumen metabolome

*Correspondence:
Xuefeng Guo
gxfdky@126.com
Full list of author information is available at the end of the article

Introduction

The Xinjiang Uygur Autonomous Region of China (34°–50° N, 73°–96° E) is located in northwestern China, with the northern part of the Tianshan Mountains known as the northern territory and the southern part known as the southern territory [1]. Southern Xinjiang has a characteristic continental climate with limited water supplies and a fragile ecological environment, which is dry and semiarid [2] with few forage species, which exhibits high crude fiber contents while high level of lignification. Karakul breed are native to the southern region of Xingjiang, and thus are considered to be excellently environmentally adaptive, and might exhibit great abilities to digest high-roughage diets. In contrast, Hu sheep is known to reach sexual maturity early and breed throughout the year, with high lambing rates [3]. According to early investigations in our research, Hu sheep have more difficulties in utilizing lignifiled roughage than Karakul sheep do, which could be explained by the more diverse and abundant ruminal microorganisms in the Karakul sheep [4], even though reason of this difference remains unclear. It was speculated that variations in the breeding process, such as the composition of dietary ingredients and the ratio of refined to coarse diet had resulted in difference in forage utilization capacity between Karakul sheep and Hu sheep, which needs further verification.

Ruminants rely critically on gut microorganisms to digest and convert the complex polysaccharides of plant materials that are mostly indigestible to humans into nutritive foods such as meat and milk. Among gut microorganisms, rumen microbes enrich the microbial community of bacteria, protozoa, archaea, fungi, and bacteriophages that rapidly colonize and digest plant materials in a complex and coordinated manner [5, 6].

Rumen microbial communities undergoes succession as the host growth and environment change, resulting in changes in the structure and composition of the microbial community [7–10]. In most ruminant, gastrointestinal microbes, largely acquired from the mother and the surrounding environment, rapidly colonize in the rumen [11]. Malmuthuge et al. [12] reported that rumen microbial colonization begins at parturition, with the highest abundance of *Veillonella*. In the subsequent genus, *Acidaminococcus Rogosa*, *Prevotella*, *Clostridium*, *Bacteroides*, *Eubacterium*, *Streptococcus*, *Bifidobacterium*, *Ruminococcus*, which accounts for 88.7% of the total rumen microbiota in calves. Furthermore, during the first week post-birth, fiber-degrading bacteria had already colonized the rumen of suckling calves, similar foundings were observed in lambs [8]. Yin et al. [13] noted that solid feed-digesting bacteria exist in the rumen prior to the introduction of solid feed introduction, with Firmicutes being the dominant phylum in primiparous animals. As the animals aged, this was gradually supplanted by the anaplastic phylum as the predominant phylum [10]. Fiber levels and sources directly affect rumen microflora that colonizated [11]. Weanned lambs that fed alfalfa 10 dyas earlier exihibit a fermentation agent by stimulating the proliferation of fiber-degrading bacteria, including unclassified_Spirochaeidae and Treponema, and promoted the presence of some fermentative and short-chain fatty acid-producing bacteria, resulting in improved feed intake and production performance [14].

This study focuses on newborn Karakul sheep as the research object and introduces Hu sheep lambs as control. The rearing period ranged from 15 ~ 150 days with progressive feed after weaning. In the second stage, a diet with cottonseed hulls were included. The rumen microbial bacterial structure, dominant bacteria and metabolite metabolism of both sheep breeds were evaluated through 16S rRNA sequencing and metabolomics. The pathways and patterns were combined with rumen fermentation parameters and cellulase activity for dynamic analysis to explore whether the breeds would affect the rumen fermentation indicators, microbial community structure and metabolites of Hu sheep and Karakul sheep.

Materials and methods

The experiment was conducted at the Tarim University training base in Alar city (80°–81° E, 40° N), Xinjiang, China, from August to December 2023. The experiment randomly selected two breeds, Hu sheep ($n=18$) and Karakul sheep ($n=18$), with similar weights and ages from the local herd. All sheep were descendants of the same paternal line within their respective breeds. To minimize the influence of external environmental, individual, and dietary factors, all sheep were raised in separate stalls under unified management. The age range of the selected sheep was limited to 15 days, with a weight range of 8.5 ~ 9 kg. To simulate the feeding and management mode under actual production conditions, the entire feeding period was divided into three stages: stage I (weaning period: 15 to 30 days), stage II (supplementary feeding period: 31 to 90 days), and stage III (complete feed stage: 91 ~ 150 days). During stage I, the main diet of the sheep was a basic diet composed of lamb pellets (60%) and green hay (40%) (China Charoen Pokphand Group Xinjiang Branch., consisted of corn, soybean meal, whey powder, stone powder, sodium chloride, vitamins, and amino acids. The measured nutritional levels of the basic diets were as follows: crude protein content was 13.00%, neutral detergent fiber content was 40.03%, and acid detergent fiber content was 27.23%.). Subsequently (stage II, stage III), they began to be officially fed the supplementary feeding period diet (08:00 and 18:00). Throughout the experiment, the sheep had free access to

food and drinking water. At the final day of each stage, animals were fasted for 24 h (withholding water for 2 h). The feeding process and slaughtering experiments adhered to the regulations of the "Ethical Review Guidelines for Laboratory Animal Welfare" (CHN) regulations. The ingredients and nutritional content of the mixed feed used in the progressive feeding period in the test are detailed in Table 1.

Rumen content sampling

Prior to the end of the three stages, 6 experimental sheep of each breed were selected, anesthetized first, and then slaughter for sampling. A 50 mL sample of rumen contents was collected from each sheep immediately after slaughter, and the pH was measured immediately. The sample was filtered with two layers of gauze to measure the concentration of MCP. After filtering with four layers of gauze, it was used to measure NH_3-N concentration and VFA concentration; separately collected 50 mL of rumen contents for high-throughput sequencing and metabolomics determination. Another portion of the rumen digesta was stored in a − 20 ℃ refrigerator for the determination of enzyme activity.

Table 1 Dietary composition and nutritional level (air-dried basis)

Item	Stage II Supplementary feeding period	Stage III Complete feeding Period
Diets composition (%)		
Corn	20	20
Soybean meal	8.7	8.7
Bran	4	4
Cottonseed hull	65	65
Salt	0.8	0.8
Calcium carbonate powder	0.5	0.5
Premix[1]	1	1
Total	100	100
Nutrient levels[2]		
DM%	91.37	91.37
ME MJ/kg	9.84	9.84
CP%	9.12	9.12
NDF%	61.88	61.88
ADF%	52.36	52.36

1. The premix (per kilogram of feed content): VA 1800 IU, VD 600 IU, VE 30 mg, Fe 65 mg, Se 0.15 mg, Cu 10 mg, Mn 28 mg, Zn 45 mg

2. ME was a calculated value, while the others were measureed values. The determination of dry matter (DM), crude protein (CP), acid detergent fiber (ADF), and neutral detergent fiber (NDF) contents in the diet is based on the standards outlined in GB/T 6435-2014(CHN), GB/T 6432-2018(CHN), and GB/T 20806-2022(CHN)

Ruminal fermentation parameters, MCP, and cellulase activity were determined

Identifying the MCP and parameters of ruminal fermentation, we used a portable pH test pen to measure pH (PH 800, SMART SENSOR, Hong Kong, China).The colorimetric approach was used to measure the concentration of NH_3-N [15]. The concentration of VFA (acetate, propionate, butyrate, pentanoate) was determined using liquid chromatography [16]; MCP concentration was determined using the Coomassie brilliant blue method [17].

The cellulase activity kit (Jingmei Biotechnology Co., Ltd., Jiangsu, China) was used to determine neutral xylanase (NEX), β-glucosidase (β-GC), and endothelial enzymes in the samples, endo-β−1, 4-glucanase/cellulase (EGse) enzyme activity. For detailed steps, please refer to the kit's instructions.

16S rRNA sequencing

First, Trimmomatic (version 0.33) was used to apply quality filtering to the raw data. The parameters included a 50 bp window. Trimmomatic (version 0.33) was used to apply quality filtering to the source data. Cutadapt (version 1.9.1) was used if the mean quality score within the window was below twenty, in which case the back-end bases were removed from the window. After determining and eliminating primer sequences, splice paired-end reads were obtained using USEARCH (version 10) and chimeras (UCHIME, version 8.1) were eliminate using UCHIME (version 8.1). After that, to obtain high-quality CCS (Circular Consensus Sequencing) sequences (UCHIME, version 8.1), we utilized the Lima (v1.7.0) program to identify the CCS sequences of various samples using barcode sequences and eliminated chimeras. OTU clustering: To cluster sequences at a 97% similarity level (default), we used USEARCH version 10.0. 0.005% of all sequences were used as the threshold by default to filter OTUs. ASV evaluation, after quality inspection, denoise the data using QIIME2 (version 2020.6) and the DADA2. The threshold used to filter ASVs was set to 0.005% of all sequence numbers that have been sequenced by default. The samples were sent to Tsingke Biotech (Beijing, China) to extract DNA and perform high-throughput 16S rDNA sequencing using the Illumina Novaseq platform (Illumina, San Diego, CA, USA).

Rumen metabolomics analysis

Metabolomic determination was performed using a Vanquish (Thermo Fisher Scientific) ultra-high performance liquid chromatograph and a Waters ACQUITY UPLC BEH Amide (2.1 mm × 50 mm, 1.7 μm) liquid chromatography column. A 100 μL sample was pipetted into

the EP tube, followed by the addition of 400 μL extraction solution (methanol: acetonitrile = 1:1 (v/v), containing isotope-labeled internal standard mixture), vortexed and mixed for 30 s; sonicated for 10 min (ice-water bath); incubated at − 40 °C for 1 h. The sample was centrifuged at 4 ℃, 12,000 rpm (13,800 × g, radius 8.6 cm) for 15 min, and the supernatant was transferred to a sampling bottle for analysis and run it on the machine for detection. An equal amount of supernatant was taken from all samples and mixed into QC samples for testing on the machine. Phase A of liquid chromatography was aqueous phase, containing 25 mmol/L ammonium acetate and 25 mmol/L ammonia water, and phase B was acetonitrile. Sample tray temperature: 4 °C, injection volume: 2 μL. The Orbitrap Exploris 120 mass spectrometer can collect primary and secondary mass spectrometry data under the control of control software (Xcalibur, version: 4.4, Thermo). The detailed parameters were as follows: Sheath gas flow rate: 50 Arb, Aux gas flow rate: 15 Arb, capillary temperature: 320 ℃, full ms resolution: 60, 000, MS/MS resolution: 15, 000, collision energy: SNCE 20/30 /40, spray voltage: 3.8 kV (positive) or − 3.4 kV (negative).

Statistical analysis

The high-throughput sequencing results were based on OTUs and their abundance results, and the alpha diversity index was obtained by the QIIME2 calculation and plotted using R software. QIIME2 was applied in order to generate the beta diversity distance matrix to plot PCA analysis based on the distance matrix. After the raw data of the metabolomics, ProteoWizard program transformed the test results into the mzXML format, metabolite identification was carried out using the collaboratively written R package. The database used was BiotreeDB (V3.0), and then the self-written R package was utilized for visual analysis. Other data were examined using IBM SPSS (26.0) software to determine their significance independent sample *t* test. $P < 0.05$ indicates significant difference, whereas $P > 0.05$ indicates no significant difference.

Result

Comparative analysis of rumen fermentation parameters, MCP content and cellulase activity of Hu sheep and Karakul sheep at different stages

As shown in Fig. 1, the NH_3-N concentrations of Hu and Karakul sheep differ significantly across the three stages. The NH_3-N concentrations of Hu sheep in stages I and II were significantly lower than those in Karakul sheep. In contrast, the MCP content exhibited an opposite trend, being higher in Hu sheep. In stage III, the NH_3-N concentration of Hu sheep was substantially higher than that of Karakul sheep, while the pentanoate content in Hu sheep during this stage was notably lower than that of Karakul sheep. However, other rumen fermentation indicators, including acetate, propionate and TVFA did not show significant differences, though they exhibited a general downward trend.

Figure 2 illustrates the cellulase activity in the rumen of Hu sheep and Karakul sheep at three stages. In stage I, the NEX activity of Hu sheep was substantially higher than that of Karakul sheep. However, the difference

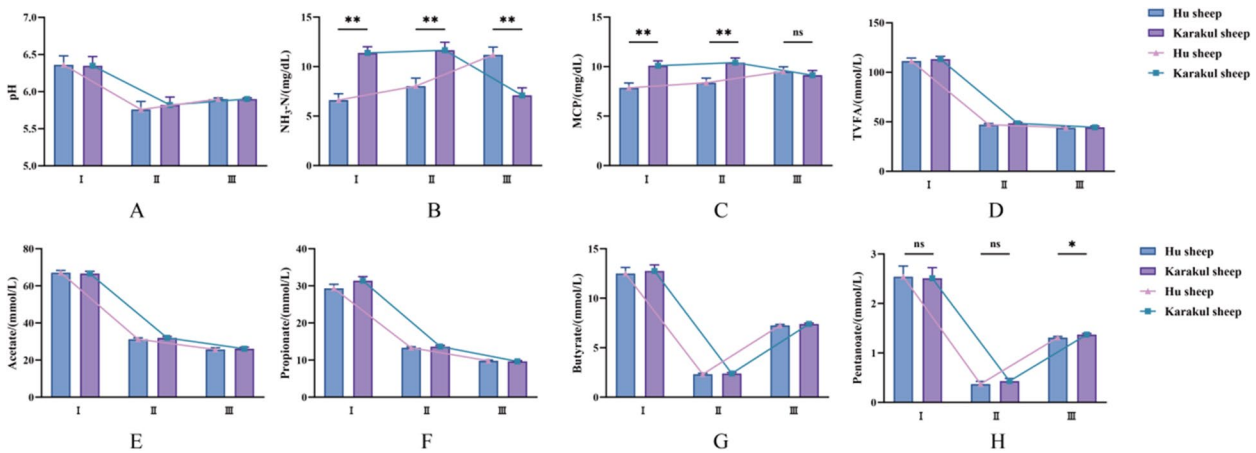

Fig. 1 Rumen fermentation parameters and MCP content of Hu and Karakul sheep at different stages. **A** Rumen pH values and trend lines of Hu and Karakul sheep at three stages. **B** Rumen NH_3-N concentration and trend line of Hu and Karakul sheep in three stages. **C** Rumen MCP content and trend lines of Hu and Karakul sheep in three stages. **D** Rumen TVFA content and trend lines of Hu and Karakul sheep in three stages. **E** Rumen acetate content and trend lines of Hu and Karakul sheep in three stages. **F** Rumen propionate content and trend lines of Hu and Karakul sheep in three stages. **G** Rumen butyrate content and trend lines of Hu and Karakul sheep in three stages. **H** Rumen pentanoate content and trend lines of Hu and Karakul sheep in three stages. ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$

between stages II and III were not statistically significant ($P > 0.05$); the β-GC and EGse activities across three stages were not significantly different. In stages I and II, the β-GC and EGse activities in Hu sheep were lower compared to Karakul sheep, whereas in stage III, the activities were higher in Hu sheep compared to Karakul sheep.

Comparative analysis of the ruminal microbial bacteria structure of Hu sheep and Karakul sheep at different stages

OTU analysis

According to the OTUs results obtained by clustering, the common and distinct OTUs between Karakul and Hu sheep were analyzed across the three stages. The Venn diagram results were shown in Fig. 3. In stage I, the number of OTUs that Hu sheep shared with Karakul sheep was 610, the number of OTUs unique to Hu sheep was 3706, and the quantity of OTUs exclusive to Karakul sheep was 3040. In stage II, Hu and Karakul sheep shared 620 OTUs, with Hu sheep having 2744 unique OTUs, and Karakul sheep also showing 2744 OTUs unique to them. In stage III, Hu and Karakul sheep in shared 989 OTUs. Hu sheep had 2665 unique OTUs to them, while Hu sheep Karakul sheep had 2645 OTUs exclusive to their ruminal microbiota.

Alpha diversity analysis of rumen bacteria

Statistics on the alpha diversity indices in samples from stages I, II, and III showed that the differences in alpha diversity between stage I and stage III were not significant. However, the Simpson diversity index for Hu sheep in stage II was markedly greater than that of Karakul sheep. The other alpha diversity indices showed not significantly differences between the two sheep breeds across the three stages (Fig. 4).

Relative abundance of rumen bacteria

According to the annotation results, it was found that in the three stages, *Bacteroidota* and *Firmicutes* were predominant bacterial phyla in the rumen of both Karakul and Hu sheep. In stage I, the proportional abundance of *Bacteroidota* in Hu sheep was notably lower than in Karakul sheep. In stage II, the relative abundance of *Spirochaetota* in Hu sheep was markedly higher than that in Karakul sheep. In stage III, the relative abundance of *Synergistota* and *Proteobacteria* in Hu sheep were appreciably lower than that in Karakul sheep. However, the relative abundances of other species at the phylum level did not significantly changes across different stages.

As shown in Fig. 5, the bacterial genera in the rumen of Hu and Karakul sheep were mainly composed of *uncultured_rumen_bacterium*, *Prevotella*, *Rikenellaceae_RC9_gut_grou*p, and some smaller genera. In stage I, the relative abundance of *unclassified_Clostridia_UCG_014* in Hu sheep was markedly higher than that in Karakul sheep; in stage II, the proportional abundance of *Prevotella* in Hu sheep was appreciably higher than that in Karakul sheep; however, the proportional abundance of *Quinella* in Hu sheep was

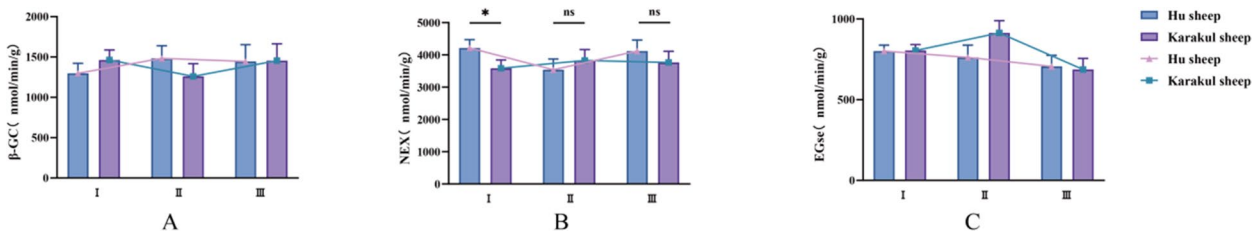

Fig. 2 Cellulase activity of Hu and Karakul sheep at different stages. **A** Rumen β-GC activity and trend line of Hu and Karakul sheep in three stages. **B** Rumen NEX activity and trend lines of Hu and Karakul sheep in three stages. **C** Rumen EGse activity and trend lines of Hu and Karakul sheep in three stages. ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$

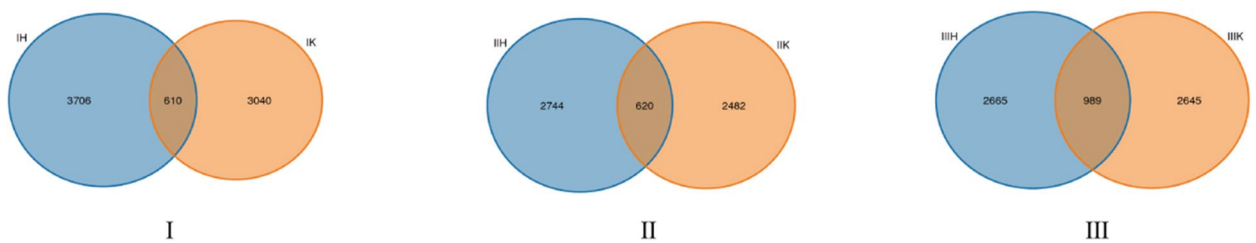

Fig. 3 Venn diagram of the OTUs analysis of Hu sheep and Karakul sheep at different stages

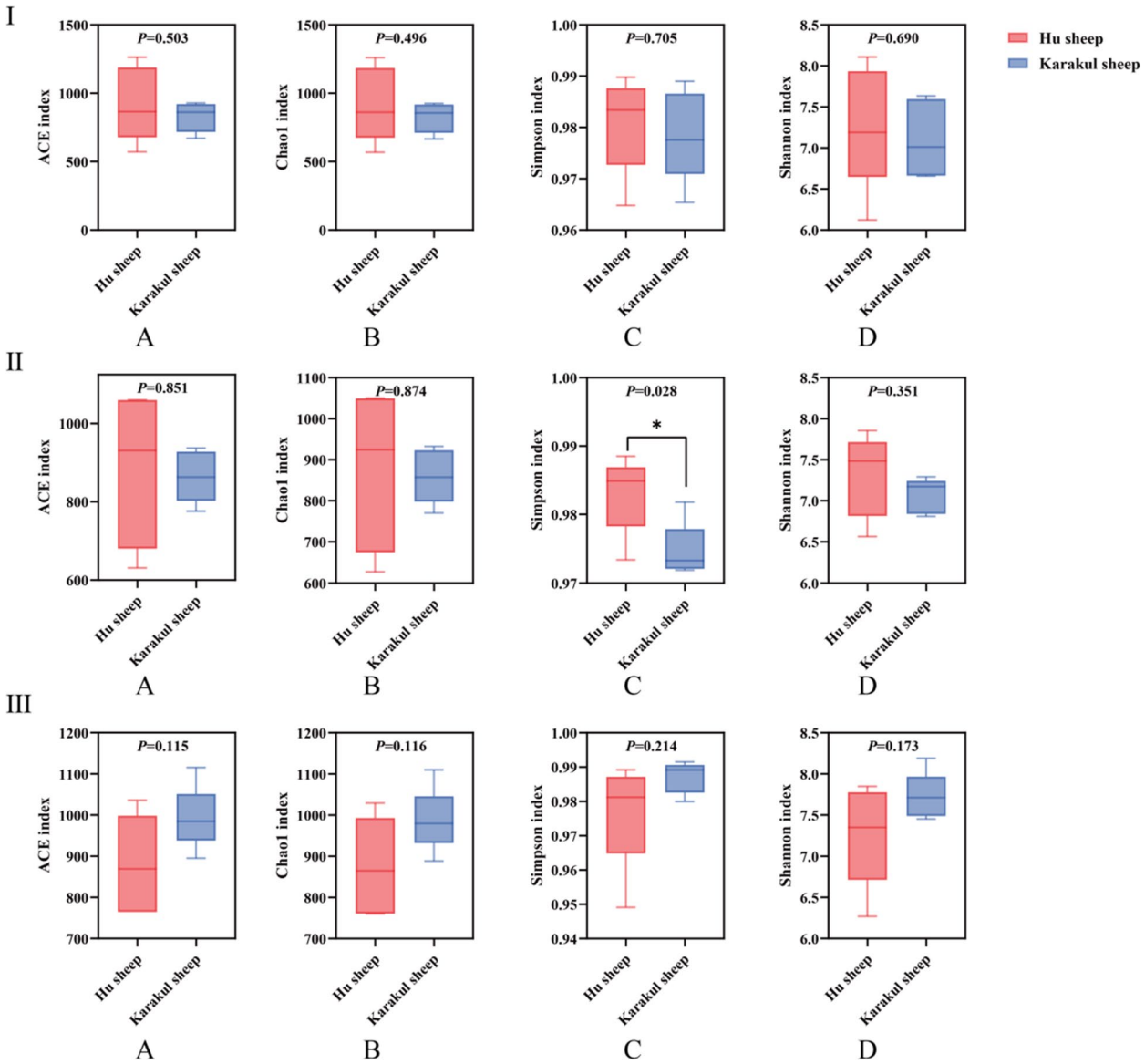

Fig. 4 Box plot of differences between groups for alpha diversity analysis. **A** ACE index, **B** Chao1 index, **C** Simpson index, **D** Shannon index. *$P < 0.05$

appreciably lower than that in Karakul sheep. The relative abundance of other species at the genus level did not change significantly at different stages.

It is also evident from Fig. 5 that the proportional abundance of *uncultured_rumen_bacterium* in stage I Hu sheep was appreciably lower than that of Kararkul sheep. However, the proportional abundance differences of other species at the species level in different stages were not significant. The overall abundance and structure of the rumen microbial community at phylum, genus and species levels gradually stabilized across three stages, reaching a relatively stable stage by stage III.

LEfSe analysis

Figure 6 exhibits the LEfSe (LDA Effect Size) analysis showing the histogram and evolutionary cladogram of the influence of differential bacterial groups. The results showed that in stage I, five different taxonomic levels of bacteria were found, and their LDA values higher than 4. Among these, two species were found in Hu sheep, while three species were found in Karakul sheep. In stage II, thirteen different taxonomic levels of bacteria with LDA values higher than 4 were found, including eight species in Hu sheep and five species in Karakul sheep. In stage III, five different taxonomic levels of bacteria were identified, all with LDA values

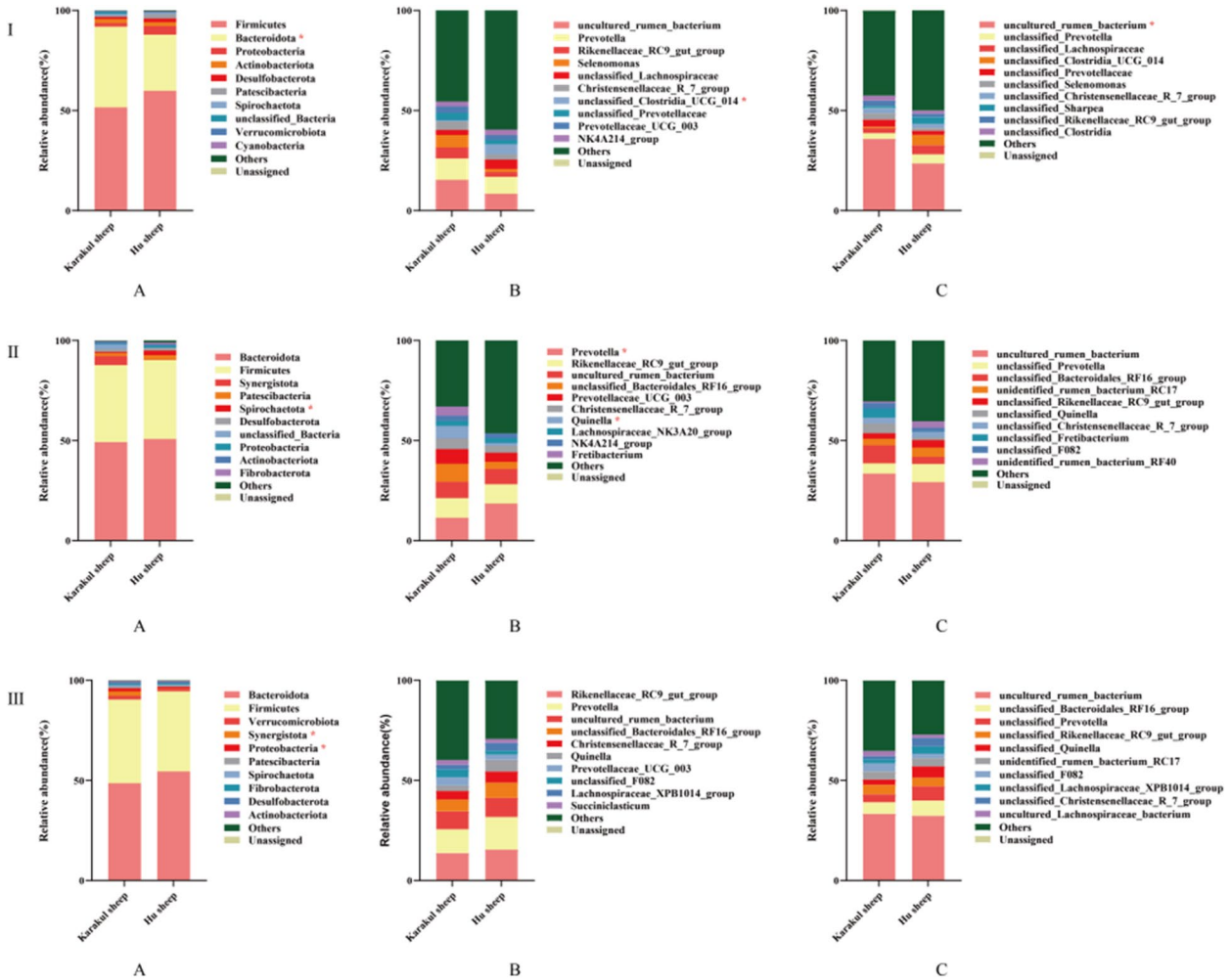

Fig. 5 Relative abundance of rumen bacteria in Hu sheep and Karakul sheep at different stages. **A** The phylum-level relative abundance of rumen bacterial. **B** The genus-level relative abundance of rumen bacterial. **C** The species-level relative abundance of rumen bacterial. *$P < 0.05$

higher than 4. Of these, two species were present in Hu sheep, and three species were found in Karakul sheep.

Identification of rumen metabolites and analysis of differential metabolites

Identification of ruminal metabolites

The PCA score plot for all samples across the three stages, as shown in Fig. 7, falls within the 95% confidence interval, confirming the validity of the experimental data. The OPLS-DA score plot, shown in Fig. 8, demonstrates clear separation between Hu sheep and Karakul sheep samples at all three stages, with all data points falling within the 95% confidence interval. The results of the permutation test between groups (HI vs. KI: R2Y = 0.83, Q2 = −0. 67 < 0; HII vs. KII: R2Y = 0.89, Q2 = −0.63 < 0; HIII vs. KIII: R2Y = 0.95, Q2 = 0.11) show that the model is valid, stable, and dependable.

Differential metabolites and their correlation analysis

Through LC–MS analysis, using the VIP>1, P&lt 0.05 limit, a total of 15,233 metabolites were discovered in rumen fluid samples from the three stages. In stage I, 1301 differential metabolites were detected, mainly consisting of fatty acids and their conjugates, benzene and substituted derivatives, carboxylic acids and derivatives, diterpenoids, triterpenes, small peptides, and alkaloids. In stage II, 1857 differential metabolites were detected, primarily including small peptides, glycerophospholipids, nucleosides, flavonoids, and alkaloids. In stage III, 439 differential metabolites were detected, mainly composed of glycerophospholipids and nucleosides.

Based on the clustering analysis, the differential metabolites across the different stages were further analyzed. The Venn analysis results were shown in Fig. 9. In stage I, the number of unique differential metabolites were 610.

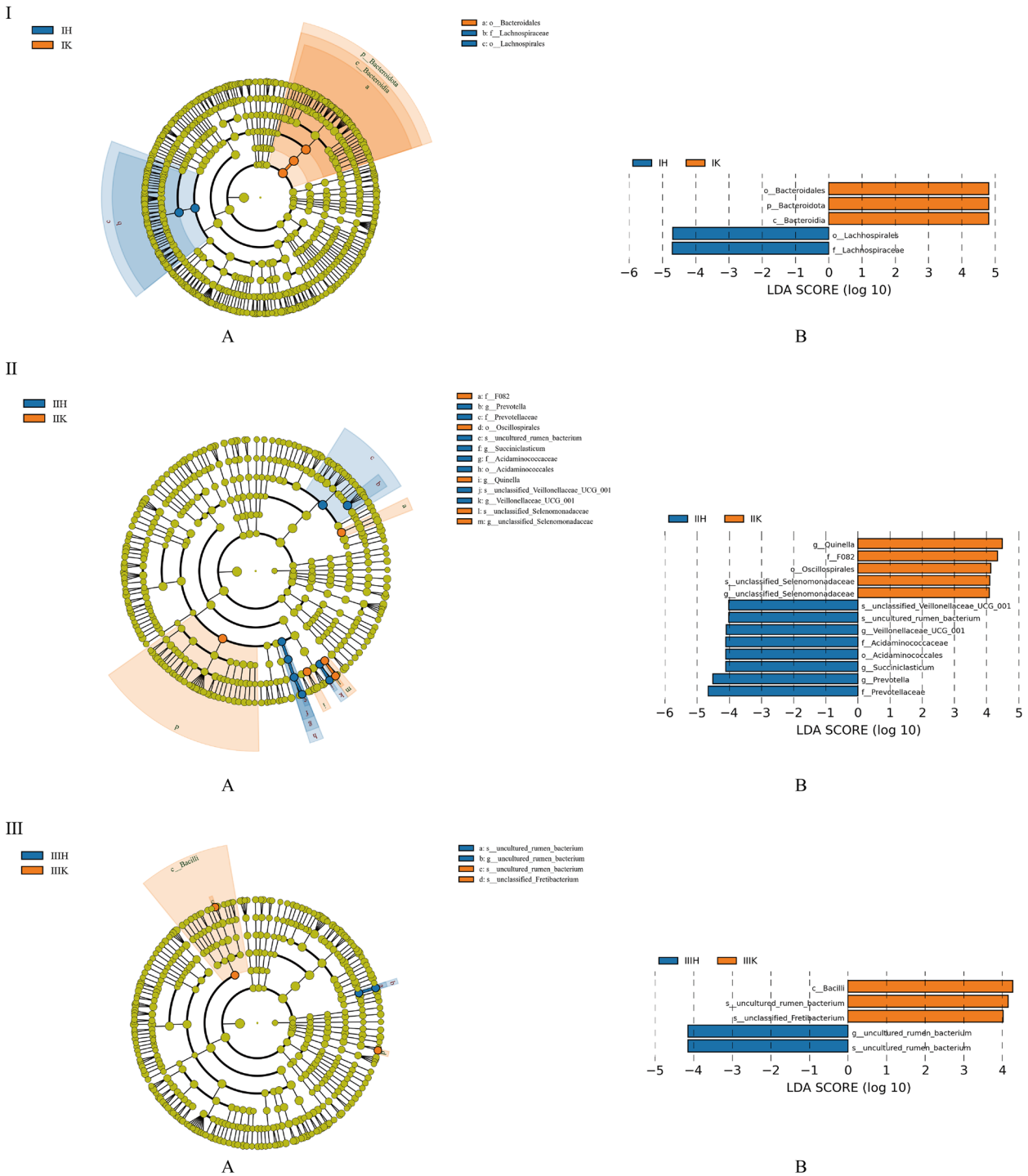

Fig. 6 The cladogram and influence distribution histogram of differential bacterial cladogram and influence distribution histogram showing significant differences in rumen bacteria between Hu sheep and Karakul sheep at different stages. **A** Evolutionary cladogram. **B** Influence distribution histogram

There were six differential metabolites shared between stages II and III, and 10 metabolites specific to stage II. In stage II, there were 236 distinct differentiated metabolites, and there were four differential metabolites in stage III. In stage III, the exclusive quantity of differential metabolites is peculiar count of 53. Furthermore, the heat map

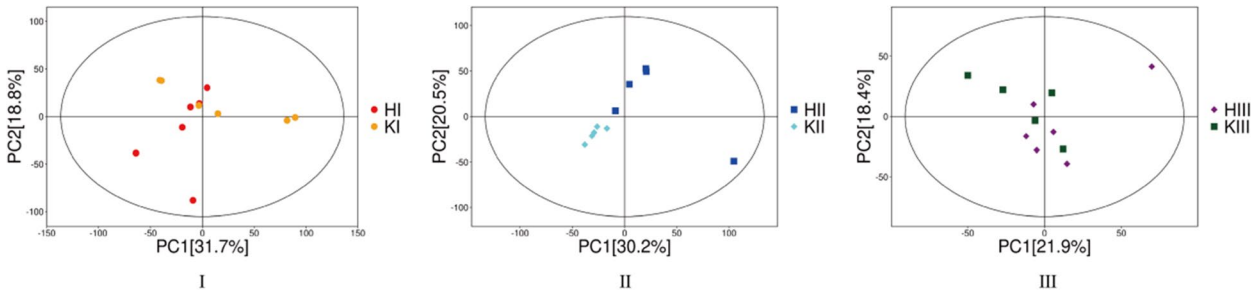

Fig. 7 Scatter plot of PCA scores of rumen metabolites of Hu sheep and Karakul sheep at different stages. The scores of the first and second ranked main components are symbolized by the ordinate PC2 and the abscissa PC1, respectively

analysis of differential metabolites demonstrated significant variation in metabolites' expression across the groups, suggesting that the rumen fluid's metabolite synthesis in Hu sheep and Karakul sheep differed at each stages. In addition, the correlation heat maps of metabolites at different stages (Figs. 10 and 11) showed that there were obvious positive and negative correlations between different metabolites, indicating that there were interactions in the rumen microbial metabolic pathways of Hu sheep and Karakul sheep at different stages.

Differential metabolite identification and enrichment analysis

In the rumen, intricate chemical reactions and its management took place in tandem, regulated by various genes and proteins that form intricate pathways and networks. Systemic alterations in the metabolome were ultimately the result of their mutual influence and regulation. In this research, we found 1301 distinct metabolites in the fluid of Hu and Karakul sheep's rumen in stage I. Compared to Hu sheep, Karakul sheep in stage I exhibited 188 significantly down-regulated metabolites in the rumen (FC < 0.5, $P < 0.05$) and 1113 significantly up-regulated differential metabolites (FC > 2, $P < 0.05$). In stage II, the rumen fluid contained a total of 1857 differentiable metabolites of Hu and Karakul sheep. In Karakul sheep's rumen fluid during stage II, 1330 metabolites were highly upregulated (FC > 2, $P < 0.05$) and 527 metabolites were significantly downregulated (FC < 0.5, $P < 0.05$). In stage III, a total of 439 differential metabolites were identified in the rumen fluid of Hu sheep and Karakul sheep. Compared to Hu sheep in stage III, there were 361 significantly downregulated differential metabolites in the rumen fluid of Karakul sheep's (FC < 0.5, $P < 0.05$), and 78 significantly upregulated differential metabolites (FC > 2, $P < 0.05$).

To further explore the functional mechanisms these differential metabolites across the three stages, this study analyzed the influencing factors of metabolic pathways (Fig. 12). In the topological analysis, each square represents a metabolic pathway. The size of the square indicates the pathway's impact factor—larger squares denote higher impact factors. The *P* value of the enrichment analysis is represented by the square color (using the negative natural logarithm, or -ln(p); the lower the *P* value, the deeper the color, and the more significant the degree of enrichment. The results showed that most of the metabolites in stage I were mainly involved in metabolism of purine and fatty acid, fatty acid biosynthesis, and biosynthesis of unsaturated fatty acids (Fig. 12(I)). In stage II, most metabolites were mainly involved in metabolism of purine, arginine, proline and tyrosine, and aminoacyl-tRNA biosynthesis (Fig. 12(II)). In stage III, most of the metabolites were mainly involved in primary bile acid biosynthesis, metabolism of pyrimidine, sphingolipid, and propanoate (Fig. 12(III)).

Relationship between rumen bacteria and metabolites and rumen characteristics

The dynamic changes in the rumen microbial community were reflected in variations in rumen metabolites, which in turn affect the rumen characteristics of ruminants. Examination the correlation between bacterial populations and rumen metabolites and rumen characteristics can help to comprehensively understand the composition, function, and pathways of metabolism of rumen ecosystems. Therefore, based on the stage III LEfSe analysis, we identified five biomarkers with rumen characteristics and key differential metabolites in metabolic pathways. The results showed that NEX activity has a very significant negative correlation with the biomarker *c_Bacilli* in Karakul sheep and the biomarker *g_uncultured_rumen_bacterium* in Hu sheep. Additionally, the biomarker *s_uncultured_rumen_bacterium* in Hu sheep showed considerable negative correlations with MCP and pH. The biomarker *c_Bacilli* in Karakul sheep had a significant negative correlation with propionic acid, *s_uncultured_rumen_bacterium* had a

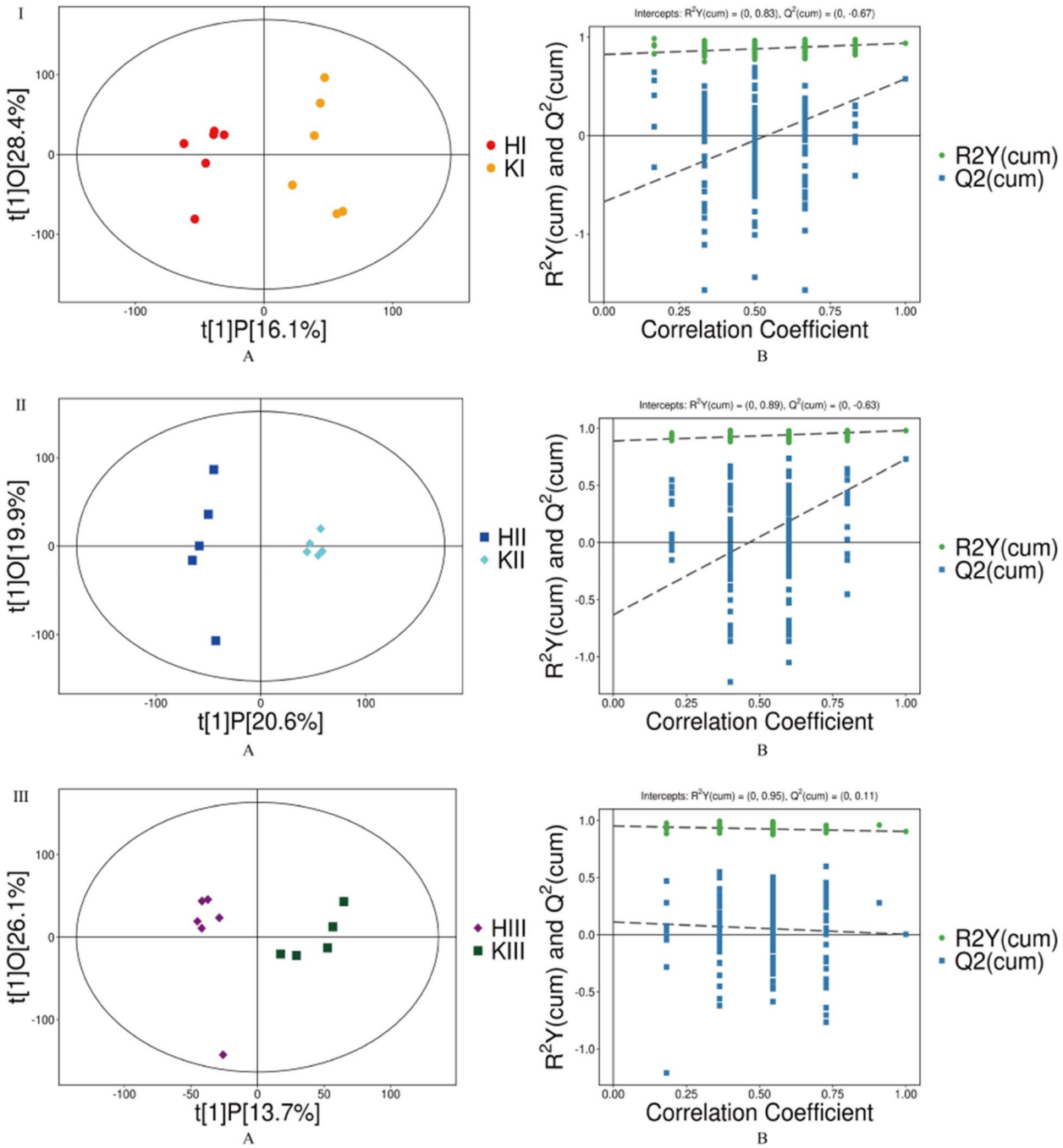

Fig. 8 Rumen metabolite analysis using orthogonal partial least squares discriminant analysis (OPLS-DA) in Hu sheep and Karakul sheep at different stages. **A** Scatter plot obtained by the OPLS-DA model. The anticipated principal component score of the first principal component, which illustrates the variation between sample groups, is represented by the abscissa t[1]P. The score for the orthogonal main component is expressed by the ordinate t[1]O. **B** The abscissa of the OPLS-DA permutation test reflects the permutation retention of the test. The two dashed lines indicate the regression lines for R2Y and Q2, respectively, while the ordinate shows the values of the permutation tests for R2Y (green circle) and Q2 (blue square)

significant negative correlation with Sphinganine, and *g_uncultured_rumen_bacterium* has a significant negative correlation with taurochenodesoxycholic acid and taurodeoxycholic acid. Furthermore, the biomarker *g_uncultured_rumen_bacterium* in Hu sheep was significantly positively correlated with dCMP and significantly

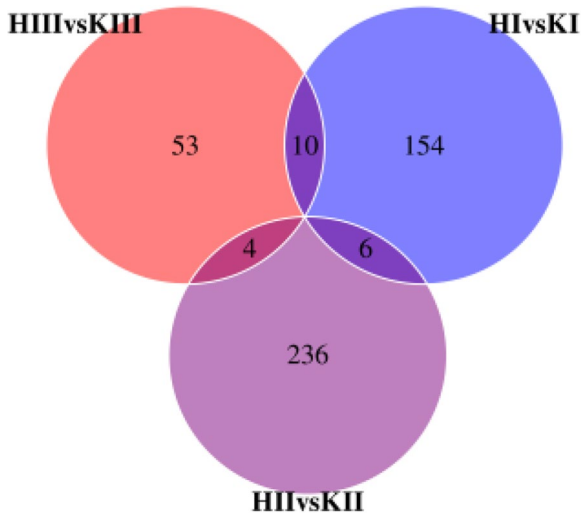

Fig. 9 Venn diagram of differential metabolites in the rumen of Hu sheep and Karakul sheep at different stages

negatively correlated with Sphinganine. The biomarker *s_uncultured_rumen_bacterium* was significantly positively correlated with deoxyadenosine monophosphate and deoxyuridine, *N*-acetyl-glucosamine 1-phosphate, 5′-deoxyadenosine, deoxyadenosine, and deoxyuridine (Figs. 13 and 14).

Discussion

Differences in animal breeds and ages are important factors affecting the growth of rumen microbiota. While, the structure of rumen microbial communities were similar across different breeds, there were some variations in the proportional abundance of various bacteria. These differences, particularly those caused by age, are mainly because the gradually development of the rumen microbial community [18]. The interaction between the host, the microbial community and their metabolites were essential for the physical wellbeing, nutritional metabolism and feed resource utilization efficiency of sheep. This study investigated how feeding cottonseed hulls affected the metabolome and bacterial community in the rumen of Hu sheep and Karakul sheep at different stages. One of the key signals of rumen fermentation is the pH, which also influences rumen microbial activity, nutrient digestion and metabolism, and the production and absorption of VFA [19, 20]. The normal range of rumen pH is between 5.5 and 7.0 [21]. Hu and Karakul sheep in the current research showed pH levels that were all within the typical range at each of the three stages, indicating that the sheep breed and diet structure do not affect the rumen pH values. Rumen NH_3-N concentration is an important indicator of rumen fermentation capacity and is the end product of protein degradation in the rumen. Therefore, changes in ammonia nitrogen content reflect changes in feed protein degradation efficiency and microbial activity [22]. The synthesis of MCP can reflect the ability of microorganisms to absorb and utilize NH_3-N [23]. We found significant differences in NH_3-N content between Hu sheep and Karakul sheep across three stages. The NH_3-N content of Hu sheep in stages I and II was very significantly lower than that of Karakul sheep, while in stage III, NH_3-N content was

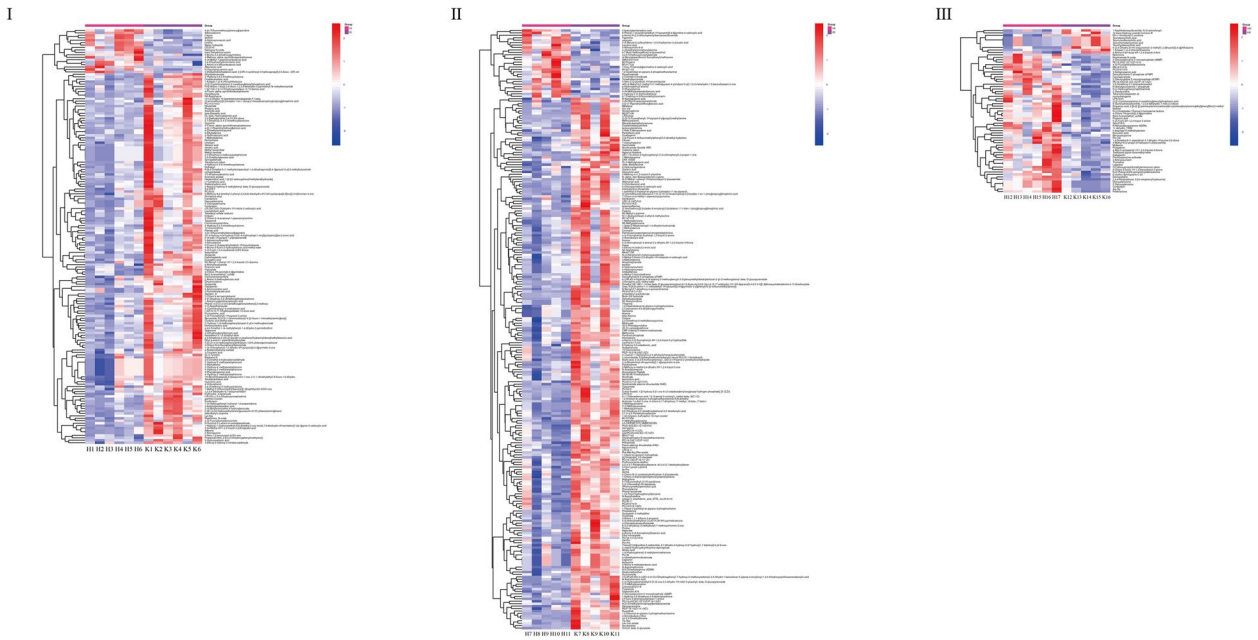

Fig. 10 Heat map of hierarchical clustering analysis of rumen metabolites of Hu sheep and Karakul sheep at different stages

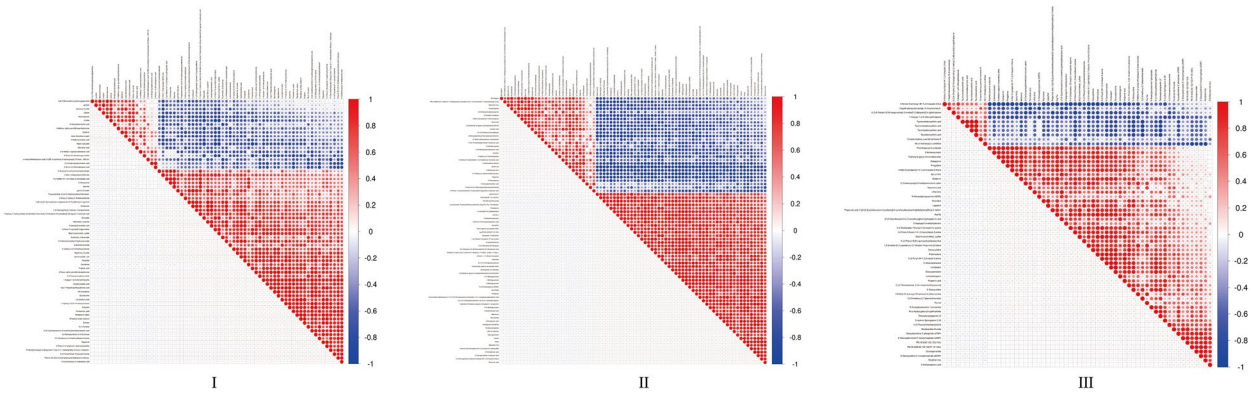

Fig. 11 Heat map of correlation between rumen metabolites of Hu sheep and Karakul sheep at different stages

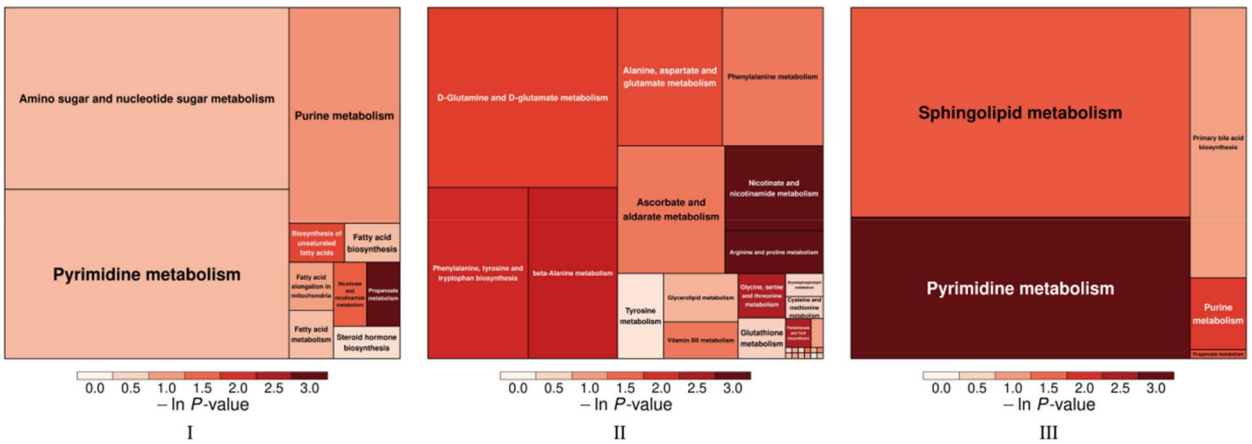

Fig. 12 Enrichment analysis of ruminal metabolic pathways in Hu sheep and Karakul sheep at different stages

higher in Hu sheep compared to Karakul sheep. The MCP content of Hu sheep in stages I and II were markedly lower than that of Karakul sheep, indicating that in stages, Hu sheep had a better utilization rate of NH_3-N. This study found that breed had a significant effect on the rumen ammonia nitrogen concentration under the same feeding conditions. In contrast, the breeds of Yiling cattle and Angus × Yiling cross cattle showed no effect on the rumen ammonia nitrogen concentration under the same feeding mode [24], which may be caused by the overall feeding cycle.

VFA are the primary energy source for ruminants, generated by the fermentation of organic matter in the feed and can supply 70% to 80% of the total energy needed [25]. VFA is mainly composed of acetate, propionate, butyrate and pentanoate, with their proportion mainly affected by the feed [26]. This study demonstrated that, in stage III, Hu sheep had substantially lower pentanoate contents compared to Karakul sheep, while no significant differences in VFA indicators were observed in other stages. The reason for the difference in pentanoate content between Hu sheep and Karakul sheep in stage III may be due to differences in rumen flora; it can be explained that the VFA content of Hu sheep and Karakul sheep is not affected by breed. Several studies have shown that rumen bacteria, fungi and protozoa jointly participated in the digestion process of cellulose in the rumen, in which anaerobic fungi played an important role. These fungi release a number of enzymes, such as cellulase, neutral xylanase, glycoside hydrolases, and others, which broken down polysaccharides, and reflect the cellulose degradation ability in the rumen, playing a vital role in the digestion of roughage by ruminants [27–29]. Early weaning can promote the proliferation of rumen microorganisms and increase the contents of carboxymethyl cellulase, neutral xylanase, cellulase, and amylase in the rumen [30, 31]. In our study, the neutral xylanase activity of Hu sheep was appreciably higher than that of Karakul sheep in stage I, but the difference was not significant in stages II and III. The neutral xylanase activity of Hu sheep and Karakul sheep increased with age, which is consistent with the above research results.

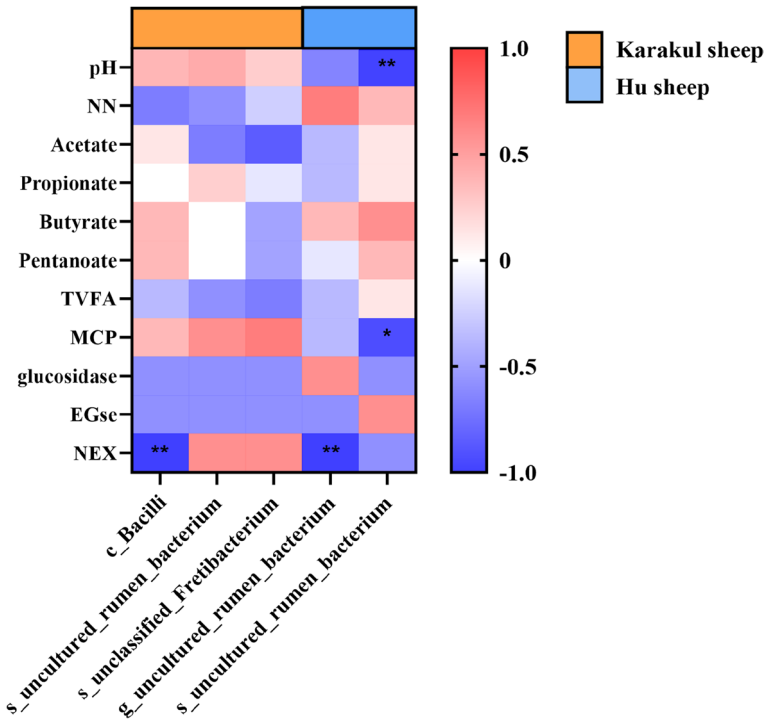

Fig. 13 Correlation matrix between the differential bacterial genera found in the rumen and its features. Positive correlation is displayed by red, and negative correlation is displayed by blue. *$P < 0.05$; **$P < 0.01$

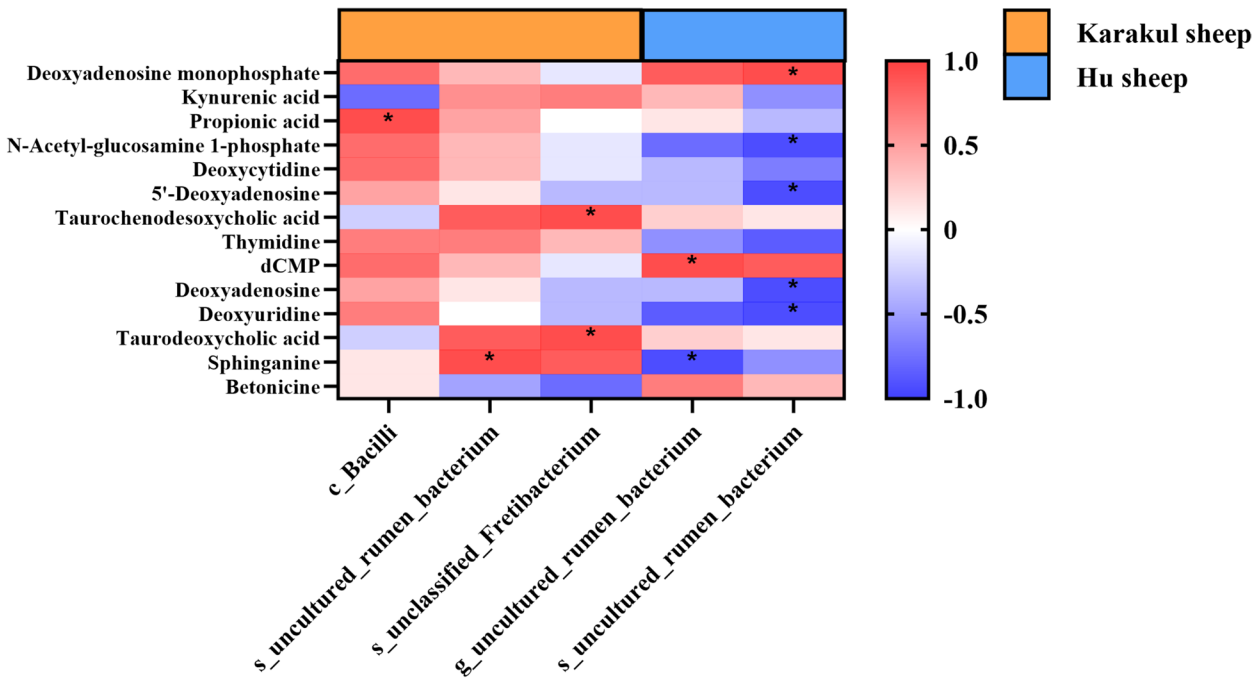

Fig. 14 Correlation matrix of differential rumen bacterial genera and differential rumen metabolites. Positive correlation is displayed by red, and negative correlation is displayed by blue. *$P < 0.05$; **$P < 0.01$

This study described the ruminal bacterial communities of Hu and Karakul sheep across three stages. The Shannon and Simpson indices were used to quantify species diversity, while the Chao1 and ACE indices directly represented species richness. Previous research results have shown that the most abundant bacterial phyla in the rumen were *Firmicutes* and *Bacteroides*, and their relative abundance accounts for more than 90% of the rumen bacteria. Changes in the food concentration ratio had little effect on the quantity of Bacilli, presumably owing to rumen microorganisms adapt to changes in dietary concentration levels through self-regulation [32–34]. Similarly, the alpha diversity results of this study showed that the Simpson index of Hu sheep in stage II is notably higher than that of Karakul sheep, while other indicators were not significantly different at different stages. This explains why there is no significant difference in alpha diversity indicators (Chao1, ACE, and Shannon index) between the three stages. At the phylum level in stage II, the abundance of *Spirochaetota* in Hu sheep was notably higher than that in Karakul sheep, which may also explain the significant difference in Simpson index between two breeds. The abundance of *Bacteroides* in Hu sheep in stage I was markedly lower than that in Karakul sheep, but there was no discernible variation between stages II and III. The main bacterial phylum changed from *Firmicutes* to *Bacteroides*, which was in line with Zhang's research finding [10].

The results of this study showed that *Prevotella*, *Rikenellaceae_RC9_gut_group*, and *uncultured_rumen_bacterium* were the dominant bacterial genera in the three stages and the most active bacterial genera in the rumen. They not only participated in the degradation process of cellulose, but also interact with proteins and soluble bacteria in the rumen, contributing to the degradation of carbohydrates and hemicellulose [35, 36]. *Quinella* primarily contributes to the breakdown of carbohydrates and the metabolism of lactic and propionic acids in the rumen [10, 37]. In the second stage, *Prevotella* in Hu sheep was notably higher than that in Karakul, while the genus *Quinella* in Hu sheep was substantially lower than that in Karakul sheep. This suggests that Hu sheep may have an advantage in digesting non-fiber carbohydrate diets in stage II.

Under normal circumstances, ruminants eat a diet rich in fiber, which is converted into various substitutes for maintenance and production with the help of rumen microorganisms, but when eating a diet rich in fermentable carbohydrates, it will lead to changes in rumen microflora, accompanied by changes in rumen metabolic pathways [38, 39]. Yi [40] investigated how the rumen fluid microbiota and metabolites of yaks were affected by three distinct ratios of food concentrate to forage (50:50, C50 group; 65:35, C65 group; and 80:20, C80 group). The findings demonstrated that modifications in the dietary concentrate ratio had a major impact on the rumen metabolites' lipid metabolism and purine metabolism, protein digestion and absorption, amino acid metabolism, and carbohydrate metabolism. The results of this study showed that most of the metabolites in stage I were mainly involved in metabolic pathways such as purine metabolism, biosynthesis of unsaturated fatty acids, and propanoate metabolism; in stage II, most metabolites were mainly involved in arginine and proline metabolism, purine metabolism, glycine, serine and threonine metabolism, and nicotinic acid and nicotinic acid metabolism; most metabolites in stage III were mainly involved in metabolic pathways such as pyrimidine metabolism and purine metabolism, which was consistent with the above research results. We found that in stage I, mainly fatty acids and their derivatives were significantly up-regulated; in stage II, mainly small peptides and glycerophospholipids were significantly upregulated; but in stage III, mainly nucleosides and glycerophospholipids were significantly downregulated. Fatty acids and their derivatives were mainly involved in propionate metabolism and lipid metabolism; small peptide metabolites were mainly involved in starch biosynthesis, fatty acid metabolism, and protein digestion and absorption; glycerophospholipid metabolites were mainly involved in metabolism and lipid metabolism. Starch metabolism; nucleoside metabolites were mainly involved in the biosynthesis, metabolism and lipid metabolism of starch. Wang [41] discovered that the tryptophan metabolism pathway is responsible for controlling the nutritional absorption and growth performance of Tibetan sheep when their diet is high in protein. The data above suggest that via enhancing lipid and amino acid metabolism in the rumen, various diets influence the growth and productivity performance of Tibetan sheep. VFA or NH3-N can be used by rumen microbes as a source of nitrogen for the generation of amino acids. This implied that higher NH3-N and acetic acid in the rumen supply more substrate for amino acid production. Chen's study [42] found that hepatocytes produced lipid droplets in response to D-ribose. In Hu sheep, significantly upregulated metabolites in stages I and II were mainly associated with lipid metabolism and fatty acid metabolism, but they were all significantly down-regulated in stage III, suggesting that the complete feeding of cottonseed hulls promotes lipid metabolism and fatty acid metabolism through rumen microorganisms.

Increasing evidence suggest that rumen microorganisms were closely related to metabolites in the rumen [43, 44]. Based on the stage III Lefse analysis, five biomarkers were found in the rumen to clarify the primary

distinctions among various rumen species. A correlation study between biomarkers, rumen characteristics, and rumen metabolites was performed, because variations in these microbes may impact the host phenotype [45]. Two biomarkers were identified in Hu sheep, both of which were unclassified bacteria. The bacterium *g_uncultured_rumen_bacterium* was significantly negatively correlated with NEX and sphinganine. Rumen were influenced by the interactions between microorganisms like bacteria and fungus and the hosts, which broken down fiber and produce cellulases (including BG enzymes, C1 enzymes, Cx enzymes, and NEX enzymes) to degrade more complex structures [46, 47]. Sphinganine is mainly involved in energy metabolism and lipid metabolism, while s_uncultured_rumen_bacterium is significantly negatively correlated with deoxyadenosine and deoxyuridine. Deoxyadenosine and deoxyuridine were mainly involved in energy metabolism. The data of this study showed that the high abundance of *g_uncultured_rumen_bacterium* and *s_uncultured_rumen_bacterium* in Hu sheep led to decrease in their NEX content. Sphinganine, deoxyadenosine and deoxyuridine were significantly down-regulated; three biomarkers found in Karakul sheep, all of which were unclassified bacteria. Among them, *s_uncultured_rumen_bacterium* was significantly positively correlated with Sphinganine, and *s_unclassified_Fretibacterium* was significantly positively correlated with taurochenodesoxycholic acid and taurodeoxycholic acid. Sphinganine, taurochenodesoxycholic acid, and taurodeoxycholic acid were all involved. Lipid metabolism, taurochenodesoxycholic acid, and taurodeoxycholic acid were metabolites of bile acids. They were mainly responsible for the emulsification of lipids and the absorption of fat-soluble vitamins [43]. From this, it can be concluded that while there were differences in the expression of metabolic pathways between Hu sheep and Karakul sheep; these differences do not significantly impact rumen fermentation characteristics and the microbial composition of these sheep.

Conclusion

Overall, the breeds of sheep were not the primary element influencing the composition of the bacterial population and rumen fermentation. Although the metabolic pathways of rumen microbial metabolites differ among individual sheep, but the functions of rumen microbial metabolite among were generally consistent, indicating similar primary functions despite species differences. These results lay the foundation for greater understanding of the intricate connection between breed and rumen microbial function in two sheep breeds tested in the study. This also supports our previous observations, where Hu sheep and Karakul sheep were fed the same diet, showed differences in bacterial communities due to variations in husbandry and dietary habits. However, this research has limitation, particularly in clarifying the specific functions of rumen microbial biomarkers and determining whether the metabolic pathways of corresponding metabolites were affected by genetic factors. Further investigation is needed to explore the connections between the rumen metabolome and the interaction mechanisms between different breeds and the rumen microbiota.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-024-02020-7.

Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions

Rumen samples were collected by Jishu Wang, Guangxu E, Yanlong Zhang, and Tiantian Bai for experimental purposes. Xuefeng Guo and Junfeng Liu discussed the data and provided suggestions throughout the experiment. Data analysis and bioinformatics/statistical analyses were carried out by Jishu Wang. Xuefeng Guo supervised the entire experiment. The manuscript was written by Jishu Wang, with input and suggestions from Meng Zhang, Xuanxuan Pu ,Subir Sarker and Long Cheng. All authors participated in revising the manuscript, and approved the final version.

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Data availability

PRJNA1137859.

Declarations

Ethics approval and consent to participate

All experimental procedures were approved by the Animal Research Ethics Committee of Tarim University, and were conducted in accordance with the Guidelines for the Care and Use of Research Animals in China (GB14925-2001) (PRC, 2001).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details
[1]College of Animal Science and Technology, Tarim University, Alar, Xinjiang 843300, PR China. [2]Key Laboratory of Tarim Animal Husbandry Science and Technology of Xinjiang Production and Construction Group, Alar, Xinjiang 843300, PR China. [3]Key Laboratory of Livestock and Forage Resources Utilization Around Tarim, Ministry of Agriculture and Rural Affairs, Alar, Xinjiang 843300, PR China. [4]College of Life Science and Technology, Tarim University, Alar, Xinjiang 843300, PR China. [5]Biomedical Sciences & Molecular Biology, College of Medicine and Dentistry, James Cook University, Townsville, QLD 4811, Australia. [6]Australian Institute of Tropical Health and Medicine, James Cook University, Townsville, QLD 4811, Australia. [7]Faculty of Science, Dookie Campus, The University of Melbourne, Melbourne, VIC 3647, Australia.

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