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High-Grain Diet Feeding Altered Blood Metabolites, Rumen Microbiome, and Metabolomics of Yaks

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Abstract: Currently, information available on the comprehensive changes in the rumen bacteria and metabolites of yaks fed high-grain diets is limited. This study aimed to investigate the effects of high-grain diet feeding on the blood metabolites, rumen microbiome, and metabolomics of yaks by using 16S rDNA gene sequencing and liquid chromatography–mass spectrometry (LC/MS). Here, fourteen healthy male yaks (body weight, 249.61 ± 8.13 kg) were randomly assigned to two different diets: a hay diet (0% grain, CON, n = 7), or a high-grain diet (70% grain, HG, n = 7). At the 74th day of treatment, blood and ruminal fluid samples were collected for the blood metabolites, rumen microbiome, and metabolomics analyses. The HG diet increased lipopolysaccharides (LPS), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), haptoglobin (HPT), serum amyloid-A (SAA), interleukin-1β (IL1-β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) serum concentrations ($p < 0.05$). Compared with the CON diet, the HG diet decreased rumen pH ($p < 0.05$), and increased total volatile fatty acids concentration, and proportion of butyrate ($p < 0.05$). The relative abundance of Firmicutes and Saccharibacteria were higher ($p < 0.05$), while Bacteroidetes was lower ($p < 0.05$) in the HG group than those in the CON group. At the genus level, the relative abundance of *Christensenelaceae_R-7_group*, *Ruminococcaceae_NK4A214_group*, *Lachnospiraceae_NK3A20_group*, and *Acetitomaculum* were higher than in those in the HG diet ($p < 0.05$). Compared with the CON group, the HG diet increased the concentrations of biogenic amines (histamine, tyramine, and putrescine), common amino acids (phenylalanine, threonine, serine, etc.), and arachidonic acid (prostaglandin H2, prostaglandin E2, 12(S)-HPETE, etc.). Collectively, these findings demonstrate that the HG diet altered the microbiota and metabolites, as well as potentially damaged their rumen health and induced inflammation in yaks.

Keywords: yak; high-grain diet; rumen microbiota; rumen metabolites; blood metabolites

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1. Introduction

Yak (*Bos grunniens*) is a uniquely herbivorous species of livestock on the Tibetan plateau, contributing milk, meat, and transport for the local herders [1]. Grazing is the conventional means of the yak's feeding, and they mainly inhabit extreme environments with low temperatures, decreased oxygen, and high altitudes, resulting in a severe shortage of quality and availability of forage, particularly in winter [2]. Moreover, the livestock population of the Tibetan plateau pastures has been rising, including the number of yaks [3], which would increase grassland degradation [4]. Hence, nutritional strategies such as supplementary feed and indoor feeding have emerged to reduce pasture pressure and improve yak growth performance [5,6].

A high-grain diet is essential for beef and dairy cattle to maximize growth, productivity, and high-quality meat or milk [7]. However, excessive fermentable carbohydrates

produce high concentrations of organic acids (volatile fatty acids and lactic acid) in the rumen [8,9], resulting in a low rumen pH, increasing the probability of subacute rumen acidosis, and decreasing long-term production [10,11]. A growing number of studies have focused on the effects of high-grain diets on rumen microbial composition and health in ruminants [12,13]. Hua et al. [14] evaluated the rumen microbiome and metabolism of goats fed a high-grain diet, showing that it reduced microbiota diversity and caused disorders of metabolism in the rumen. Additionally, feeding a high-grain diet enhanced inflammatory responses in ruminants, such as increased concentrations of acute proteins and inflammatory factors [15]. A series of studies have showed that changes in diet altered rumen microbiota and the metabolomics of yaks [6,16]. For instance, Zhang et al. [17] investigated the effect of dietary protein levels on ruminal microbiota and metabolites in yaks; the results showed that a high-protein diet with higher abundance of starch-degrading bacteria increased the concentrations of organic acid and amino acids metabolites, which revealed a close relationship between rumen microbes and metabolites. However, to the best of our knowledge, there is limited information on the effects of a high-grain diet on the inflammatory response, rumen microbiome and metabolomics of yaks.

Therefore, this study considered both the ruminal microbiome and the metabolome to investigate bacterial community diversity and metabolite composition of yaks fed a high-grain diet or hay diet. We also evaluated the variation in blood metabolites in response to yaks fed a high-grain diet, with inflammatory properties in particular. We hypothesized that HG diets impact the blood metabolites, ruminal microbiota and metabolites, which in turn affect the yak's health.

2. Materials and Methods

All experimental procedures and animal experiments were performed following the guidelines of the relevant Ethics Committee. This study was approved by the Institutional Animal Care and Use Committee of Qinghai University (Protocol number: QHU20200617).

2.1. Animals, Diets, and Experimental Design

The feeding trial was conducted from July to September 2020 at the commercial yak farm of Laozaxi Ltd. in Guinan County, Qinghai Province, China. In brief, fourteen 3-year-old male yaks with similar body conditions (249.61 ± 8.13 kg) were adapted to an hay-only diet for two weeks and then randomly divided into two treatment groups: one group was fed a hay diet (0% grain, CON, $n = 7$), and the other group was fed a high-grain diet (70% grain, HG, $n = 7$). The ingredients and nutrient composition of the two diets are shown in Table 1. The experimental period lasted for 60 d. Each yak was fed 4.0 kg/d of dry matter diet for a whole day, equally divided at 06:00 and 18:00, separated by fencing, with free access to water.

Table 1. Ingredient and nutrient composition of the diets.

Item	Diet	
	Hay	High-Grain
Diet ingredient, % DM		
Oat hay	75.46	35.00
Alfalfa	19.53	0.00
Corn	0.00	39.75
Wheat	0.00	30.25
$CaHPO_4$	1.50	1.50
NaCL	1.00	1.00
Premix	2.50	2.50
Nutrient levels		
Crude protein, % DM	10.49	10.73
Crude fat, % DM	3.55	3.59
Neutral detergent fiber, % DM	53.32	29.23
Acid detergent fiber, % DM	34.48	14.55
Crude ash, % DM	9.68	6.36

The premix provided the following, per kg of the diet: VA 3500 IU, VD 1000 IU, VE 40 IU, Mn 40 mg, Fe 50 mg, Cu 10 mg, Zn 40 mg, Se 0.3 mg. Nutrients were measured values.

2.2. Sample Collection

At the end of the experiment, blood samples were collected from the jugular vein into 5 mL evacuated tubes (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) without anticoagulant after 2 h of the morning feeding, then centrifuged at 3500× *g* for 15 min at 4 °C to obtain serum samples, frozen with liquid nitrogen, and stored at −80 °C until analysis. Meanwhile, we collected approximately 100 mL ruminal fluid from each yak through a flexible oral stomach tube (Wuhan Colibri Herding Technology Company, Wuhan, China), and extracted rumen fluid pH was quickly measured by a portable pH meter (Testo205; Testo AG, Schwarzwald, Germany). Fifty milliliters of ruminal fluid was filtered through four layers of sterile gauze and then flash-frozen in liquid nitrogen for the determination of VFAs. The remaining 50 mL of ruminal fluid insert was immediately frozen in liquid nitrogen for use in sequencing and metabolomics analyses. On arrival at the experimental laboratory, all samples were transferred to a −80 °C freezer (Qingdao Haier Group Company, Qingdao, China).

2.3. Blood Metabolites and Rumen Fermentation Parameters Analyses

The concentrations of aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were analyzed with an automatic biochemical analyzer (Beckman Coulter AU 480; Brea, CA, USA). Blood samples' glutamyltransferase (GGT), haptoglobin (HPT), serum amyloid-A (SAA), interleukin-1β (IL1-β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) concentrations were measured by ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and the serum Lipopolysaccharides (LPS) concentrations were determined using a Limulus amebocyte lysate assay (Xiamen Houshiji, Ltd., Xiamen, China).

Concentrations of VFAs (acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate) were measured with gas chromatographs (GC-2014; Shimadzu, Japan), in accordance with a previous study by Zhang et al. [18]. Briefly, 5 mL thawed ruminal fluid was mixed with 0.5 mL of 25% ortho-phosphoric acid and centrifuged at 15,000× *g* for 15 min at 4 °C. Then, the supernatant was injected into an AT-FFAP capillary column. The determination conditions were as follows: column temperature (FFAP 30 m × 0.32 mm × 0.5 um) of 140 °C, inlet temperature of 250 °C, detector temperature of 250 °C, splitting ratio of 30:1, and the high-purity nitrogen flow was 40 mL/min.

2.4. DNA Extraction, Sequencing, and Bioinformatic Analysis

Total genome DNA was extracted from ruminal fluid samples using the E.Z.N.A Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA), according to the manufacturer's instructions. The quality of extracted DNA was detected with 1% agarose gel electrophoresis. The extracted DNA concentration and purity were determined using a NanoDrop 2000 c (Thermo Scientific, Wilmington, DE, USA). PCR amplification of the V3–V4 high variant region, with universal primers 338F: (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR reaction system was as follows: 5 μL of KOD FX Neo Buffer, 2 μL of dNTP (2 mmol/L), 0.3 μL primer (10 μmol/L), 50 ng of template DNA, and 0.2 μL of KOD ROD polymerase. The reaction parameters were as follows: pre-denaturation at 95 °C for 5 min, followed by denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 1 min for a total of 25 cycles, and final extension at 72 °C for 7 min. PCR products were mixed and recovered using 2% agarose gel electrophoresis and quantified using a QuantusTM Fluorometer (Promega, Fitchburg, WI, USA). DNA fragments were sequenced on a MiSeq PE300 platform (Beijing Baemai Biotechnology Co. Ltd., Beijing, China).

The original sequences' data were quality-controlled using fastp software (v.1.2.11) [19], and the singleton and non-target sequences were removed from the analysis. A series of high-quality sequences were clustered into Operational Taxonomic Units (OTUs) based on a 97% similarity level [20]. Each 16S rRNA gene sequence was annotated for species classification with an RDP classifier [21] against the SILVA 16S rRNA gene database (V138), with a comparison threshold of 70%. Bacterial community diversity and richness were

analyzed using two indicators (Chao1 and Shannon indices), performed with Mothur (v.1.30.1). For beta diversity analysis, principal coordinate analysis (PCOA) was performed with R package ropls (v.1.6.2). In addition, PICRUSt 2 analysis was conducted to predict the function of rumen bacteria in all samples [22].

2.5. Metabolic Profile Analysis

Fourteen ruminal fluid samples were analyzed with a liquid chromatography mass spectrometry platform. After rumen fluid samples were thawed at room temperature, 100 μL of each sample was mixed with 400 μL of methanol:acetonitrile (4:1, *v/v*); and then vortexed for 1 min. The LC/MS analyses were carried out with an Agilent7890 gas chromatography system (Agilent Technologies, Santa Clara, CA, USA); coupled with an Acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm, Waters, Waltham, MA, USA). During chromatography, samples were eluted using positive (ESI+) and negative (ESII−) mobile phases. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (methanol) with the gradient described below at flow rates of 0.40 mL/min, and a column temperature was of 40 °C. The following solvent gradient was used: 2% eluent B,1.5 min; 2–100% eluent B, 12.0 min; 100% eluent B, 14.0 min; 100–2% eluent B, 14.1 min; and 2% eluent B, 17 min. The ESI source conditions were set as follows: ion source gas 1 at 3.52 kg/cm^2, ion source gas 2 at 3.52 kg/cm^2, curtain gas at 1.76 kg/cm^2, source temperature of 500 °C, and Ion Spray Voltage Floating (ISVF) of 5500 V or −4500 V in the positive or negative modes, respectively.

Meanwhile, the mass spectrometry data were analyzed using ChromaTOF43X software and the LECO-FiehnRtx5 (Shang Ltd., Shanghai, China) database [23]. The original data were processed using SIMCA software (v.14, Umetrics AB, Umea, Sweden) for principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). The significantly differential metabolites (DEMs) were screened by combining the differential multiple, the *p*-value of the t-test, and the VIP value of the OPLS-DA model, with p-value < 0.05 and VIP > 1. The enrichment of metabolic pathways and the analysis of discrepant metabolic pathways were performed with the MetaboAnalyst v.4.0 online procedure and the Bos taurus Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database.

2.6. Statistical Analysis

The statistical analysis of blood metabolites and rumen fermentation parameters data was performed using SPSS 25.0 (SPSS Inc., Chicago, IL, USA), with an independent sample t-test. The significance determinations of the relative abundance of microbiota and the predicted KEGG pathways (%) were tested with the non-parametric Kruskal-Wallis test (SPSS 25.0 Inc., Chicago, IL, USA). Correlations between different metabolites and bacterial communities were calculated with Spearman's correlation analysis using an R program, and p-values < 0.05 indicated significant differences.

3. Results

3.1. Blood Metabolites

The blood metabolites of the yaks are shown in Table 2. The serum concentrations of AST, GGT, HPT, SAA, IL1-β, IL-6, TNF-α, and LPS were greater in the HG group than in the CON group ($p < 0.05$). However, there was no significant difference in the serum concentration of ALT observed between the CON and HG groups ($p = 0.490$).

Table 2. Effects of high-grain diet on the blood metabolites of yaks.

Items [b]	Diet [a]		SEM [c]	*p*-Value
	CON	HG		
AST, mmol/L	32.28	39.71	1.53	0.009
ALT, mmol/L	80.14	84.57	3.05	0.490
GGT, U/L	8.11	12.20	0.49	<0.001
HPT, ng/mL	42.83	50.88	1.93	0.031
SAA, ug/mL	23.26	28.15	0.78	<0.001
IL1-β, ng/L	42.96	51.89	1.45	<0.001
IL-6, ng/L	397.14	446.35	8.72	0.001
TNF-α, ng/L	169.94	195.25	4.86	0.004
LPS, EU/mL	0.62	1.17	0.08	<0.001

[a] Diet: CON, hay group; HG, high-grain group. [b] AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HPT, haptoglobin; SAA, serum amyloid-A; IL1-β, interleukin-1β; IL-6, interleukin-6; LPS, Lipopolysaccharides. [c] SEM: standard error of the mean (n = 7).

3.2. Rumen Fermentation Parameters

The effects of high-grain feeding on the rumen fermentation characteristics in yaks are listed in Table 3. Compared with a hay diet, the high-grain diet significantly increased the TVFA concentration and proportions of isobutyrate, butyrate, and isovalerate ($p < 0.05$), whereas the tendency towards higher proportions of acetate was observed in the CON group ($p = 0.052$). However, the proportions of propionate and valerate and ratio of acetate to propionate showed no significant differences ($p > 0.05$). Moreover, we found a lower ruminal pH value in the HG group ($p < 0.05$).

Table 3. Effects of high-grain diet on the rumen fermentation parameters in yaks.

Items [b]	Diet [a]		SEM [c]	*p*-Value
	CON	HG		
Ruminal pH	6.54	5.73	0.12	<0.001
TVFA, mmol/L	60.01	81.36	3.28	<0.001
VFAs, molar % of TVFA				
Acetate, %	64.17	60.75	1.58	0.052
Propionate, %	22.28	22.81	1.34	0.699
Isobutyrate, %	0.76	1.00	0.10	0.026
Butyrate, %	10.61	12.56	0.50	0.002
Isovalerate, %	0.81	1.28	0.13	0.003
Valerate, %	1.38	1.13	0.19	0.200
Acetate: propionate ratio	2.94	2.68	0.46	0.301

[a] Diet: CON, hay group; HG, high-grain group. [b] TVFA, total volatile fatty acids. [c] SEM: standard error of the mean (n = 7).

3.3. Rumen Bacterial Diversity Analysis

In total, 1,239,494 16S rRNA gene sequences were generated from all rumen liquid samples. After subsampling each sample to an equal sequencing depth (35,363 reads per sample) and clustering, a total of 3493 OTUs were identified at a sequence-similarity level of 97%. There were 1983 common OTUs among the two groups, and there were 914 and 597 unique OTUs in the CON and HG groups, respectively (Figure 1A). The Chao1 and Shannon alpha diversity indices are presented in Figure 1C,D. According to the Chao1 value (1879.26 ± 101.08 vs. 1487.67 ± 127.17, $p < 0.05$), the Shannon index value (7.86 ± 0.28 vs. 7.13 ± 0.12, $p < 0.05$) indicates the significant influence of HG feeding on the ruminal microbial diversity and richness of the yaks. Notably, we found lower diversity and richness of the ruminal microbial community in the HG group than in the CON group. In addition, we used the PCOA plots with unweighted UniFrac matrix distances, which clearly demonstrated that the microbial community structure had a significant difference between the CON and HG groups (Figure 1B).

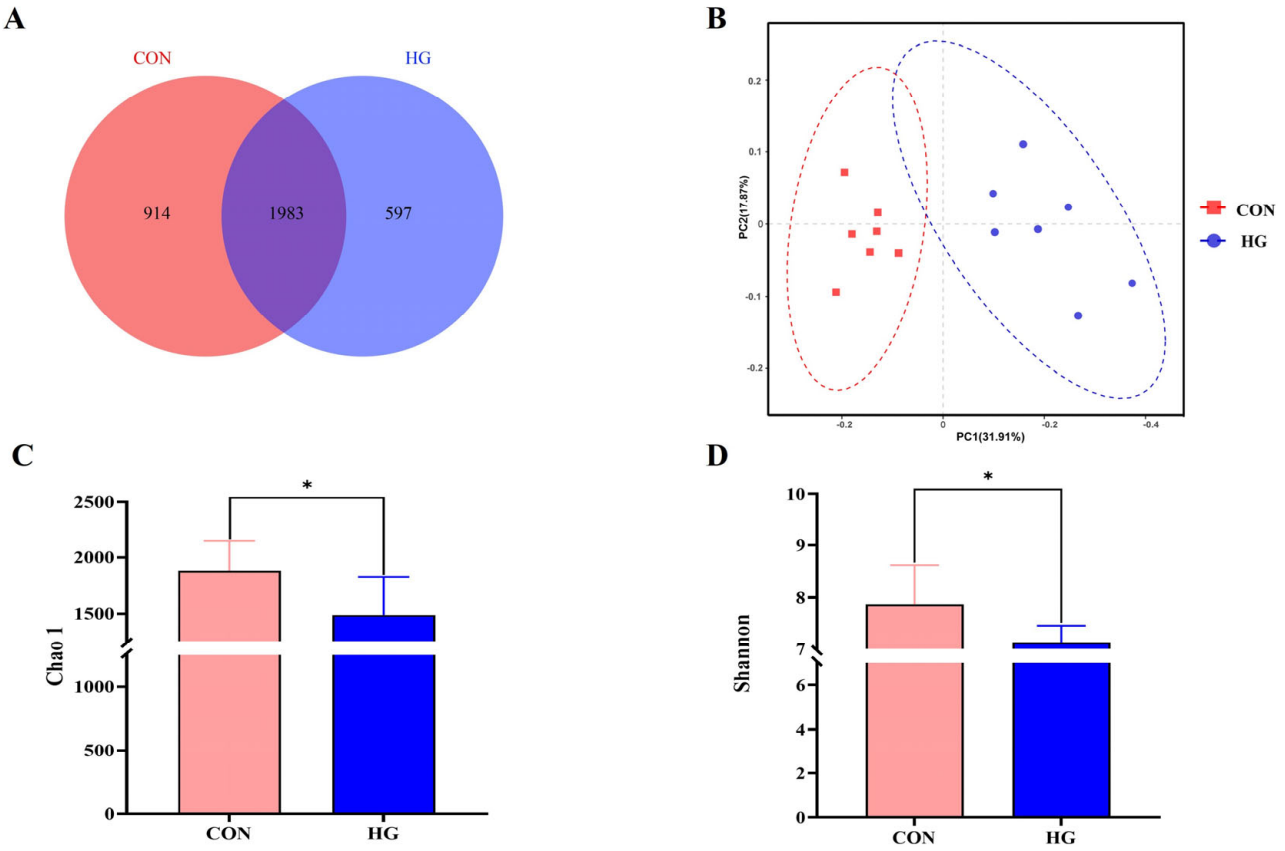

Figure 1. The operational taxonomic unit (OTU) numbers, ruminal bacterial diversity and richness, and principal coordinate analysis (PCoA) of yaks fed different diets. CON, hay group; HG, high-grain group. (**A**) Venn diagram of OTUs in the ruminal microbiota; (**B**) principal coordinate analysis (PCoA); (**C**) Chao1 index used to estimate ruminal bacterial richness; and (**D**) Shannon index used to estimate ruminal bacterial diversity. * $p < 0.05$.

3.4. Differences in Bacterial Community Composition between Two Groups

The taxonomic analysis of the reads revealed a total of 24 bacterial phyla; Firmicutes and Bacteroidetes were the dominant phyla, accounting for 62.44% and 28.51% in the CON group, and 72.02% and 20.25% in the HG group, respectively (Figure 2A). At the genus level, 256 genera were identified in the rumen liquids of the yaks. Among them, *Christensenellaceae R-7_group* (12.56%, 14.21%), *Prevotella_1* (7.10%, 3.65%), *Rikenellaceae_RC9_gut_group* (7.60%, 5.98%), *Ruminococcus_2* (5.46%, 1.77%), *Ruminococcaceae_NK4A214_group* (5.02%, 7.49%), *Lachnospiraceae_NK3A20_group* (4.61%, 9.04%), and *Ruminococcaceae_UCG-014* (2.4%, 3.10%) were the predominant genera in the CON and HG groups, respectively (Figure 2B).

At the phylum level (Figure 2C), the relative abundances of Firmicutes and Saccharibacteria were significantly higher in the HG group ($p < 0.05$) than in the CON group, while the CON group had a higher relative abundance of Bacteroidetes compared with the group HG ($p < 0.05$). At genus level (Figure 2D), feeding yaks with a high-grain diet significantly increased the relative abundance of *Christensenelaceae_R-7_group*, *Ruminococcaceae_NK4A214_group*, *Lachnospiraceae_NK3A20_group*, and *Acetitomaculum* relative to the CON group ($p < 0.05$). On the contrary, the relative abundance of *Prevotella_1*, *Rikenellaceae_RC9_gut_group*, *Ruminococcus_2*, and *Butyrivibrio_2* were significantly higher in the CON group than in the HG group ($p < 0.05$).

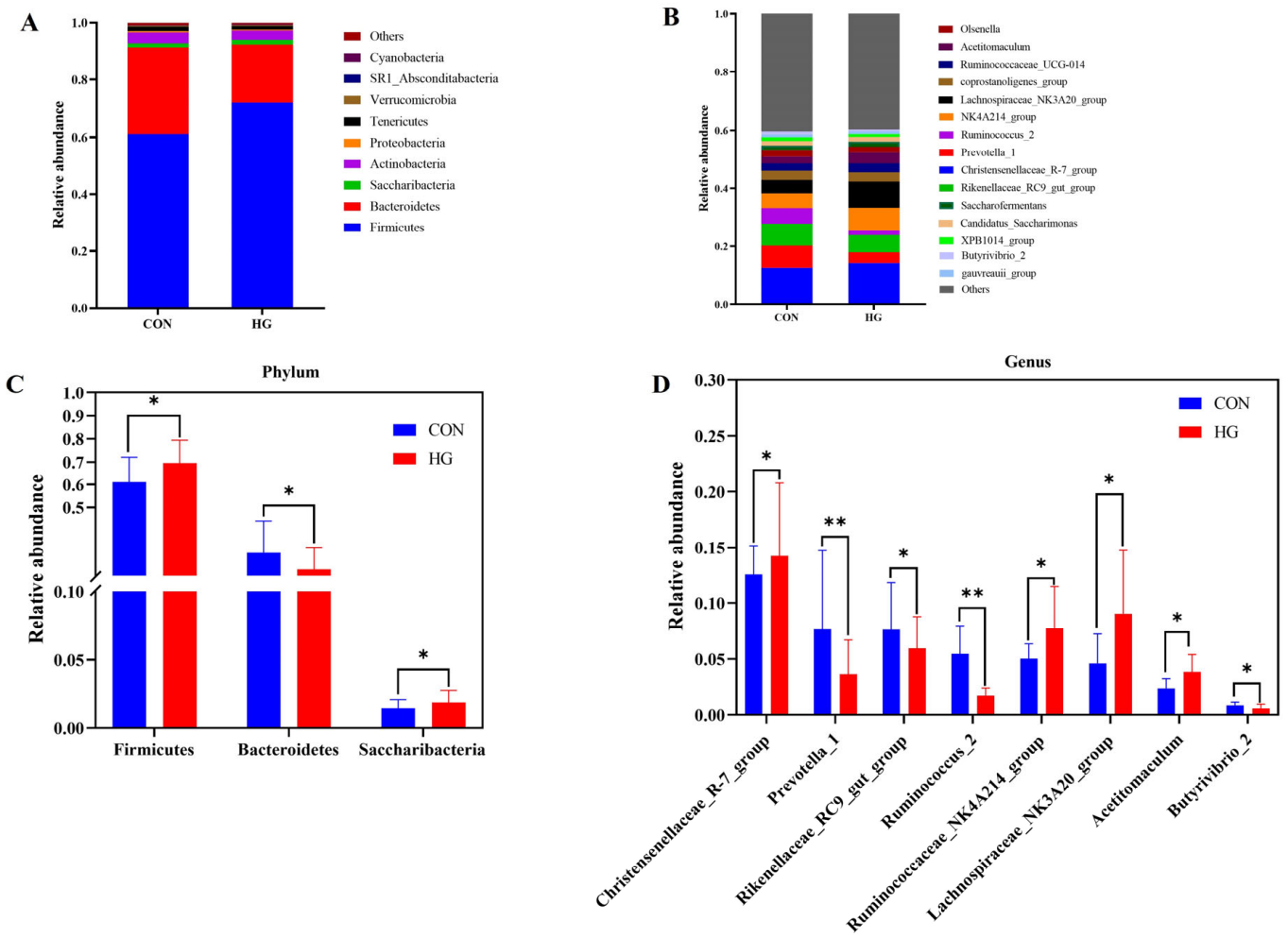

Figure 2. Bacterial community composition and significant differences of yaks fed different diets. CON, hay group; HG, high-grain group. (**A**) Phylum level; (**B**) genus level; (**C**) the bacteria with significant differences at the phylum level; and (**D**) the bacteria with significant differences at the genus level. * $p < 0.05$; ** $p < 0.01$.

3.5. Predicted Functional of the Rumen Bacterial Community

PICRUSt 2 was used to predict the potential functions of CON and HG yak rumen microbiota; 45 gene families were identified at KEGG 2. Among the 45 KEGG gene families, functions in amino acid metabolism (12.11%), carbohydrate metabolism (10.28%), metabolism of cofactors and vitamins (10.02%), metabolism of other amino acids (6.98%), replication and repair (6.07%), energy metabolism (4.88%), and lipid metabolism (4.22%) were the dominant metabolic pathways. We used a principal component analysis of the KEGG pathway which revealed different clusters in the CON and HG samples (Figure 3A). As shown in Figure 3B, the relative abundances of folding, sorting and degradation, translation, energy metabolism, replication and repair, metabolism of other amino acids, and amino acid metabolism were significantly increased in HG yaks compared with the CON yaks ($p < 0.05$).

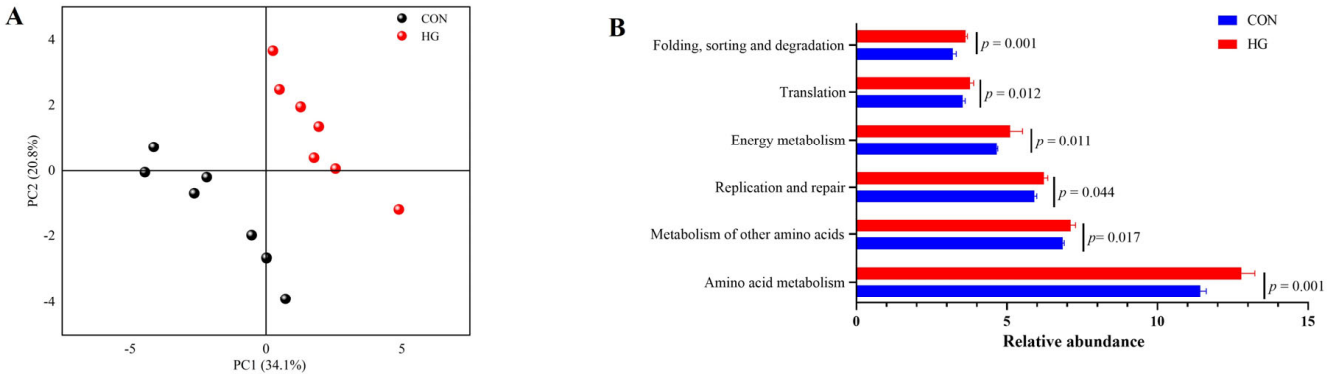

Figure 3. Functional predictions of rumen microbiota of yaks fed different diets. CON, hay group; HG, high-grain group. (**A**) A principal component analysis of KEGG pathways, and (**B**) the functional predictions of rumen microbiotas with significant differences at the KEGG 2.

3.6. Rumen Metabolomics Profiles and Metabolic Pathway Analyses

Overall, based on an analysis of ruminal content metabolome determined by LC-MS, we conducted PCA analysis and tested the validity of the data (Supplementary Materials Figure S1). Furthermore, to better reveal the differences between ruminal metabolites of CON and HG yaks, we used the OPLS-DA model, including positive and negative ionization. The corresponding R^2Y-values of positive and negative models were 0.987 and 0.908, indicating that the model was stable and reliable (Figure 4A,C). Cross-validation and permutation tests for the model in positive and negative ion modes showed that the Q^2Y-values were −0.173 and −0.267, respectively (Figure 4A,C), suggesting that the OPLS-DA model was valid. Furthermore, we found clear separation between the CON and HG groups and all the samples in the score plots were within the 95% Hotelling T2 ellipse (Figure 4B,D).

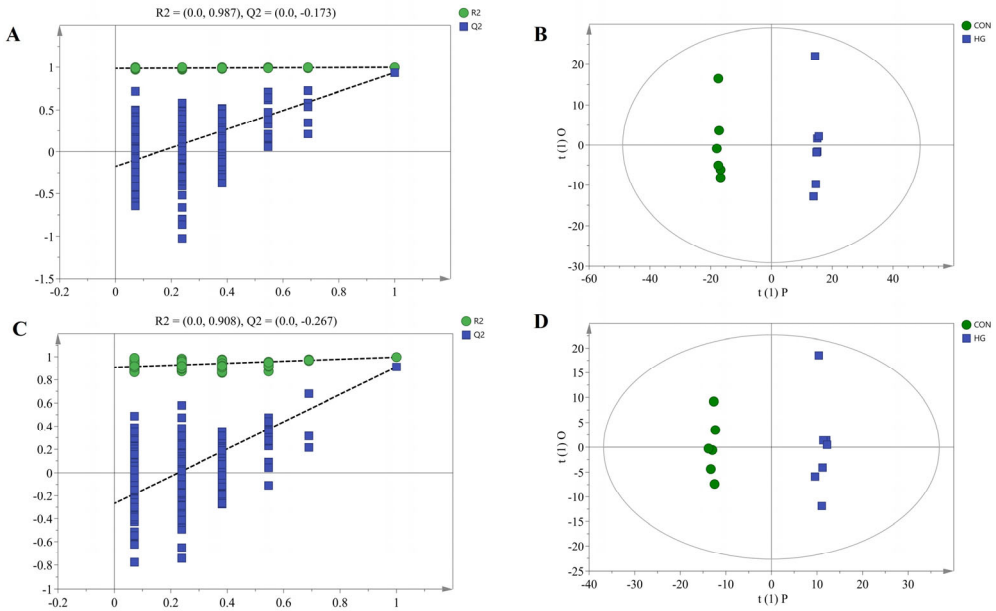

Figure 4. Corresponding validation plots and OPLS-DA score plots of yak rumen metabolites in the CON and HG groups following (**A**,**B**) positive and (**C**,**D**) negative mode ionization.

As shown in Supplementary Materials Table S1, a total of 1226 metabolites (785 positively ionized metabolites and 441 negatively ionized metabolites) were identified in the CON and HG groups. With a VIP threshold of 1.0 and $p < 0.05$, we in total identified 472 differential metabolites, including 288 positively ionized metabolites and 184 nega-

tively ionized metabolites. These different metabolites were mainly involved in amino acids, peptides, and analogues, lipids and lipid-like molecules, nucleosides, nucleotides, and analogues, and amines. Table 4 presents the primary differential metabolites in the rumen fluids of the HG and CON yaks. Compared with the CON group, the HG group had 26 ruminal metabolites; significantly upregulated ruminal metabolites included amino acids (phenylalanine, serine, methionine, and proline), lipids (alpha-linolenic acid, urocanic acid, and oleic acid), nucleosides/nucleotides (deoxyinosine, guanosine, and xanthosine) and amines (histamine, tyramine, and putrescine). Compared with the CON group, the HG group had eight significantly reduced ruminal metabolites, such as glutamic acid, methionine, arachidonic acid and normetanephrine. To further understand the multiple pathways that alternated in response to hay or high-grain diets in the yaks; we analyzed the function of pathways associated with differential metabolites in line with the Kyoto Encyclopedia of Genes and Genomes (KEGG). Figure 5 displays the seven significantly impacted pathways ($p < 0.05$) identified in the CON and HG groups, related to purine metabolism, arachidonic acid metabolism, histidine metabolism, aminoacyl-tRNA biosynthesis, arginine and proline metabolism, tyrosine metabolism, and the biosynthesis of unsaturated fatty acids.

Table 4. Identification of significant differential metabolites in the ruminal fluids of HG and CON yaks.

Metabolites	VIP [a]	FC [b]	*p*-Value	Type [c]	Metabolic Classes
L-Glutamic acid	1.259	0.598	0.002	Down	Amino acids, peptides, and analogues
Formiminoglutamic acid	1.203	3.143	0.004	Up	
Phenylalanine	1.143	1.883	0.008	Up	
Serine	1.179	2.702	0.005	Up	
Methionine	1.331	0.267	<0.001	Down	
Threonine	1.1735	2.730	0.006	Up	
Proline	1.142	2.242	0.007	Up	
Citrulline	1.142	1.873	0.0087	Up	
Gamma-Aminobutyric acid	1.170	2.088	0.006	Up	
4-Acetamidobutanoic acid	1.378	0.583	0.007	Down	
DL-Dopa	1.402	1.999	<0.001	Up	Lipids and lipid-like molecules
Alpha-Linolenic acid	1.359	3.167	<0.001	Up	
Arachidonic acid	1.407	0.301	<0.001	Down	
Urocanic acid	1.363	2.935	<0.001	Up	
PC(P-16:0/16:0)	1.537	0.480	<0.001	Down	
20-Hydroxyeicosatetraenoic acid	1.516	0.311	<0.001	Down	
Oleic acid	1.622	4.021	<0.001	Up	
Prostaglandin H2	1.323	3.597	<0.001	Up	
Prostaglandin E2	1.435	2.841	<0.001	Up	
12(S)-HPETE	1.116	2.232	0.011	Up	
Deoxyadenosine monophosphate	1.013	2.394	0.028	Up	Nucleosides, nucleotides, and analogues
Deoxyinosine	1.130	2.399	<0.001	Up	
Deoxyguanosine	1.415	2.278	<0.001	Up	
Adenosine	1.332	1.933	0.001	Up	
Xanthosine	1.050	7.064	0.014	Up	
Inosine	1.161	2.863	<0.001	Up	
Guanosine	1.188	2.571	0.007	Up	
Histamine	1.945	1.674	0.035	Up	Amines
Tyramine	1.775	8.30	0.010	Up	
Putrescine	1.401	1.448	0.039	Up	
Prostaglandin G2	1.146	2.481	<0.001	Up	Others
Hypoxanthine	1.285	1.604	0.001	Up	
Normetanephrine	1.282	0.409	0.002	Down	
Epinephrine	1.259	0.654	0.007	Down	

[a] VIP, variable importance in the projection. All differential metabolites listed here had values VIP > 1 and $p < 0.05$.
[b] FC, Fold change. FC > 1 indicates that this metabolite was more abundant in the HG yaks than in the CON yaks.
[c] Up, upregulated; down, downregulated.

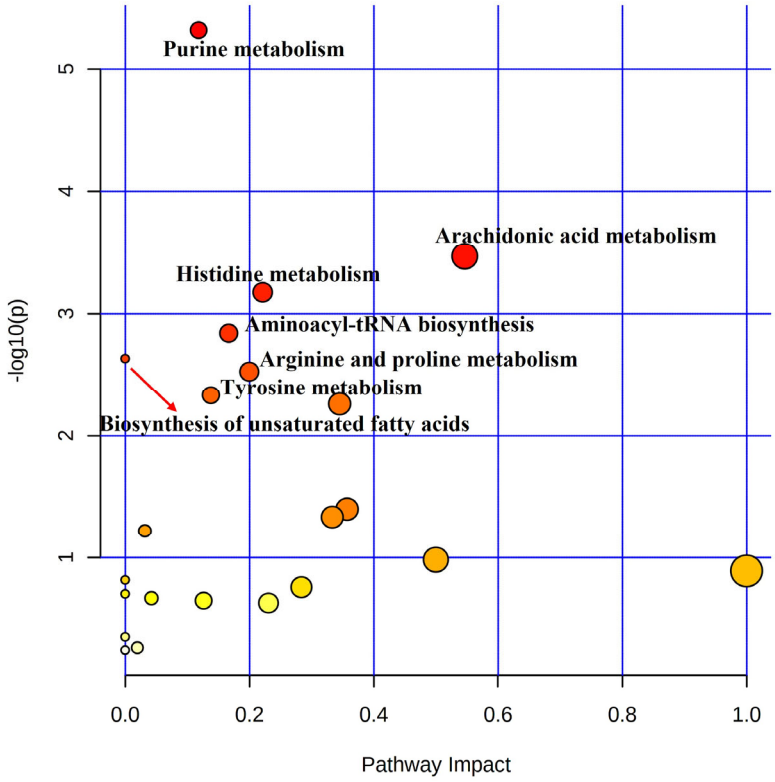

Figure 5. Metabolic pathway enrichment analysis. The manipulated metabolic pathways are based on the analysis of the differentiated ruminal metabolites of yaks fed hay or high-grain diets following the Bos taurus Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, using MetaboAnalyst v.4.0. The x-axis represents the pathway to impact, and the y-axis represents the pathway enrichment. Larger sizes and darker colors indicate greater pathway enrichment and higher pathway impact values, respectively.

3.7. Correlation between Metabolites and Rumen Bacteria

We intended to comprehensively understand the relationship between the perturbations in the ruminal microbial and metabolites after feeding the yaks hay or high-grain diets. Therefore, we conducted a correlation analysis between eight variable bacterial genera and key differential metabolites, which are presented in Figure 6. We found that the genus *Prevotella_1* was negatively correlated with inosine and xanthosine. Moreover, the proportions of isobutyrate and putrescine were negatively associated with the genus *Butyrivibrio_2,* while the *Butyrivibrio_2* was positively associated with Normetanephrine. The *Rikenellaceae_RC9_gut_group* was negatively associated with inosine. The genus *Ruminococcus_2* was positively associated with hydroxyeicosatetraenoic acid, prostaglandin G2, prostaglandin E2, PC(P-16:0/16:0), 4-acetamidobutanoic acid, methionine, while it was negatively associated with urocanic acid, citrulline, phenylalanine, alpha-linolenic acid, tyramine, dl-dopa, oleic acid, propionate, deoxyguanosine, histamine, isobutyrate, and isovalerate. The *Ruminococcaceae_NK4A214_group* was positively associated with isobutyrate, dl-dopa, and oleic acid, while it was negatively associated with methionine, 4-acetamidobutanoic acid, PC(P-16:0/16:0), and prostaglandin E2. The *lachnospiraceae_NK3A20_group* was positively associated with serine, phenylalanine, alpha-Linolenic acid, formiminoglutamic acid, and hypoxanthine, while it was negatively associated with 12(S)-HPETE, hydroxyeicosatetraenoic acid, and prostaglandin E2. *Acetitomaculum* was negatively associated with docosahexaenoic acid, hydroxyeicosatetraenoic acid, and prostaglandin E2, while it was positively associated with Serine, phenylalanine, alpha-linolenic acid, formiminoglutamic acid, and hypoxanthine.

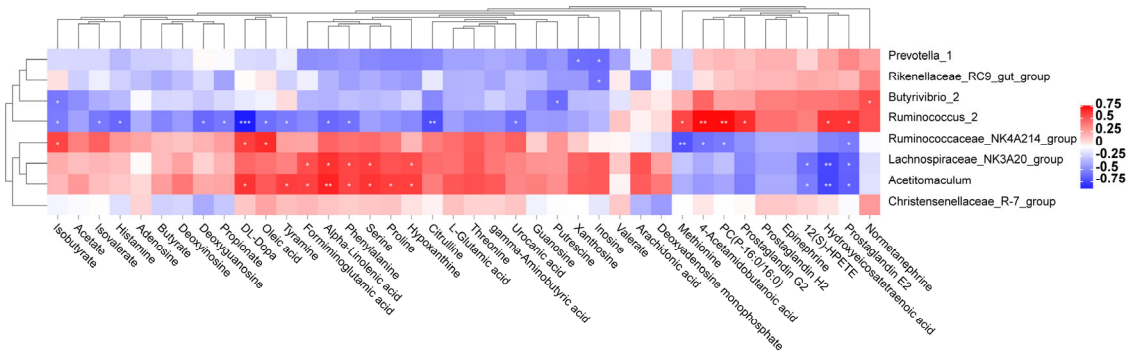

Figure 6. Correlation coefficients between rumen bacterial genera and rumen differential metabolites (derived from Table 3). Each column in the graph represents a genus, each row represents a metabolite, and each cell represents a Pearson correlation coefficient between a genus and a metabolite. Red indicates a positive correlation, and blue represents a negative correlation. p * < 0.05; ** p < 0.01; *** p < 0.01.

4. Discussion

In traditional management, yaks usually graze on a full grazing system with only herbage as feed, which causes slow growth and increased grassland degradation [4]. To address these issues, the indoor feeding of yaks has been applied in the farming industry, including feeding on high-concentrate diets. In similar studies on other ruminants, HG-diet feeding led to health problems [24] that were closely linked to rumen metabolism and microbiota [25]. However, compared with other ruminants, there is a paucity of information on the inflammatory response, rumen microbiome, and metabolomics of yaks after HG diet feeding. Therefore, in the current study, we combined 16S rDNA gene sequencing and liquid chromatography–mass spectrometry (LC/MS) analysis to investigate bacterial community diversity and metabolite characteristics of yaks fed a high-grain diet. The results of our study may provide a comprehensive understanding of the effects of the HG diet on the rumen microbiome and metabolite of yaks, as well as provide a theoretical foundation for research on HG diets for yaks.

The liver enzymes released in the blood, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyltransferase (GGT) are used to assess sensitive markers of liver function [26,27]. In the present study, we found that the HG diet increased the activity of AST and GGT, indicating that liver inflammation and damage were induced, which is consistent with the results of [28]; this may have been due to the free LPS concentration increasing with the higher proportion of dietary grain. A series of studies have demonstrated that free LPS is transferred from the gastrointestinal tract to the liver via the bloodstream, thereby inducing an inflammatory response and liver damage [29,30]. Interestingly, we observed a greater concentration of blood LPS in the HG diet, findings in line with a previous study in in dairy cows [31], which was lateral proof of increasing AST and GGT concentrations. Numerous studies have shown that high-grain feeding in ruminants promotes the secretion of inflammatory factors, such as IL-6, IL-1β, and TNF-α, and induce the production of acute-phase proteins such as HPT and SAA [32,33]. Compared with the CON group yaks, the HG group yaks showed significant increases in acute-phase proteins, including HPT and SAA, and higher blood IL-6, IL-1β, and TNF-α concentrations, as reported by similar studies in dairy cows [34,35]. Overall, the HG diet induced an inflammatory response and reduced the liver's metabolic function in yaks.

Increasing evidence is showing that high-grain diets provide ruminants with more readily-fermentable carbohydrates, resulting in the production of large amounts of VFAs and lactic acid, which directly lead to a reduction in rumen pH and increase the risk of rumen acidosis [36,37]. In our study, higher concentration TVFA and proportions of isobutyrate, butyrate, and isovalerate were identified in the HG group, which is in agreement with the results of Bevans et al. [38] and Nagata et al. [39]; these changes

resulted in a significant decrease in ruminal pH. Moreover, we observed a significantly lower rumen pH value (5.73) in the HG yaks. A similar study by Zebeli et al. [40] reported that rumen pH was below 5.8, reducing feed conversion efficiency to VFA and microbial protein for animal production. Curiously, the proportion of propionate did not differ statistically between the two groups; this result is inconsistent with previous studies [7,12]. The lack of consistent results was likely the result of differences in nutrient levels (energy, CP, etc.) and breed between the studies.

Rumen microbial disorders induced by HG diets have been confirmed by a series of studies, but study on the effects of the HG diet on the rumen microbiome in yaks has produced a paucity of information. The structure and function of the rumen microbiome are affected by the host animal's age, diet, and antibiotic regime, as well as the animal's care and management, with diet being the dominant factor [41]. In our study, PCoA analysis indicated microbial differences between the CON and HG groups, consistent with other reports [42]. The Chao1 index was used to analyze gut microbiota species richness, while the Shannon index indicated the gut microbiota diversity. Consistent with many previous studies [43,44], our study confirmed that the HG diets had the potent ability to reshape the rumen microbial community by reducing community richness and diversity. We found that the dominant phyla in the yak's rumen were Firmicutes, Bacteroidota, and Actinobacteria, which is consistent with previous studies on yaks [5,16]. Meanwhile, that research also found that a high-grain diet of ruminants increased the abundance of Firmicutes, but decreased the abundance of Bacteroidota. Previous studies have shown that a high-acidity environment would inhibit gram-negative bacteria belonging to Bacteroidotas [43,45]. Meanwhile, Kaoutari et al. [46] stated that Firmicutes was more efficient at degrading starch than Bacteroidotas. Albertsen et al. [47] reported that Saccharibacteria, a phylogenetic lineage of uncultured bacteria, was capable of converting polysaccharides into ethanol, acetate, hydrogen, and carbon dioxide. In our study, the relative abundance of Saccharibacteria was higher in the rumens of HG-diet yaks, showing it may be involved in the degradation of polysaccharides.

Christensenelaceae_R-7_group belongs to the family *Christensenellaceae*, and produces propionate and butyrate with the ability to digest starch [48]. Our result also demonstrated this finding; a higher abundance of *Christensenelaceae_R-7_group* was found in the HG group, which may be reason the high-grain diet provides a greater proportion of starch for yaks. In the HG diet group in this study, the content of dietary resistant starch increased, which may have been caused by the higher abundance of *Ruminococcaceae_NK4A214_group* in the HG group, which is in line with the results of a similar report by Fan et al. [49]. Members of *Lachnospiraceae* have been shown to degrade plant cellulose and polysaccharides, which are indigestible by hosts, and to convert them into VFA such as acetate, butyrate, and propionate [50,51]. In our study, the abundance of *Lachnospiraceae_NK3A20_group* was significantly increased in the HG group compared with the CON group, which partly explained the higher concentration of TVFA in the HG group. Furthermore, the HG diet significantly increased the abundance of *Acetitomaculum*, which is consistent with the results of a similar study by Liu et al. [51]. Research has demonstrated the ability of *Acetitomaculum* to degrade carbohydrates and convert them into propionate and butyrate in the rumen [52,53], and this was further confirmed in our study. Conversely, the abundance of *Prevotella_1* was significantly reduced in the HG group, which was consistent with the results of previous studies [54,55]. The relative abundance of *Prevotella_1*, belonging to the Prevotellaceae family, was negatively correlated with rumen pH in a study by Mao et al. [43]. Zhang et al. [15] reported that *Rikenellaceae_RC9_gut_group*, belonging to the *Rikenellaceae* family, demonstrated the ability to degrade crude fiber in the rumen. Furthermore, Zened et al. [56] found that the abundance of *Rikenellaceae_RC9_gut_group* increased with increasing dietary neutral detergent fiber levels. Accordingly, the abundance of *Rikenellaceae_RC9_gut_group* in the CON group was significantly higher than in the HG group in the current study; this may have been related to the higher roughage content in the CON diet. *Ruminococcus* had been reported to be the predominant genus to degrade

fiber [57]. In our study, the HG group had a greater content of *Ruminococcus_2*, which was similar to the results of previous studies [15,28]. A study by Wang et al. [58] reported a lower relative abundance of Butyrivibrio_2 in the HG group compared with the CON group, which was consistent with the results of our study. Moreover, to further explore the effect of high-grain diets on rumen microbial function in yaks, we used PICRUSt2 to predict the function of yak rumen microbial communities in the two treatment groups. The results indicated that the HG diet significantly increased some rumen metabolism pathways (such as energy metabolism, replication and repair, metabolism of other amino acids, and amino acid metabolism). These functional alterations indicated that the HG group's rumen microbiota could have been in a subnormal state of health, with relative volatility, enhanced metabolism, and faster turnover. Unfortunately, our results are based on determinations of the rumen fluid bacteria that are limited due to experimental conditions; results generally have a degree of variation compared to whole rumen sample, as has been demonstrated by previous studies [59,60]. Therefore, further studies need to explore the variation of rumen bacteria of original rumen digesta in yaks fed a high-grain diet. Moreover, the extraction of ruminal microbial DNA was by a kit method in our study, and Henderson et al. [59] and Chen et al. [61] have concluded that kit methods obtained poor DNA yields and non-representative community compositions compared to traditional methods; contrastingly, according to the report of Vaidya et al. [62], the kit DNA extraction method was able to meet the requirements for sufficient quality and quantity DNA with PCR, showing that our microbial results would be credible.

It is well-known that rumen and host health are affected by altered concentrations of various rumen metabolites [23,42]. Hence, to further understand the effects of the HG diet on the rumen metabolism of yaks, LC–MS metabolome analysis was used to evaluate metabolites in the rumen fluid of our CON and HG yaks. We observed clear separations based on PCA and OPLS-DA analysis among samples from the CON and HG groups, proving that the different dietary treatments significantly affected their rumen metabolism. It has been well documented that high-grain diet feeding could contribute to the accumulation of amino acids in the rumens of ruminants [63]. In our study, we consistently found that the HG diet significantly improved the content of five common amino acids (phenylalanine, threonine, serine, etc.) in the rumen compared with the CON diet. In ruminants, amino acids are mainly derived from the degradation of dietary proteins and microproteins obtained by rumen microbiota. Previous studies have shown that the abundances of starch-degrading microbiota were increased in relatively high starch diet groups, and these microbiota increases may facilitate the degradation of rumen proteins [42,64]. Additionally, previous studies have shown that rumen microbiota could synthesize amino acids de novo using acetate, propionate, or other carbon sources and nitrogen compounds, such as ammonia, as nitrogen sources [65,66]. In the present study, the acetate and propionate concentrations were higher in the HG group, and they may have promoted the greater synthesis of amino acids. In addition, we observed enriched pathways, including histidine metabolism, aminoacyl-tRNA biosynthesis, arginine and proline metabolism, and tyrosine metabolism, which also validated the above-mentioned results. Glutamine is an essential nutrient for the metabolism of gastrointestinal mucosal cells, and it plays a critical role in maintaining the structural integrity of the mucosal epithelium of the gastrointestinal tract [67]. Interestingly, we observed a decrease in glutamine concentration in the rumens of HG yaks, which may have indicated damage to the integrity and barrier function of the rumen epithelium after HG diet feeding.

In addition to amino acids, the results of our study on biogenic amines also provided evidence that HG diets may be detrimental to rumen health. Here, the concentrations of histamine, tyramine, and putrescine were significantly increased in the HG group, which was consistent with the results of previous studies [23,68]. Biogenic amines originate from the decarboxylation of certain amino acids (such as arginine, histidine, and ornithine) via microbial action, and the amount of biogenic amines has been found to be closely associated with microbiota and environment in the rumen [69]. An in vitro study by Bailey et al. [70]

reported that a lower ruminal pH could enhance the enzymatic activity of biogenic amines through rumen microorganisms, which accelerated the decarboxylation of amino acids and promoted the production of biogenic amines in the rumen. Moreover, relevant studies have revealed that HG diets elevate biogenic amine concentrations, particularly those of histamine, which may cause damage to the epithelial barrier of the rumen; these consequences are closely associated with ruminal acidosis [69,71]. Hence, increased biogenic amine concentrations may exacerbate the risk of rumen acidosis in yaks fed an HG diet. Notably, higher concentrations of several metabolites (prostaglandin H2, prostaglandin E2, 12(S)-HPETE, and prostaglandin G2) involved in the metabolism of arachidonic acid metabolism were observed in the HG group. The arachidonic acid metabolic pathway is mainly used for the synthesis of inflammatory mediators and mediates the production of various inflammatory factors, such as monocyte chemotactic protein-1 (MCP1), tumor necrosis factor (TNF), and interleukin-1β (IL-1β), which is positively associated with the inflammation of the host. Andersen et al. [72] found that endotoxin and arachidonic acid metabolites of pre-hepatic origin may be involved in the pathogenesis of ruminal acidosis, which is in agreement with our findings. Therefore, we speculate that the increased concentration of arachidonic acids in our study may have been due to the HG diets, which led to damage to the rumen epithelium. Additionally, prostaglandin E2 and 12(S)-HPETE were both positively associated with *lachnospiraceae_NK3A20_group* and *Acetitomaculum*; these findings indicated that arachidonic acid metabolism might be related to close changes in the acidity of the rumen. However, in the current study, we only conducted a preliminary investigation of the effects of high-grain diets on yaks by using rumen microbiome and metabolomics analyses. Further studies should aim to investigate the serum metabolome and rumen morphology of yaks fed HG diets.

5. Conclusions

In summary, feeding yaks an HG diet resulted in changes in rumen fermentation characteristics, specifically, an increase in the concentration of TVFA, and a decrease in rumen pH and a decreased richness and diversity of the ruminal microbiota, with a lower abundance of *Prevotella_1*, *Rikenellaceae_RC9_gut_group*, *Ruminococcus_2*, and *Butyrivibrio_2*. In addition, HG feeding led to increases in the concentrations of biogenic amines (histamine, tyramine, and putrescine), common amino acids (phenylalanine, threonine, serine, etc.), and arachidonic acids (prostaglandin H2, prostaglandin E2, 12(S)-HPETE, etc.). Moreover, HG feeding significantly increased serum LPS, AST, GGT, HPT, SAA, IL1-β, IL-6, and TNF-α levels. Overall, the high-grain diet changed fermentation characteristics, microbiota, and metabolites in the rumens of the yaks, and increased the risk of inflammation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9030215/s1, Table S1: Positively and negatively ionized metabolites between the CON and HG groups; Figure S1: PCA analysis on two groups.

Author Contributions: Conceptualization, D.D., X.W. and X.X.; methodology, D.D. and X.W.; software, D.D. and S.W.; validation, D.D., C.G. and S.C.; formal analysis, D.D.; investigation, D.D. and S.C.; resources, X.X.; data curation, X.X.; writing—original draft preparation, D.D.; writing—review and editing, D.D.; visualization, D.D.; supervision, D.D.; project administration, X.W.; funding acquisition, X.W. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All experimental procedures and animal experiments were performed following the guidelines of the relevant Ethics Committee. This study was approved by the Institutional Animal Care and Use Committee of Qinghai University (Protocol number: QHU20200617).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request, and the sequencing data are available from NCBI. The BioProject number is PRJNA899488.

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Conflicts of Interest: The authors declare no conflict of interest.

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