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Illumina Sequencing and Metabolomic Analysis Explored the Effects of the Mixed Silage of Rice Straw and Chinese Cabbage Waste on Fecal Microorganisms and Metabolites in Hu Sheep

Chuang Li [1,2,†], Zhiqi Lu [2,†], Ruxin Qi [2], Zhenbin Zhang [2], Yue Lu [2], Muhammad Hammad Zafar [2], Kailun Yang [1,*] and Mengzhi Wang [2,3,*]

[1] College of Animal Science, Xinjiang Agricultural University, Urumqi 830091, China; chuangliyzu@163.com
[2] Laboratory of Metabolic Manipulation of Herbivorous Animal Nutrition, College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China; zqlu73@163.com (Z.L.); ruxinqi@126.com (R.Q.); 15861273716@163.com (Y.L.); hammadzafar075@gmail.com (M.H.Z.)
[3] State Key Laboratory of Sheep Genetic Improvement and Healthy Production, Xinjiang Academy of Agricultural Reclamation Sciences, Shihezi 832000, China
* Correspondence: ykl@xjau.edu.cn (K.Y.); mzwang@yzu.edu.cn (M.W.)
† These authors contributed equally to this work.

Abstract: Silage is the most important component of a ruminant diet and has important production and health significance in ruminant production. The aim of the research was to investigate how the mixed silage of Chinese cabbage waste and rice straw (mixed silage) impacts the fecal microorganisms and metabolites in Hu sheep using Illumina sequencing and metabolomic analysis. A total of 16 Hu sheep (8 rams and 8 ewes) weighing about 39 kg and 5.5 months old were used as experimental sheep and divided into two groups (4 rams and 4 ewes, n = 8) using the principle of randomized trials: the control group with peanut sprouts, corn husks, and sorghum husks as roughage and the silage group with the mixed silage as roughage. There were no significant differences in the average daily gain (ADG), dry matter intake (DMI), or feed conversion rate (FCR) between the control group and the mixed silage groups ($p > 0.05$). Microbiome results showed that 15 microorganisms such as *Ruminococcaceae UCG 010*, *Breznakia*, *Erysipelothrix*, *Desulfovibrio*, *Succiniclasticum*, and *Shuttleworthia* were significantly different between the two groups. In addition, metabolomics showed that the mixed silage modulated the concentrations and metabolic pathways of metabolites in the manure. Significantly different metabolites were mainly enriched in amino acid anabolism ("glycine, serine, and threonine metabolism", "valine, leucine, and isoleucine biosynthesis", "arginine biosynthesis", etc.), nucleic acid metabolism (pyrimidine metabolism). In conclusion, the addition of mixed silage to the diet of Hu sheep can alter the structure of the hindgut microflora and regulate the metabolism of amino acids and nucleotides, which affects health performance.

Keywords: Chinese cabbage waste; fecal microorganisms; growth performance; Hu sheep; metabolomics; mixed silage; rice straw

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1. Introduction

Between 2005 and 2050, global demand for meat and milk will increase by 57% and 48%, respectively, which is a huge challenge for the livestock industry [1]. Forage resources of sufficient quality and quantity are necessary to meet human demand for animal products. However, this is a great challenge for most countries. Therefore, researchers are turning to unconventional feed ingredients. Most non-conventional feeds have limited nutritional value, and some modifications should be considered before feeding to ruminants for optimal performance. Silage is an effective and widely used technology for long-term preservation of feed, with the characteristics of low cost and easy operation [2].

Silage is the most important component of ruminant diets and has important production and health implications in ruminant production by maintaining good rumen functional status and reducing the risk of diseases such as rumen acidosis and crumpled stomach displacement [3].

The nutritional value of rice straw is low, with protein content only accounting for 2–5% of dry matter (DM), fiber and lignin content exceeding 50%, and DM digestibility being low, resulting in feed intake of 1.5–2.0% [4]. Therefore, rice straw can be used as a good source of roughage for ruminants only after further treatment. Typically, rice straw stalks are harvested and collected after wilting and drying to yellow stalks. During the air-drying process, the water in the rice straw is almost completely evaporated, so the free sugar in the air-dried rice is difficult to obtain. Therefore, lower water-soluble carbohydrates and a higher lignin content will seriously affect the quality of silage [5]. In this regard, vegetable waste has high water-soluble carbohydrates and moisture content, which can make up for the shortcomings of rice straw in silage [6]. Previous research results showed that when the mixed feed of broccoli byproducts and wheat straw (ratio 69:31) in the DM diet does not exceed 200 g/kg, the growth performance and apparent digestibility of nutrients and rumen fermentation parameters of Fashandy lambs will not be affected [7].

Fecal samples are not only convenient to collect, but also have a unique metabolome of nutrients from feed, gut microbiota, and host products [8]. Due to the emergence of high-throughput sequencing technology, we have a deeper understanding of the composition, structure, and interactions of microorganisms in the gastrointestinal tract of livestock and poultry [9]. The gut microbiota plays a crucial role in the regulation of carbohydrates, amino acids, lipids, vitamins, and mineral metabolism [10,11], along with influencing the immune system of the host [12,13]. Studies have shown that dietary changes can alter the microflora structure of the gut [14,15]. Studies have explored the impact of gut microbiota on metabolic function through metabolomics approaches. Metabolomics is a high-throughput sequencing technology, following genomics and proteomics, that provides new insights into the effects of diet, drugs, and disease, and aims to characterize and quantify all small molecules in a sample [16]. It has been used in recent years to detect plasma, fecal, rumen, and tissue metabolite biomarkers in humans and animals [17–20].

There is limited information on the effect of the mixed silage as an unconventional roughage on the fecal microbiota and metabolites in Hu sheep. Therefore, the purpose of this study was to explore the effects of the mixed silage on hindgut microbiota composition and fecal metabolites in Hu sheep using 16s RNA gene sequencing technology and LC-MS metabolomics.

2. Materials and Methods

2.1. Experimental Diet, Experimental Animals, and Feeding

The mixed silage was made based on previous research [15]. A total of 16 Hu sheep (8 rams and 8 ewes) weighing about 39 kg and 5.5 months old were used as experimental sheep and divided into two groups (4 rams and 4 ewes, n = 8) using the principle of randomized trials: the control group with peanut sprouts, corn husks, and sorghum husks as roughage and the silage group with the mixed silage as roughage. The feed formulation was designed based on the nutritional requirements of mutton sheep (NRC, 2007) [21]. The entire experiment spanned 35 days, consisting of a preliminary 7-day pre-feeding phase followed by an official 28-day trial period. All the Hu sheep in the experiment were raised together in one pen, and prior to the study, the sheep enclosure underwent cleaning and disinfection. Both control and silage groups were fed 50% roughage and 50% concentrate in equal portions at 8:00 and 18:00. Table 1 provides the dietary composition and nutrient levels for both the control and silage groups. Before entering the sheep house, all test sheep received uniform deworming and immunization. Throughout the experiment, a feeding management system with unlimited access to food and water was implemented.

Table 1. Experimental diet formula and nutrition level (DM basis/%).

Items	Treatment [1]	
	Control	Silage
Ingredients (% of DM)		
Peanut seedling	30	-
Corn husk	15	-
Sorghum shell	5	-
Mixed Silage	0	50
Corn	34	34
Soybean meal	7	5.5
Bran	7.5	8
Corn gluten meal	-	1
$NaHCO_3$	0.5	0.5
Premix contained [2]	0.5	0.5
Salt	0.5	0.5
Total	100	100
Nutrient composition (% of DM)		
Digestive energy/DE (MJ/kg) [3]	13.52	14.73
Metabolizable energy/ME (MJ/kg) [4]	18.93	20.62
Crude protein, CP	15.08	15.11
Ash	4.36	12.33
Neutral Detergent Fiber, NDF	47.64	48.23
Acid Detergent Fiber, ADF	23.71	27.17
Ca	0.48	0.45
P	0.38	0.39

Note: [1] Control: control group, the control group with peanut sprouts, corn husks, and sorghum husks as roughage; Silage: silage group, the silage group with the mixed silage as roughage. [2] Premix contained (per kg): VA 70~130 kIU, VD 315~30 kIU, VE $\geq$ 130 kIU, Fe 0.4~0.8 g, Mn 0.5~1.0 g, Zn 1.5~3.0 g, Cu 0.1~0.2 g, Se 4~8 mg, Ca 8~16%, P $\geq$ 1%, NaCl 5~10%. [3,4] DE and ME were estimated according to NRC (2007). The others were measured values. The experimental design of this study was the same as that of Li et al. [15].

2.2. Sample Collection and Measurement

2.2.1. Growth Performance

During the experiment, the quantity of feed and remaining feed for every sheep were documented, and DMI was calculated. Each sheep was weighed before the start of the formal trial and at the end of the experiment before the morning feed to record its body weight, and ADG was calculated. Feed efficiency is expressed as FCR, which is the ratio of DMI to ADG.

2.2.2. Fecal Sample Collection

On the eve of concluding the feeding experiment, random rectal fecal samples were gathered from six Hu sheep in each group, with each individual sample weighing approximately 30 g. The samples were rapidly placed in liquid nitrogen to avoid the influence of environmental microorganisms on the samples. Subsequently, they were transferred to a −80 °C refrigerator for storage and used for the determination of fecal microorganisms and metabolomes.

2.2.3. 16S rRNA Microbial Community Analysis

Total DNA was extracted from 12 fecal samples using the HiPure Soil DNA Kit soil DNA extraction kit (Beijing Tiangen Biochemical Technology Co., Ltd., Beijing, China). The A260/A280 values were measured by an ultramicro-volume spectrophotometer (NzanoDrop-1000, Thermo Fisher Scientific Co., Ltd., Waltham, MA, USA), and electrophoresis was carried out using a 1% agarose gel to check the integrity and purity of the extracted DNA. Primers 341F (5′-CCTACGGGGGNGGCWGCAG-3′) and 806R (5′-GGACTACHVGGGGTWTCTAAT-3′) were used for PCR amplification of the V3 to V4 region of the 16S rDNA gene. The PCR amplification system was a 30 μL reaction system, which included 15 μL of 2 × Phanta Master Mix, 1 μL of Bar-PCR primer F (10 μM), 1 μL of Primer R (10 μM), 10 ng of Genomic

DNA, and ddH_2O. Thermal cycling conditions were pre-denaturation at 95 °C for 5 min, followed by denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s for 27 cycles; the final extension was at 72 °C for 10 min. After the PCR is completed, the products are extracted by 2% agar gel electrophoresis and further purified and quantified. The Illumina Novaseq PE250 platform was used to sequence the amplified products (Genepioneer Biotechnologies Co., Ltd., Nanjing, China).

The raw sequenced sequences were quality controlled and spliced using fastp (V 0.20.0) and FLASH (V 1.2.7) software, respectively [22,23]. The quality sequences were clustered at a 97% similarity level according to UCLUST (V.1.2.22q) software to obtain each OTU. The taxonomic information of the species corresponding to each OTU can be obtained by comparing the OTUs with the SILVA rRNA database [24].

OTU-based dilution curves were used to evaluate sequencing coverage depth and were accomplished with the R (V 4.0.2) "vegan". Fecal microbial Venn diagrams were calculated with R (V 4.0.2) "VennDiagram" and visualized with "ggplot2". Microbial richness (ACE and Chao1 indices), microbial diversity (Shannon and Simpson indices), and microbial coverage (goods_coverage) were calculated using the R (V 4.0.2) "picante". Principal Coordinate Analysis (PCoA, weighted UniFrac distance) was used to assess the colony structure of the different microorganisms, and its visualization was done with the R "ggplot2" package [25]. The top ten dominant bacterial groups in phyla and genera were also visualized with R (V 4.0.2) "ggplot2". Analysis of similarity (ANOSIM) is used to determine the similarity of groups, with "0" indicating indistinguishable and "1" indicating distinguishable. LDA effect size (LEfSe) analysis was applied to screen for signature differential flora in feces of Hu sheep fed different roughages. The Harvard University online analysis platform "http://huttenhower.sph.harvard.edu/galaxy (accessed on 20 December 2021)" was used to complete the LEfSe analysis. We believe that this bacterial group differed between the two groups, when the linear discriminant analysis (LDA) effect value was greater than 3.

2.2.4. Untargeted Metabolomics Based on Liquid Chromatography-Mass Spectrometry and Data Processing

The untargeted metabolome of fecal samples was analyzed by liquid chromatography-mass spectrometry (Waters, UPLC; Thermo, Q Exactive) platforms. A total of 50 mg of fecal sample was weighed into a 1.5 mL centrifuge tube, and 800× µL of 80% methanol was added and ground for 90 s at 65 Hz, and ground for 90 s. After sufficient mixing, the sample was sonicated for 30 min at 4 °C and then allowed to stand at −40 °C for 1 h with vortex oscillation for 30 s. The sample was then separated from the sample for 1 h with the vortex oscillator. The sample mix needs to be centrifuged (12,000 rpm) for 15 min, but it needs to be left at 4 °C for 30 min first. All of the supernatant solution was pipetted into a centrifuge tube at −40 °C for 60 min, and then centrifuged again at 4 °C at 12,000 rpm for 15 min, next, 200 µL of the supernatant was transferred to a vial, with 0.14 mg/mL dichlorophenylalanine of the internal standard being added, and stored at −80 °C to be analyzed on the machine.

A small amount of treated fecal extract was taken and separated by chromatography through a chromatograph. The model of the HPLC column was ACQUITY UPLC HSS T3 (2.1 × 100 mm, 1.8 µm). Chromatographic separations were performed in positive ion mode (ESI+) and negative ion mode (ESI−) using a preheated SuperGold C18 column (100 × 4.6 mm, 3 µm i.d.). Water and 0.05% acetonitrile with 0.1% formic acid formed solvent A. Acetonitrile mixed with 0.1% formic acid formed solvent B. The samples were eluted in the mobile phase, consisting of solvent A and solvent B at a flow rate of 0.3 µL/min. The elution of the samples was divided into a total of three steps, with the ratios of mobile phase A to mobile phase B being 95%:5%, 5%:95%, and 95%:5%, respectively; and the elution times for each step were 1 min, 25.5 min, and 29.6 min, respectively. QC samples need to be tested along with the test samples for monitoring and evaluating the stability and reliability of the system's test data. The Q Exactive HF-X (Thermo Fisher

Scientific, Waltham, MA, USA) mass spectrometer operates in positive/negative mode, with each sample detected under positive (ESI+) and negative (ESI−) ion mode conditions. Mass spectrometry parameter conditions differ in the ESI+ and ESI− modes. The mass spectrometry parameter conditions were different in positive and negative modes. In ESI+ mode, the heater temperature was 300 °C, the sheath gas flow rate was 45 arb, the auxiliary gas flow rate was 15 arb, the tail gas flow rate was 1 arb, the electrospray voltage was 3 kV, the capillary temperature was 350 °C, and the S-Lens RF Level was 30%. In ESI− mode, only the electrospray voltage (3.2 kV) and the S-Lens RF Level (60%) were changed; all other conditions remained the same. Peak extraction, baseline correction, and peak matching based on mass spectrometry data were performed using ChromaTOF software (V4.3x, LECO), and these peaks were matched to the LECO/Fiehn Metabolomics Library database to identify metabolites.

The normalized data were analyzed and visualized by multivariate analysis in SIMCA (V 14.1) software. The horizontal coordinates of the supervised partial least squares (OPLS-DA) score plot indicate the score values of the main components of the orthogonal signal correction (OSC) process, from which intergroup differences can be seen; the vertical coordinates indicate the score values of the orthogonal components of the OSC process; and intragroup differences (differences between samples within a group) can be seen on the vertical coordinates. The R2 and Q2 values of the OPLS-DA model were used to assess the model's validity. VIP values were obtained from the OPLS-DA model, and p values were calculated by a one-way ANOVA with a t test. Differential metabolites were screened with VIP > 1 and $p < 0.05$. KEGG-enriched metabolic pathways were accomplished through the MetaboAnalyst online open-source website "https://www.metaboanalyst.ca (accessed on 7 July 2022)". $p < 0.05$ was used as the criterion for having a significant functional pathway among the differential metabolites.

2.3. Data Analysis

Growth performance data were statistically analyzed using an independent t-test in SPSS 20.0 software (IBM Corporation, Armonk, NY, USA), and differences were considered significant at $p < 0.05$. Fecal microbiota and metabolites were correlated using Spearman's rank correlation, and R and p values were calculated using the R (V 4.0.2) "Psych", and correlations were considered when $p < 0.05$ and $R > |0.8|$. Correlation heatmaps were visualized using the R (V 4.0.2) "ggcorrplot".

3. Results

3.1. Growth Performance

The effects of the mixed silage on the growth performance of Hu sheep are shown in Table 1. There were no differences in the ADG (Figure 1A), DMI (Figure 1B), or FCR (Figure 1C) between the control and silage groups ($p > 0.05$).

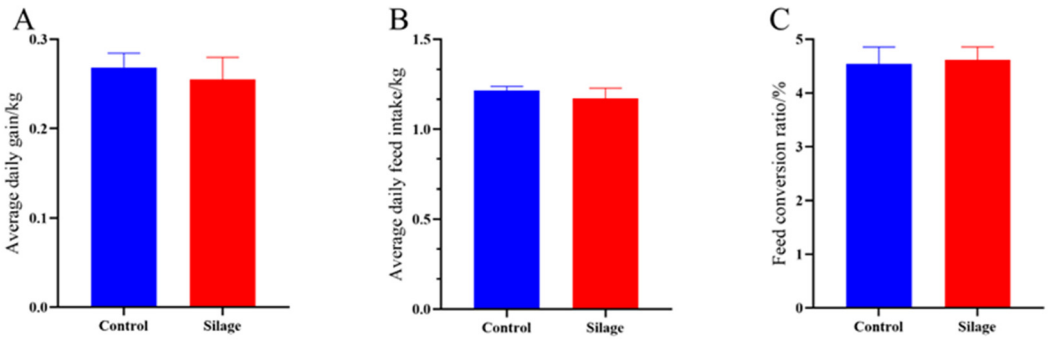

Figure 1. Effects of the mixed silage on growth performance in Hu sheep ($n = 6$). (**A**) Average daily feed intake; (**B**) average daily gain; (**C**) feed conversion ratio. Control: control group, the control group with peanut sprouts, corn husks and sorghum husks as roughage; Silage: silage group, the silage group with the mixed silage as roughage.

3.2. Fecal Microbiota Structure

In the fecal microbiome, a total of 1,111,942 reads were obtained. Based on 97% sequence similarity, an average of 894,173 valid data points were obtained after quality control, yielding 1839 OTUs. The results of the rarefaction curves (Supplementary Figure S1A) showed that with the gradual increase of sequences, the curve was in the rising stage, and a large number of microorganisms were found in the sample. As the number of measured sequences continued to increase, the dilution curve had gradually flattened, indicating that sequencing depth could cover most of the microbial groups in the sample and could be used for later experiments. The venn diagram results showed (Supplementary Figure S1B) that the number of OTUs in the control group was lower than the silage group, with 1380 OTUs shared between the two groups. In addition, the control group had exclusive access to 107 OTUs, and the silage group had exclusive access to 352 OTUs. The results of alpha diversity (Supplementary Figure S2) showed that the replacement of roughage in the control group by the mixed silage did not affect the Richness, Shannon, Simpson, Chao1, ACE, and goods_coverage indices of the microorganisms in manure ($p > 0.05$). The PCoA results (Figure 2A) showed that the contribution of the first and second principal coordinates of PCoA was 33.55% and 15.51%, respectively. Anosim analyses showed that the fecal microflora structure of Hu sheep was affected by the mixed silage ($p = 0.011$). At the phyla level (Figure 2B), *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the dominant phyla in the both groups, followed by *Spirochaetes*, *Verrucomicrobia*, *Planctomycetes*, *Patescibacteria*, *Fibrobacteres*, *Kiritimatiellaeota*, and *Actinobacteria*. At the genera level (Figure 2C), *Ruminococcaceae UCG-005* and the *Christensenellaceae R-7 group* were the dominant genera in the control group, and the *Christensenellaceae R-7 group* and the *Rikenellaceae RC9 gut group* were the dominant genera in the silage group. The results of LEfSe analysis (Figure 2D) showed that a total of 15 differentiated microorganisms were obtained, of which four were in the control group, namely *Ruminococcaceae UCG_010*, *Breznakia*, *Erysipelothrix*, and *Desulfovibrio*, and eleven in the silage group, which were mainly distributed in the *Bacilli*, *Clostridiaceae1*, *Pseudomonadales*, *Succiniclasticum*, and *Shuttleworthia*.

3.3. Fecal Metabolomics

Quality control (QC) samples were used throughout the trial to assess the stability and reproducibility of the data. The quality of the data is directly proportional to the correlation of the QC samples. The values for the R-type 2 ESI+ and ESI− polarity modes are 1 and 0.79, respectively (Supplementary Figure S3). OPLS-DA with supervision was used to distinguish differences in metabolic profiles between control and silage groups, and samples from control and silage groups were significantly separated in ESI+ or ESI− mode (Figure 3A,C). In addition, there was more aggregation between samples within the silage group than in the control group. These results suggested that silage can alter fecal metabolites in Hu sheep. After 200 response replacement tests, OPLS-DA calculated the regression intercept (Q2), and we considered the model to be valid when Q2 was less than zero. From (Figure 3B,D), the Q2 value is −0.199 for the ESI+ model (Figure 3B) and −0.858 for the ESI− model (Figure 3D). Therefore, we concluded that the model was reliable and stable.

The metabolites of the feces from the control and silage groups were analyzed by LC-MS, and 582 and 612 peaks were retained after preprocessing the raw data for ESI+ and ESI− modes, resulting in the identification and quantification of 217 positive and 231 negative compounds. Among these compounds, 19 positively ionized compounds and 59 negatively ionized compounds were differentiated (VIP > 1 and $p < 0.05$), and these metabolites were mainly distributed among amino acids, peptides, and analogues, benzoic acids and derivatives, bile acids, alcohols and derivatives, carbohydrates and carbohydrate conjugates, carbonyl compounds, eicosanoids, fatty acids and conjugates, isoflav-2-enes, pyrimidine 2′-deoxyribonucleosides, tetrahydrofuran lignans, and others (Table 2).

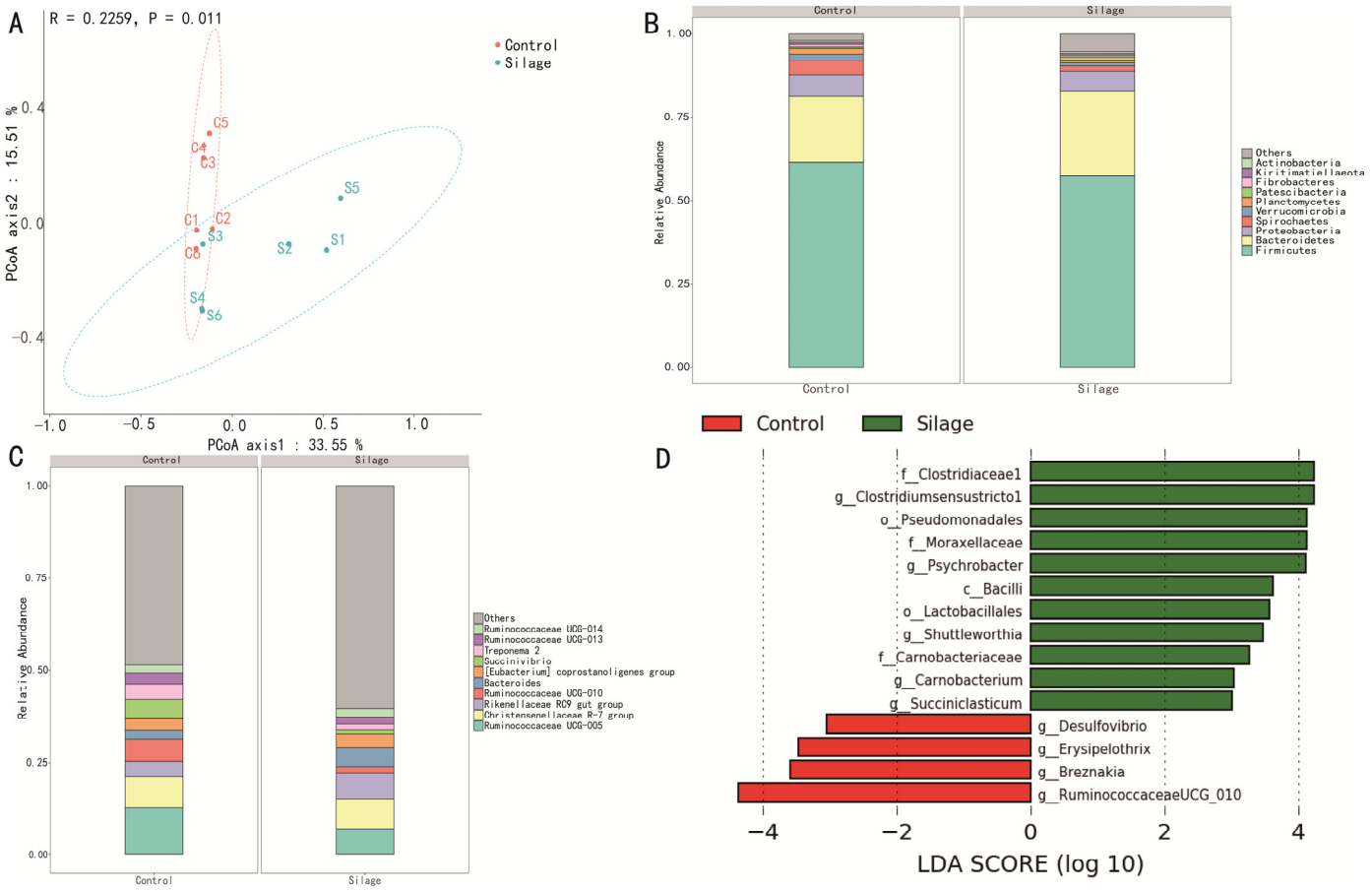

Figure 2. The structural composition of fecal microflora in the control group and silage group (n = 6). (**A**) Principal coordinate analysis (PCoA) demonstrated the separation of microbial communities in the feces of Hu sheep fed two diets based on the Bray–Curtis dissimilarity matrix. The top ten phyla (**B**) and genera (**C**) of fecal microbiota in the control group and silage group. (**D**) Discriminant analysis of fecal microbial OTU of Hu sheep fed with diet in the control group and silage group, with LDA score > 3. Control: control group, the control group with peanut sprouts, corn husks, and sorghum husks as roughage; Silage: silage group, the silage group with the mixed silage as roughage.

Table 2. Contents of differential metabolites in feces (n = 6).

Metabolites	Group [1]		VIP [2]	p	FC [3]	Trend	Mode
	Control	Silage					
Amino acids, peptides, and analogues							
DL-Alanine	0.079	0.242	1.403	0.018	3.071	Up	ESI+
Glycine	0.003	0.011	1.637	0.001	3.633	Up	ESI+
LEVODOPA	0.004	0.008	1.430	0.013	1.950	Up	ESI+
N,N-Dimethylglycine	0.012	0.003	1.277	0.042	0.268	Down	ESI+
Ala-Ile	0.065	0.027	1.119	0.045	0.407	Down	ESI−
Arginine	0.053	0.004	1.299	0.006	0.079	Down	ESI−
D-ASPARTATE	0.644	0.093	1.375	0.002	0.145	Down	ESI−
gamma-Glutamylleucine	0.083	0.027	1.407	0.001	0.328	Down	ESI−
L-Histidine	0.055	0.007	1.147	0.046	0.136	Down	ESI−
L-Phenylalanine	1.630	0.474	1.165	0.040	0.291	Down	ESI−
L-Valine	0.077	0.018	1.197	0.022	0.231	Down	ESI−
N-Acetylglutamic acid (NAG)	0.641	0.230	1.220	0.012	0.359	Down	ESI−
N-Isobutyrylglycine	1.345	0.017	1.206	0.015	0.012	Down	ESI−
N-Tigloylglycine	0.861	0.003	1.199	0.025	0.004	Down	ESI−

Table 2. *Cont.*

Metabolites	Group [1]		VIP [2]	*p*	FC [3]	Trend	Mode
	Control	Silage					
Benzoic acids and derivatives							
2-Hydroxyhippuric acid	0.544	0.007	1.199	0.028	0.013	Down	ESI−
5-Methoxysalicylic acid	0.018	0.005	1.135	0.049	0.277	Down	ESI−
Butylparaben	0.231	0.063	1.144	0.019	0.275	Down	ESI−
Bile acids, alcohols, and derivatives							
Cholic Acid	0.015	0.009	1.419	0.011	0.578	Down	ESI+
Glycochenodeoxycholate	0.014	0.001	1.274	0.045	0.040	Down	ESI+
GLYCOCHOLATE	0.005	0.002	1.411	0.011	0.441	Down	ESI+
Carbohydrates and carbohydrate conjugates							
N-Acetylneuraminic acid	0.018	0.133	1.487	0.006	7.483	Up	ESI+
D-SACCHARIC ACID	0.166	0.053	1.425	0.001	0.318	Down	ESI−
N-Acetylmannosamine	8.252	2.333	1.232	0.010	0.283	Down	ESI−
N-Acetylmuramic Acid	0.636	0.141	1.425	0.001	0.222	Down	ESI−
Carbonyl compounds							
Acetophenone	0.243	0.035	1.627	0.001	0.145	Down	ESI+
4-Hydroxybenzaldehyde	0.844	0.276	1.424	0.001	0.327	Down	ESI−
Eicosanoids							
Prostaglandin B1	0.007	0.079	1.272	0.007	11.976	Up	ESI−
Resolvin E1	0.011	0.054	1.376	0.002	4.752	Up	ESI−
Fatty acids and conjugates							
3,3-Dimethylglutaric acid	0.642	0.110	1.577	<0.001	0.172	Down	ESI−
3-Methylglutaric acid	0.050	0.224	1.154	0.032	4.455	Up	ESI−
Arachidic acid	0.322	1.498	1.126	0.016	4.655	Up	ESI−
Lauric acid	0.061	0.027	1.224	0.006	0.454	Down	ESI−
Isoflav-2-enes							
Daidzein	0.176	0.050	1.147	0.024	0.283	Down	ESI−
Genistein	2.530	0.442	1.376	0.002	0.175	Down	ESI−
Pyrimidine 2′-deoxyribonucleosides							
2′-Deoxyuridine	0.002	0.006	1.564	0.003	3.150	Up	ESI+
Thymidine	0.018	0.051	1.330	0.030	2.752	Up	ESI+
Tetrahydrofuran lignans							
Enterolactone	0.018	0.110	1.118	0.029	6.082	Up	ESI−
matairesinol	0.163	0.015	1.202	0.010	0.091	Down	ESI−
Others							
Benzothiazole	1.248	1.073	1.295	0.033	0.860	Down	ESI+
Metaxalone	0.003	0.001	1.319	0.029	0.219	Down	ESI+
9-Fluorenone	5.195	0.812	1.172	0.017	0.156	Down	ESI−
Abietic acid	0.005	0.016	1.135	0.034	3.514	Up	ESI−
Biotin	0.038	0.082	1.157	0.047	2.143	Up	ESI−
Bis(4-hydroxyphenyl)methane	0.028	0.074	1.168	0.022	2.634	Up	ESI−
cirsimaritin	0.033	0.002	1.508	<0.001	0.074	Down	ESI−
delta7-Dafachronic acid	0.090	0.047	1.104	0.048	0.525	Down	ESI−
Ecgonine	0.381	0.062	1.137	0.050	0.162	Down	ESI−
Piceatannol	0.010	0.059	1.393	0.001	6.047	Up	ESI−
Pseudouridine	1.798	0.457	1.160	0.033	0.254	Down	ESI−
Resveratrol	0.071	0.172	1.129	0.031	2.420	Up	ESI−
santin	0.019	0.085	1.286	0.009	4.525	Up	ESI−
URIDINE	1.502	0.455	1.120	0.043	0.303	Down	ESI−
3-Hydroxyphenylacetic acid	0.108	0.021	1.519	<0.001	0.199	Down	ESI−
adrenosterone	0.044	0.001	1.329	0.002	0.025	Down	ESI−
3-Indoxyl sulphate	2.911	0.004	1.160	0.042	0.001	Down	ESI−
Epinephrine	0.190	0.000	1.203	0.024	0.002	Down	ESI−
Isobutyric acid	0.085	0.019	1.208	0.020	0.220	Down	ESI−
Bisphenol A	0.003	0.045	1.409	0.001	16.963	Up	ESI−
Glabranine	1.708	0.069	1.608	<0.001	0.040	Down	ESI−
Stearamide	0.022	0.010	1.365	0.016	0.448	Down	ESI+
Chrysin	0.027	0.001	1.553	<0.001	0.045	Down	ESI−

Table 2. *Cont.*

Metabolites	Group [1]		VIP [2]	*p*	FC [3]	Trend	Mode
	Control	Silage					
Ferulic acid	0.005	0.012	1.375	0.023	2.257	Up	ESI+
afzelechin	0.020	0.116	1.388	0.002	5.671	Up	ESI−
3-(2-Hydroxyethyl)indole	0.073	0.001	1.171	0.035	0.019	Down	ESI−
D-(+)-Tryptophan	0.459	0.165	1.162	0.041	0.360	Down	ESI−
Equol	0.072	0.003	1.478	<0.001	0.043	Down	ESI−
Dihydrojasmonic Acid	0.306	0.128	1.269	0.011	0.419	Down	ESI−
13-HPODE	2.260	4.175	1.091	0.034	1.847	Up	ESI−
Vanillin	0.025	0.005	1.362	0.003	0.192	Down	ESI−
Formononetin	0.069	0.003	1.522	<0.001	0.045	Down	ESI−
2-Hydroxyphenylacetic acid	0.060	0.004	1.318	0.003	0.060	Down	ESI−
2′-Deoxyinosine	0.029	0.065	1.360	0.021	2.221	Up	ESI+
Uric acid	0.671	0.083	1.165	0.038	0.124	Down	ESI−
4-Pyridoxic acid	0.060	0.026	1.294	0.042	0.426	Down	ESI+
Cytosine	0.045	0.099	1.520	0.004	2.218	Up	ESI+
Acetylcholine	0.151	0.030	1.295	0.043	0.200	Down	ESI+
4-Methyl-2-oxovaleric Acid	1.376	0.415	1.162	0.013	0.302	Down	ESI−

Note: [1] Control: control group, the control group with peanut sprouts, corn husks, and sorghum husks as roughage; Silage: silage group, the silage group with the mixed silage as roughage. [2] VIP: variable importance in projection; [3] FC: fold change.

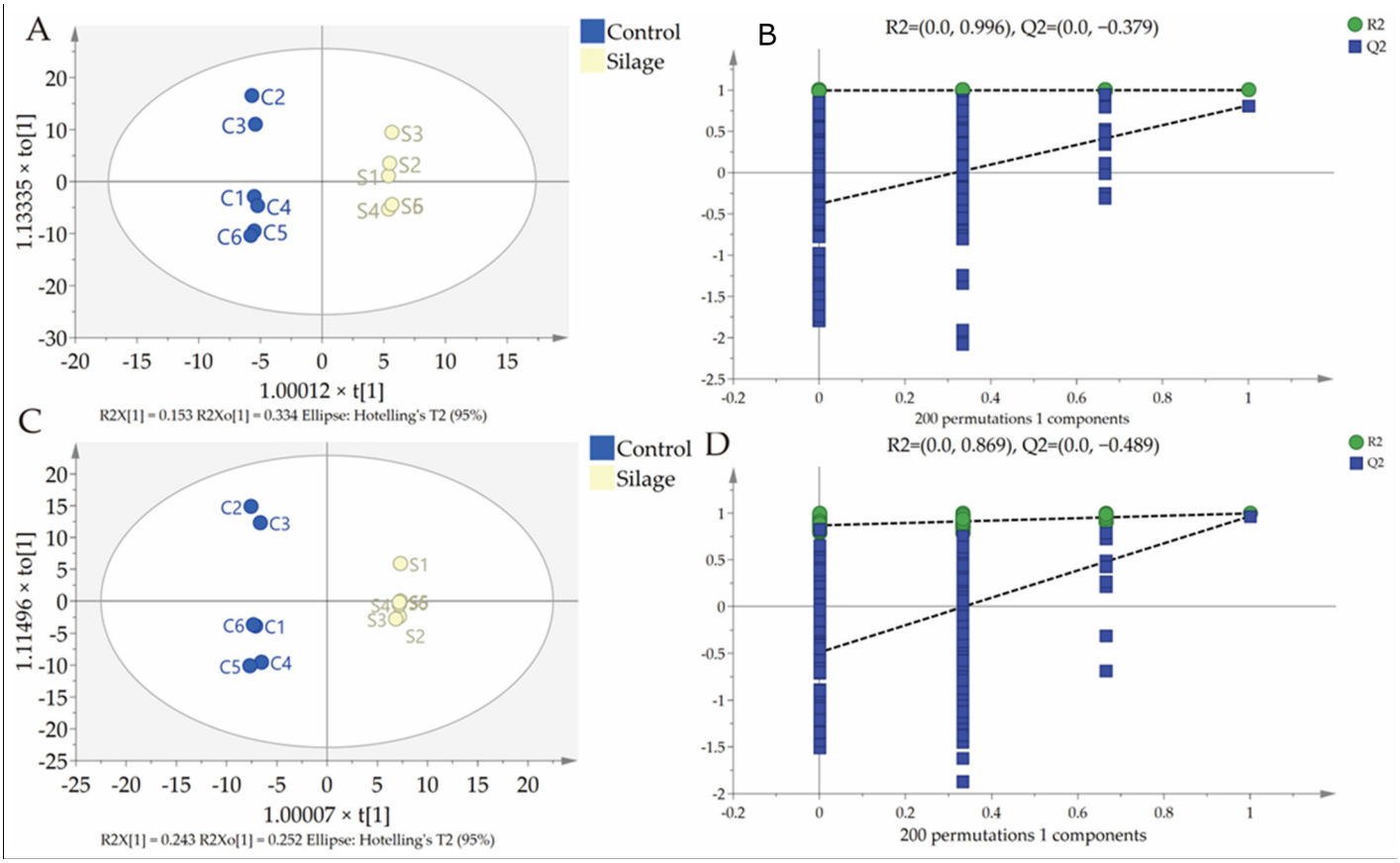

Figure 3. OPLS-DA score plot and response permutation test of fecal microbial flora structure in the control group and silage group. (**A**) OPLS-DA score plot of positive ion metabolites. (**B**) OPLS-DA response permutation test for positive ion metabolites. (**C**) OPLS-DA score plot of negative ion metabolites. (**D**) OPLS-DA response permutation test of negative ion metabolites.

3.4. KEGG Enrichment Analysis

Enrichment analysis based on fecal differential metabolism metabolites (KEGG) showed that the mixed silage altered nine metabolic pathways (Figure 4). In the ESI+ mode (Figure 4A), the mixed silage diet altered four stored metabolic pathways, namely primary bile acid biosynthesis, pyrimidine metabolism, and glycine, serine, and threonine metabolism ($p < 0.05$). In the ESI− mode (Figure 4B), five metabolic pathways were altered, namely aminoacyl-tRNA biosynthesis, valine, leucine, and isoleucine biosynthesis, phenylalanine metabolism, arginine biosynthesis, and phenylalanine, tyrosine, and tryptophan biosynthesis ($p < 0.05$).

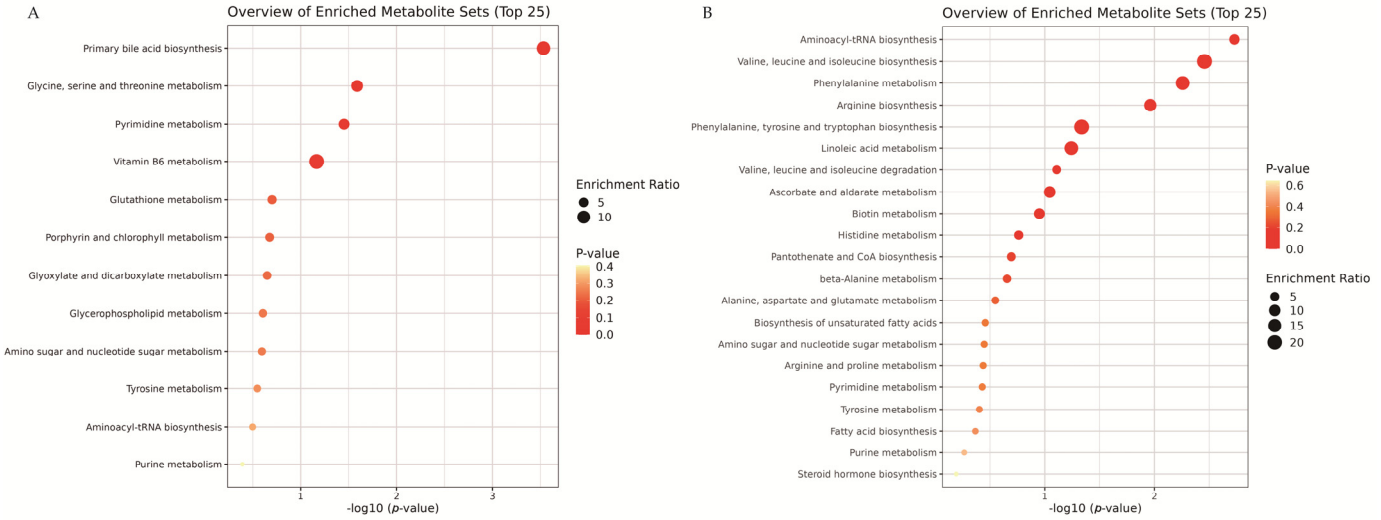

Figure 4. Enrichment analysis (KEGG) of metabolic pathways of differential metabolites in the feces of Hu sheep fed control and silage diets. (**A**) ESI+. (**B**) ESI−.

3.5. Analysis of Differential Metabolites and Fecal Microbial Correlations

The correlation analysis between differential metabolites and differential microorganisms is shown in Figure 5. *Ruminococcaceae UCG_010* and L-valine, D-saccharic acid, lauric acid, NAG, metaxalone, vanillin, 3-hydroxyphenylacetic acid, D-aspartate, 3-indoxyl sulfate, gamma-glutamylleucine, L-phenylalanine, L-histidine, arginine, 3′2-hydroxyethyl indole, and 2-hydroxyhippuric acid were positively correlated, while they were negatively correlated with ferulic acid, santin, and 13-hpode ($p < 0.05$). *Breznakia* has a positive and significant correlation with N-isobutyrylglycine, daidzein, pseudouridine, butylparaben, uridine, equol, and dihydrojasmonic acid ($p < 0.05$). *Desulfovibrio* was positively correlated with pseudouridine, uridine, dihydrojasmonic acid, 2-hydroxyhippuric acid, uric acid, 3-indoxyl sulfate, ecgonine, vanillin, N-tigloylglycine, epinephrine, 3,2-hydroxyethyl indole, L-histidine, arginine, N-isobutyrylglycine, and L-valine ($p < 0.05$). *Carnobacterium* was positively correlated with DL-alanine, N-acetylneuraminic acid, glycine, and ferulic acid. *Psychrobacter* was positively correlated with 3-methylglutaric acid ($p < 0.05$).

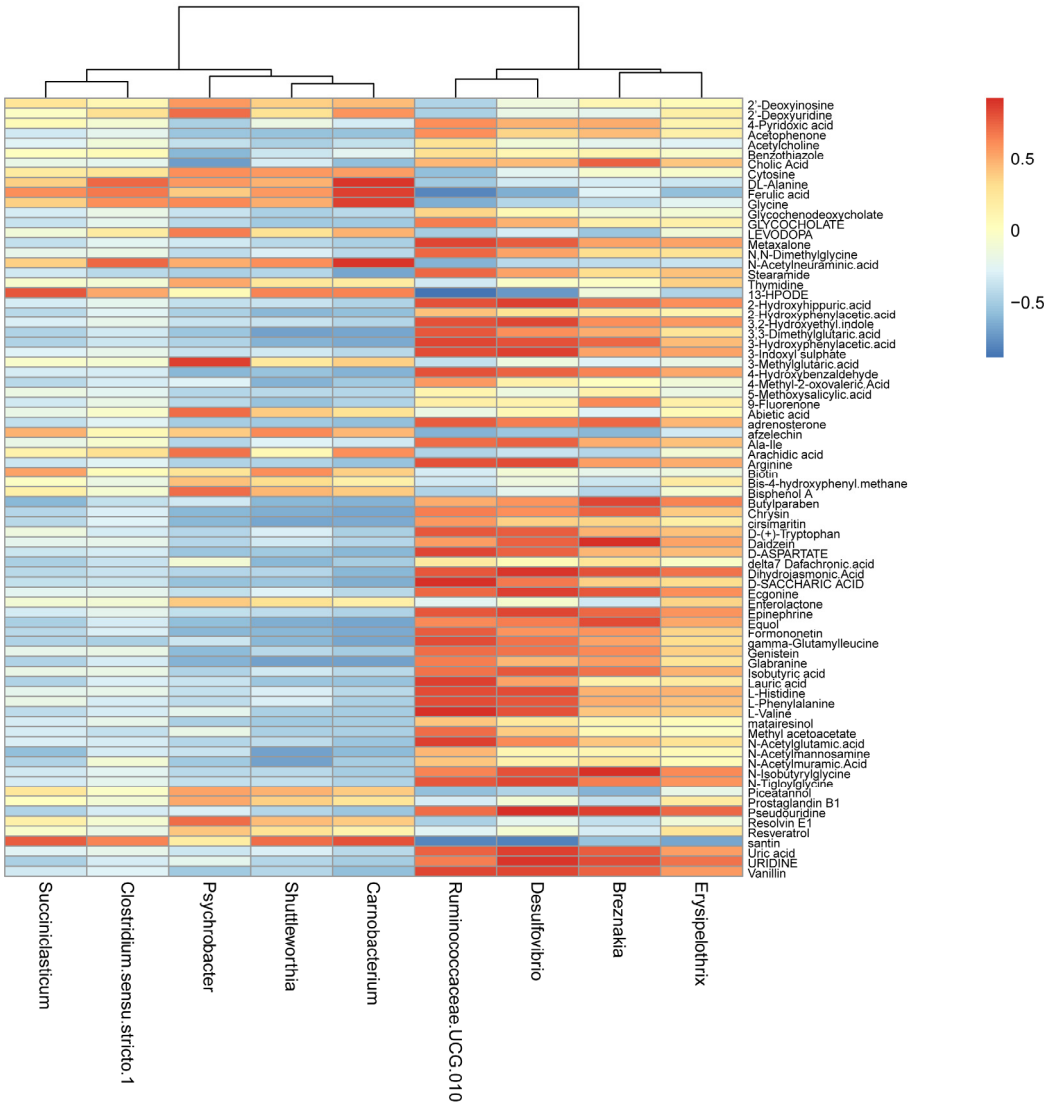

Figure 5. Spearman rank correlation between fecal microbiota and metabolites. Blue indicates negative correlations, and red indicates positive correlations.

4. Discussion

Silage can make up for the shortage of seasonal roughage and contribute to the feeding of ruminants. In this experiment, our results showed that the use of mixed silage in the diets of Hu sheep did not affect growth performance but had an effect on the structure of the microflora, metabolites, and metabolic pathways in the hindgut.

In this experiment, the difference in fecal microbial diversity and abundance between the silage and control groups was not significant, which is consistent with previous studies [26]. PCoA and Anosim analyses indicated that the mixed silage had a highly significant effect on the microflora structure of manure. At the phyla level, our results indicated that the predominant phyla in both groups was *Firmicutes*, followed by *Bacteroidetes* and *Proteobacteria*. Previous studies have shown that the main microorganisms considered to be present in sheep feces are *Firmicutes* [27]. The main phyla were shown to be *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* in a study on the evolution of mammalian gut microorganisms [13]. In the intestinal tract of ruminants, *Bacteroides* plays a very important role in the degradation of cellulose [28]. It has been shown that *Firmicutes* plays a very important role in the digestion of carbohydrates and the fermentation of organic matter [29]. The role of *Proteobacteria* is not fully understood, and further studies are needed [30]. However, studies on the diversity and function of fecal microbiota in sheep have shown that the fecal

microbiota is mainly associated with carbohydrate degradation and catabolism [27]. At the genera level, the main microorganisms within the silage group are dominated by the *Christensenellaceae R-7 group* and the *Rikenellaceae RC9 gut group*. The *Christensenellaceae R-7 group* is a genus in the family *Christensenaceae*, which is widespread in the human gut and animal intestinal tract and is closely related to the host's health [31]. The function of the *Rikenellaceae RC9 gut group* may be related to fiber degradation [32,33]. Furthermore, the proportion of *Rikenellaceae RC9 gut group* in the feces of musk deer is significantly reduced when they experience diarrhea and exacerbate it [34]. The *Christensenellaceae R-7 group* was the dominant genera within the silage group, which is more favorable for Hu sheep health.

The LEfSe results showed that at the genera level, the signature microorganisms in the silage group were *Psychrobacter*, *Succiniclasticum*, and *Carnobacterium*. *Psychrobacter* has a wide range of cryophilic bacteria, with members of the genera varying widely in cold suitability and genome, and can be isolated from antarctic soils, seawater, Siberian tundra, and the intestinal tracts of marine fish. *Psychrobacter* has a wide range of cryophiles, and members of the genera have widely varying cold suitability and genomes that can be isolated from Antarctic soil, seawater, Siberian tundra, and the intestinal tracts of marine fish, but their metabolisms are unknown [35–37]. The addition of *Psychrobacter* to the diet could enhance the activity of digestive enzymes in the gut and improve the digestive utilization of feed [26]. *Succiniclasticum* may be a normal genus in the intestinal tract of ruminants that catabolizes succinic acid to produce propionic acid, and also has some catabolic capacity for cellulose and starch [38]. The elevated relative abundance of Succiniclasticum may be related to higher levels of NDF and ADF in the mixed silage. *Carnobacterium* is widely distributed in the gastrointestinal tract of meat, fish, and poultry or in lakes and produces lactic acid, which in part inhibits the action of harmful microorganisms [39,40]. In addition, the mixed silage reduced the proportion of *Desulfovibrio*, which has a toxic effect on the intestinal epithelium and contributes to gastrointestinal disorders [41,42].

Metabolomics can explain phenotypic changes better than genomics and proteomics [43]. Our metabolomics data suggest that the mixed silage altered the concentrations of many fecal metabolites, which may be related to changes in fecal microbial abundance. The screened differential metabolites were enriched in glycine, serine, and threonine metabolism, valine, leucine, and isoleucine biosynthesis, phenylalanine metabolism, arginine biosynthesis, phenylalanine metabolism, arginine, and phenylalanine, tyrosine, and tryptophan biosynthesis. Amino acids are important for microbial growth and metabolism, and are key components of protein and peptide synthesis and regulate several metabolic pathways [44]. L-valine enters the tricarboxylic acid cycle to provide energy and has important roles in protein synthesis, cell proliferation, and signaling pathway activation [45,46]. NAG is an important allosteric activator of carbamoyl phosphate synthase. NAG can activate carbamoylphosphate synthase and promote its synthesis of carbamoyl phosphate, thus promoting the synthesis of arginine. NAG participates in the urea cycle in the liver and combines with ornithine transcarbamylase to generate citrulline in the intestine, and then citrulline is converted into an important pathway for the synthesis of arginine in the kidney [47]. L-arginine is involved in the tricarboxylic acid cycle as well as the urea cycle and can regulate energy metabolism, amino acid metabolism, and microbial metabolism in animals [48]. NAG, L-valine, and L-arginine were significantly down-regulated in the silage group, which may be related to the decrease in the proportion of *Ruminococcaceae UCG 010*.

It is noteworthy that we found that the concentrated metabolites of concentrated pyrimidine metabolism also changed significantly. In our experiments, the mixed silage significantly upregulated 2′-deoxyuridine and thymidine. Pyrimidine metabolism is a prominent feature of the hindgut, and different combinations of nucleosides have variable effects on animal growth performance [49]. Feeding broilers with yeast extract as a source of nucleotides does not affect the growth performance of broilers, but adding nucleotides to the diet can improve the performance of piglets [50,51]. In addition, the study of Ma et al. [52] showed that the accumulation of nucleosides functioned to enhance the growth

performance of Dorper sheep. Therefore, mixed silage has the potential to improve the growth performance of Hu sheep.

5. Conclusions

In conclusion, the mixed silage increased the relative abundance of microorganisms such as *Psychrobacter*, *Succiniclasticum*, and *Carnobacterium* and decreased the relative abundance of microorganisms such as *Ruminococcaceae UCG_010* and *Breznakia*. In addition, the mixed silage alters the concentration of many fecal metabolites and enriches metabolic pathways, which is more beneficial to the healthy growth of Hu sheep.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10050233/s1, Figure S1: Ruminal microbial OTUs with the different dietary groups. (A) OTU-based microbial sparsity profiles were used to assess the depth of coverage for each sample. (B) Venn diagram of fecal bacterial OTUs; Figure S2: Number of species, richness and diversity indices observed in fecal samples. Figure S3: Person Correlation of QC samples. (A) ESI+; (B) ESI−.

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Institutional Review Board Statement: All animal experiments were conducted in accordance with the Animal Protection Law, in accordance with the "Guidelines for the Care and Use of Experimental Animals" (SXXY 2015-0054) approved by the Ethics Committee of Yangzhou University.

Informed Consent Statement: The animal owners have expressed written informed consent to their animals participating in this study.

Data Availability Statement: The datasets presented in this study can be found in online repositories. The names of the reposi-tory/repositories and accession number(s) can be found below: NCBI SRA BioProject, accession no: PRJNA 1028414.

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Conflicts of Interest: The authors declare that this study was conducted without any business or financial relationships that could be considered a potential conflict of interest.

References

1. Alexandratos, N.; Bruinsma, J. *World Agriculture towards 2030/2050: The 2012 Revision*; Food and Agriculture Organization: Rome, Italy, 2012.
2. Wang, C.; Zheng, M.; Wu, S.; Zou, X.; Chen, X.; Ge, L.; Zhang, Q. Effects of gallic acid on fermentation parameters, protein fraction, and bacterial community of whole plant soybean silage. *Front. Microbiol.* **2021**, *12*, 662966. [CrossRef]
3. Wilkinson, J.; Rinne, M. Highlights of progress in silage conservation and future perspectives. *Grass Forage Sci.* **2018**, *73*, 40–52. [CrossRef]
4. Wanapat, M.; Sundstøl, F.; Garmo, T. A comparison of alkali treatment methods to improve the nutritive value of straw. I. Digestibility and metabolizability. *Anim. Feed Sci. Technol.* **1985**, *12*, 295–309. [CrossRef]
5. Weinberg, Z.; Chen, Y. Effects of storage period on the composition of whole crop wheat and corn silages. *Anim. Feed Sci. Technol.* **2013**, *185*, 196–200. [CrossRef]
6. Ren, H.; Xu, N.; Li, J.; Li, Z.; Zhao, T.; Pei, F.; Yao, X.; Sun, Y. Effects of different mixed ratio of maize straw and cabbage wastes on silage quality. *J. Biobased Mater. Bioenergy* **2015**, *9*, 88–94. [CrossRef]

7. Partovi, E.; Rouzbehan, Y.; Fazaeli, H.; Rezaei, J. Broccoli byproduct-wheat straw silage as a feed resource for fattening lambs. *Transl. Anim. Sci.* **2020**, *4*, txaa078. [CrossRef]
8. Lu, H.; Yan, H.; Almeida, V.; Adeola, O.; Ajuwon, K. Effects of dietary resistant starch content on nutrient and energy digestibility and fecal metabolomic profile in growing pigs. *J. Anim. Sci.* **2016**, *94*, 364–368. [CrossRef]
9. Kim, J.-H.; Hong, S.W.; Park, B.-Y.; Yoo, J.G.; Oh, M.-H. Characterisation of the bacterial community in the gastrointestinal tracts of elk (*Cervus canadensis*). *Antonie Van Leeuwenhoek* **2019**, *112*, 225–235. [CrossRef]
10. Al-Lahham, S.a.H.; Roelofsen, H.; Priebe, M.; Weening, D.; Dijkstra, M.; Hoek, A.; Rezaee, F.; Venema, K.; Vonk, R.J. Regulation of adipokine production in human adipose tissue by propionic acid. *Eur. J. Clin. Investig.* **2010**, *40*, 401–407. [CrossRef]
11. Ussar, S.; Fujisaka, S.; Kahn, C.R. Interactions between host genetics and gut microbiome in diabetes and metabolic syndrome. *Mol. Metab.* **2016**, *5*, 795–803. [CrossRef]
12. Jia, W.; Li, H.; Zhao, L.; Nicholson, J.K. Gut microbiota: A potential new territory for drug targeting. *Nat. Rev. Drug Discov.* **2008**, *7*, 123–129. [CrossRef]
13. Ley, R.E.; Hamady, M.; Lozupone, C.; Turnbaugh, P.J.; Ramey, R.R.; Bircher, J.S.; Schlegel, M.L.; Tucker, T.A.; Schrenzel, M.D.; Knight, R. Evolution of mammals and their gut microbes. *Science* **2008**, *320*, 1647–1651. [CrossRef]
14. Johnson, A.J.; Vangay, P.; Al-Ghalith, G.A.; Hillmann, B.M.; Ward, T.L.; Shields-Cutler, R.R.; Kim, A.D.; Shmagel, A.K.; Syed, A.N.; Personalized Microbiome Class, S.; et al. Daily Sampling Reveals Personalized Diet-Microbiome Associations in Humans. *Cell Host Microbe* **2019**, *25*, 789–802.e785. [CrossRef]
15. Li, C.; Chen, N.; Zhang, X.; Shahzad, K.; Qi, R.; Zhang, Z.; Lu, Z.; Lu, Y.; Yu, X.; Zafar, M.H. Mixed silage with Chinese cabbage waste enhances antioxidant ability by increasing ascorbate and aldarate metabolism through rumen Prevotellaceae UCG-004 in Hu sheep. *Front. Microbiol.* **2022**, *13*, 978940. [CrossRef]
16. Nicholson, J.K.; Lindon, J.C. Metabonomics. *Nature* **2008**, *455*, 1054–1056. [CrossRef]
17. Zhang, Z.; Shahzad, K.; Shen, S.; Dai, R.; Lu, Y.; Lu, Z.; Li, C.; Chen, Y.; Qi, R.; Gao, P. Altering Dietary Soluble Protein Levels With Decreasing Crude Protein May Be a Potential Strategy to Improve Nitrogen Efficiency in Hu Sheep Based on Rumen Microbiome and Metabolomics. *Front. Nutr.* **2021**, *8*, 815358. [CrossRef]
18. Yi, H.; Yang, G.; Xiong, Y.; Wu, Q.; Xiao, H.; Wen, X.; Yang, X.; Wang, L.; Jiang, Z. Integrated metabolomic and proteomics profiling reveals the promotion of Lactobacillus reuteri LR1 on amino acid metabolism in the gut–liver axis of weaned pigs. *Food Funct.* **2019**, *10*, 7387–7396. [CrossRef]
19. Plaizier, J.C.; Li, S.; Danscher, A.M.; Derakshani, H.; Andersen, P.H.; Khafipour, E. Changes in microbiota in rumen digesta and feces due to a grain-based subacute ruminal acidosis (SARA) challenge. *Microb. Ecol.* **2017**, *74*, 485–495. [CrossRef]
20. Warner, R.D.; Jacob, R.H.; Rosenvold, K.; Rochfort, S.; Trenerry, C.; Plozza, T.; McDonagh, M.B. Altered post-mortem metabolism identified in very fast chilled lamb M. longissimus thoracis et lumborum using metabolomic analysis. *Meat Sci.* **2015**, *108*, 155–164. [CrossRef]
21. National Research Council (NRC). *Nutrient Requirements of Small Ruminants, Sheep, Goats, Cervids, and New World Camelids*; National Academy Press: Washington, DC, USA, 2007.
22. Magoč, T.; Salzberg, S.L. FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **2011**, *27*, 2957–2963. [CrossRef]
23. Chen, S.; Zhou, Y.; Chen, Y.; Gu, J. fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **2018**, *34*, i884–i890. [CrossRef] [PubMed]
24. Edgar, R.C. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* **2013**, *10*, 996–998. [CrossRef] [PubMed]
25. Lozupone, C.; Knight, R. UniFrac: A new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* **2005**, *71*, 8228–8235. [CrossRef] [PubMed]
26. Sun, Y.Z.; Yang, H.L.; Ma, R.L.; Zhang, C.X.; Lin, W.Y. Effect of dietary administration of *Psychrobacter* sp. on the growth, feed utilization, digestive enzymes and immune responses of grouper *Epinephelus coioides*. *Aquacult. Nutr.* **2011**, *17*, e733–e740. [CrossRef]
27. Tanca, A.; Fraumene, C.; Manghina, V.; Palomba, A.; Abbondio, M.; Deligios, M.; Pagnozzi, D.; Addis, M.F.; Uzzau, S. Diversity and functions of the sheep faecal microbiota: A multi-omic characterization. *Microb. Biotechnol.* **2017**, *10*, 541–554. [CrossRef] [PubMed]
28. Shabana, I.; Albakri, N.; Bouqellah, N. Metagenomic investigation of faecal microbiota in sheep and goats of the same ages. *J. Taibah Univ. Sci.* **2021**, *15*, 1–9. [CrossRef]
29. Spence, C.; Wells, W.G.; Smith, C.J. Characterization of the primary starch utilization operon in the obligate anaerobe Bacteroides fragilis: Regulation by carbon source and oxygen. *J. Bacteriol.* **2006**, *188*, 4663–4672. [CrossRef] [PubMed]
30. Wang, J.; Fan, H.; Han, Y.; Zhao, J.; Zhou, Z. Characterization of the microbial communities along the gastrointestinal tract of sheep by 454 pyrosequencing analysis. *Asian Australas. J. Anim. Sci.* **2017**, *30*, 100. [CrossRef]
31. Waters, J.L.; Ley, R.E. The human gut bacteria Christensenellaceae are widespread, heritable, and associated with health. *BMC Biol.* **2019**, *17*, 83. [CrossRef]
32. Asma, Z.; Sylvie, C.; Laurent, C.; Jérôme, M.; Christophe, K.; Olivier, B.; Annabelle, T.-M.; Francis, E. Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. *FEMS Microbiol. Ecol.* **2013**, *83*, 504–514.

33. Huang, C.; Ge, F.; Yao, X.; Guo, X.; Bao, P.; Ma, X.; Wu, X.; Chu, M.; Yan, P.; Liang, C. Microbiome and metabolomics reveal the effects of different feeding systems on the growth and ruminal development of yaks. *Front. Microbiol.* **2021**, *12*, 682989. [CrossRef]
34. Li, Y.; Hu, X.; Yang, S.; Zhou, J.; Qi, L.; Sun, X.; Fan, M.; Xu, S.; Cha, M.; Zhang, M. Comparison between the fecal bacterial microbiota of healthy and diarrheic captive musk deer. *Front. Microbiol.* **2018**, *9*, 300. [CrossRef]
35. Zhang, S.; Song, W.; Yu, M.; Lin, X. Comparative genomics analysis of five Psychrobacter strains isolated from world-wide habitats reveal high intra-genus variations. *Extremophiles* **2017**, *21*, 581–589. [CrossRef]
36. Amato, P.; Christner, B.C. Energy metabolism response to low-temperature and frozen conditions in Psychrobacter cryohalolentis. *Appl. Environ. Microbiol.* **2009**, *75*, 711–718. [CrossRef]
37. Lin, X.; Cui, S.; Xu, G.; Wang, S.; Du, N.; Shen, J. Cloning and heterologous expression of two cold-active lipases from the Antarctic bacterium *Psychrobacter* sp. G. *Polar Res.* **2010**, *29*, 421–429.
38. Van Gylswyk, N. *Succiniclasticum ruminis* gen. nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. *Int. J. Syst. Evol. Microbiol.* **1995**, *45*, 297–300. [CrossRef] [PubMed]
39. Hammes, W.P.; Hertel, C. The genera lactobacillus and carnobacterium. *Prokaryotes* **2006**, *4*, 320–403.
40. Zeineldin, M.; Barakat, R.; Elolimy, A.; Salem, A.Z.; Elghandour, M.M.; Monroy, J.C. Synergetic action between the rumen microbiota and bovine health. *Microb. Pathog.* **2018**, *124*, 106–115. [CrossRef]
41. Murros, K.E.; Huynh, V.A.; Takala, T.M.; Saris, P.E. Desulfovibrio Bacteria Are Associated with Parkinson's Disease. *Front. Cell. Infect. Microbiol.* **2021**, *11*, 378. [CrossRef]
42. Xu, Y.; Shao, M.; Fang, X.; Tang, W.; Zhou, C.; Hu, X.; Zhang, X.; Su, K.-P. Antipsychotic-induced gastrointestinal hypomotility and the alteration in gut microbiota in patients with schizophrenia. *Brain Behav. Immun.* **2022**, *99*, 119–129. [CrossRef]
43. Vinayavekhin, N.; Homan, E.A.; Saghatelian, A. Exploring disease through metabolomics. *ACS Chem. Biol.* **2010**, *5*, 91–103. [CrossRef] [PubMed]
44. Mariz, L.; Amaral, P.; Valadares Filho, S.; Santos, S.; Detmann, E.; Marcondes, M.; Pereira, J.; Silva Júnior, J.; Prados, L.; Faciola, A. Dietary protein reduction on microbial protein, amino acid digestibility, and body retention in beef cattle: 2. Amino acid intestinal absorption and their efficiency for whole-body deposition. *J. Anim. Sci.* **2018**, *96*, 670–683. [CrossRef] [PubMed]
45. Michonneau, P.; Fleurat-Lessard, P.; Cantereau, A.; Crépin, A.; Roblin, G.; Berjeaud, J.M. Implication of actin in the uptake of sucrose and valine in the tap root and leaf of sugar beet. *Physiol. Plant.* **2021**, *172*, 218–232. [CrossRef] [PubMed]
46. Rose, A.J. Amino acid nutrition and metabolism in health and disease. *Nutrients* **2019**, *11*, 2623. [CrossRef] [PubMed]
47. Shigesada, K.; Tatibana, M. Enzymatic synthesis of acetylglutamate by mammalian liver preparations and its stimulation by arginine. *Biochem. Biophys. Res. Commun.* **1971**, *44*, 1117–1124. [CrossRef] [PubMed]
48. Guo, C.; Xue, Y.; Seddik, H.-E.; Yin, Y.; Hu, F.; Mao, S. Dynamic changes of plasma metabolome in response to severe feed restriction in pregnant ewes. *Metabolites* **2019**, *9*, 112. [CrossRef] [PubMed]
49. Daneshmand, A.; Kermanshahi, H.; Mesgaran, M.D.; King, A.J.; Ibrahim, S.A.; Klasing, K.C. Combination of purine and pyrimidine nucleosides influences growth performance, gut morphology, digestive enzymes, serum biochemical indices and immune functions in broiler chickens. *Anim. Feed Sci. Technol.* **2017**, *228*, 186–193. [CrossRef]
50. Jung, B.; Batal, A. Effect of dietary nucleotide supplementation on performance and development of the gastrointestinal tract of broilers. *Brit. Poult. Sci.* **2012**, *53*, 98–105. [CrossRef]
51. Superchi, P.; Saleri, R.; Borghetti, P.; De Angelis, E.; Ferrari, L.; Cavalli, V.; Amicucci, P.; Ossiprandi, M.; Sabbioni, A. Effects of dietary nucleotide supplementation on growth performance and hormonal and immune responses of piglets. *Animal* **2012**, *6*, 902–908. [CrossRef]
52. Ma, Y.; Yang, X.; Hua, G.; Deng, X.; Xia, T.; Li, X.; Feng, D.; Deng, X. Contribution of gut microbiomes and their metabolomes to the performance of Dorper and Tan sheep. *Front. Microbiol.* **2022**, *13*, 1047744. [CrossRef]