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Oxalic Acid Supplementation in Different Hemicellulose Diets Affects In Vitro Rumen Fermentation by Regulating Nutritional Digestibility, Microbial Diversity and Metabolic Pathways

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Abstract: Determining hemicellulose (HM) degradation is crucial for evaluating the nutritional value of ruminant diets. Our previous study showed that oxalic acid (OA) regulates rumen fermentation. Building on this research, the present study examined the effects of OA supplementation in different hemicellulose diets on sheep rumen fermentation, microbial diversity, and metabolite production in vitro. Diets with low and high HM levels (10.3% and 17%, respectively) and supplemented with seven concentrations of OA (0, 2.5, 5, 10, 20, 40, 80 mg/kg DM) were evaluated. Tests were conducted under both low (HM10.3%) and high (HM17%) hemicellulose conditions; however, the addition of 10 mg/kg DM oxalic acid could have better effects under low hemicellulose (HM10.3%), with higher concentrations of acetic, propionic, and butyric acids, as well as total acids. A 2 × 2 factorial design was used to collect rumen fluid after 12 h of fermentation to analyze microbial populations and metabolites. OA supplementation at 10 mg/kg DM significantly increased the relative abundances of several bacterial genera, including *Prevotella*, *Butyrivibrio*, *Ruminococcus*, *Sharpea*, *RFN20*, *Bulleidia*, *Olsenella*, and *Bifidobacterium* ($p < 0.05$). A positive correlation was observed between Butyrivibrio and Sharpea and the production of isobutyric and isovaleric acids ($p < 0.01$), indicating that these bacteria play a role in volatile fatty acid (VFA) production. Furthermore, rumen metabolites involved in mineral absorption and lipid metabolism, including α-tocopherol, L-glutamic acid, and ginkgolide B, were upregulated. In summary, supplementation with oxalic acid in HM diets alters rumen fermentation, enhances nutrient digestibility, promotes microbial diversity, and influences metabolic pathways. Thus, OA supplementation should be tailored to specific dietary conditions for optimal effects.

Keywords: oxalic acid; hemicellulose; nutrient digestibility; microbial diversity; metabolites; in vitro

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1. Introduction

Hemicellulose digestibility is a crucial factor for evaluating the nutritional value of ruminant diets. Ruminants rely on microbial communities in the rumen, including anaerobic microorganisms and extracellular enzymes, to break down polysaccharides such as cellulose and hemicellulose, which are otherwise difficult to digest. This fermentation process produces volatile fatty acids (VFAs), essential nutrients that help maintain rumen stability and overall host health [1]. For example, a study by Plata et al. showed that yeast culture (YC) supplementation in an animal diet can promote the degradation of hemicellulose [2]. Similarly, Palmieri et al. found that supplementation of oxalic acid in the diet may enhance rumen fermentation efficiency and improve fiber degradation [3]. Furthermore, our previous study identified oxalic acid as a functional substance of yeast culture, positively influencing animal growth and regulating gastrointestinal function [4].

Interestingly, oxalic acid is recognized as an antinutrient [5]. It can bind with dietary calcium (Ca) or magnesium (Mg) to form insoluble calcium or magnesium oxalate, which may lead to mineral deficiency in the form of low serum calcium or magnesium levels. Additionally, oxalic acid is implicated in renal failure as it is reported to cause sedimentation in the kidneys [6]. The effect of oxalic acid on ruminant growth and development is mainly reflected in insufficient mineral intake and oxalic acid poisoning [7]. For example, the addition of 6% oxalate to the diet had no adverse effect on sheep when introduced gradually, following a dose-by-dose approach [8]. However, Frutos et al. found that feeding 0.6 mM/kg live weight/day of oxalic acid increased the abundance of rumen bacteria that degrade active oxalic acid in goat rumen [9]. Moreover, adding free oxalic acid to the feed can cause changes in the decomposition rate of oxalic acid in rumen fluid, especially in goats, where the effect on oxalic acid breakdown is more pronounced [10].

In contrast to its negative effects, adding a certain amount of oxalic acid to the diet could improve rumen efficiency and microbial function by breaking down hemicellulose. This process may help maintain the ability of the rumen to degrade oxalic acid, improving the adaptation of ruminants to its presence. Palmieri et al. reported that roughage supplemented with organic acids improves rumen efficiency by maintaining a higher pH, balancing ammonia–nitrogen (NH_3-N), increasing the microbial synthesis of proteins and VFAs, and reducing methane (CH_4) production [11]. Oxalic acid is one of the main effective substances in yeast culture. The cell wall components in yeast culture can regulate the microbial community in rumen, promote the growth of beneficial microorganisms, and inhibit the reproduction of harmful microorganisms, so as to optimize the rumen fermentation process. Sheep can adapt to having 1% oxalic acid in their diet, but a 2% increase in oxalic acid is harmful and affects growth performance, fiber and CP digestibility, rumen function, and the microbial ecosystem within the sheep's rumen [7]. Studies have shown that the effects of oxalic acid in monogastric animals (such as pigs and poultry) are more complex, especially the absorption and deposition of minerals. Oxalic acid can combine with calcium, magnesium, and other minerals to form insoluble oxalate [12], which affects the bioavailability of these minerals, and may lead to mineral deficiency, affecting animal bone health and growth and development. For example, in poultry, oxalic acid intake can affect bone and egg-laying performance by affecting oxalic acid absorption and metabolism in the small intestine, reducing calcium absorption [13]. In terms of gut microbes, oxalic acid intake can have an impact on the microbial community, leading to the suppression of some microbial populations and the overgrowth of others. Oxalic acid may negatively affect microbial metabolic processes such as rumen fermentation and cecal fermentation in ruminants and monogastric animals by altering intestinal pH and microbial environment [14]. At the same time, oxalic acid metabolism also affects the liver and kidney

function of animals; especially in the case of long-term intake, oxalic acid accumulation may lead to kidney stones and other mineral metabolism-related diseases [6].

In this study, we hypothesize that oxalic acid supplementation in diets containing varying levels of hemicellulose will enhance rumen fermentation function, nutrient digestibility, and rumen microbial diversity. This study aimed to provide a valuable reference for the use of oxalic acid in ruminant nutrition, with potential application for improving fiber degradation and overall rumen efficiency.

2. Materials and Methods

2.1. Animals

Four healthy, quarantine-qualified male Small Tail Han sheep (36 ± 1.5 kg) with permanent rumen fistulae were housed individually, with regular fistula cleaning. They were fed daily at 8 am and 5 pm, with free access to clean water. All experiments were conducted at the College of Animal Science and Technology, Jilin Agricultural University, Changchun, China. All experimental procedures were performed in accordance with the Guidelines for the Care and Use of Experimental Animals of Jilin Agricultural University (JLAU-ACUC2024-011).

2.2. Experimental Design

A 2 × 7 full-factor experimental design with two factors was implemented, involving factors 1 and 2. Factor 1 consisted of two diets with different hemicellulose levels (10.3% and 17%), and Factor 2 was oxalic acid at seven different doses (0, 2.5, 5, 10, 20, 40, and 80 mg/kg DM), with 4 replicates in each batch, 3 batches in each group, and a total of 12 replicates. Samples were collected from three batches at three time points (0, 6, and 12 h). Subsequently, digestion parameters, fermentation parameters, and rumen contents were analyzed to determine the appropriate dose of oxalic acid to positively regulate rumen fermentation, based on previously published research [15]. Oxalic acid was obtained from the Rhawn Reagent Company (Shanghai, China).

2.3. In Vitro Experiment

Before morning feeding, rumen fluid was collected from the fixed rumen fistula using a rigid PVC tube (The diameter is 2 cm and the length is 80 cm) and quickly returned to the laboratory, where it was filtered with a multi-layer gauze. At the same time, each container is continuously injected with CO_2 for 30 s at a flow rate of 5 mL/s to maintain the appropriate amount of CO_2 to ensure an anaerobic environment for the rumen flora. According to the method of Menke and Steingass et al. [11], the filtered rumen fluid and the buffer were mixed at a 1:1 ratio. After the mixture was stirred well to create a uniform solution, CO_2 was continuously injected into the container containing the substrate and oxalic acid in advance, and the cap was tightly screwed. The container was placed in a gas bath shaking at a controlled temperature (39 °C, 80 rpm) for oscillation culturing, and the gas production was monitored and recorded using an ANKOM RF gas production system (ANKOM Technology Corp., Macedon, NY, USA).

2.4. Determination of Rumen Fermentation Parameters

The ANKOM RF gas production system was configured to measure the gas pressure (psi) and record the cumulative gas production of each ANKOM bottle. The GPM software (ANKOM GPM v11.4) gas production formula is:

$$Vy = Vx \times Ppsi \times 0.068004084 \quad (1)$$

where Vy represents the volume of gas generated (in mL). Vx stands for the volume of gas in the upper space of each bottle in mL; Ppsi denotes the cumulative pressure expressed in psi over a given period of time. Throughout this experiment, at each fermentation time point, a Sanxin MP 52304 digital pH meter (Shanghai Sanxin Instrumentation, Inc., Shanghai, China) was used according to the manufacturer's guidelines. The pH of all the bottles in a group was averaged to obtain the pH level based on fermentation time.

Using the method described by Chaney in 1962 [16], the ammonia nitrogen (NH_3-N) content of each test treatment was determined. A Cary series UV-visible spectrophotometer connected to a computer with Cary series analysis software (Cary WinUV Cary 60)(Agilent Technologies, Santa Clara, CA, USA) was used for this purpose.

The analysis of volatile fatty acids (VFA) and six major fatty acids (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid) was carried out using an Agilent 7890 A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) and a 50 m (internal diameter 0.32 mm) CP-Wax Chrompack silica-fused capillary column (Varian, Palo Alto, CA, USA). The initial and final oven temperatures were set at 65 and 190 °C, rising 20 °C per minute, split ratio: 50:1; Sample size: 1 μL [17].

After in vitro fermentation, the substrate was dried at 105 °C until absolutely dry; then, in vitro dry matter digestibility (IVDMD) was calculated. In vitro crude protein digestibility (IVCPD), in vitro ether extract digestibility (IVEED), and in vitro neutral detergent fiber digestibility (IVNDFD) were calculated using the formulas mentioned by Huang et al. [18], DeFeo et al. [19], and Goeser and Combs [20], respectively.

2.5. Determination of Rumen Microbial Diversity

Nucleic acids were extracted using an OMEGA Soil DNA Kit (D5625-01) (Omega Bio-Tek, Norcross, GA, USA). DNA was quantified using an ultraviolet spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), and the highly variable V3V4 region of the bacterial 16s rDNA gene was used as a template for sequencing. Specific primers F, ACTCCTACGGGAGGCAGCA, R, and GGACTACHVGGGTWTCTAAT were selected. NEBQ5 DNA high-fidelity polymerase was used for PCR amplification, and the PCR products were quantified on an FLx800 microplate reader (BioTek, Winooski, VT, USA) using a Quant-iT PicoGreen dsDNA Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and prepared according to the volume of the sample. The TruSeq Nano DNA LT library (Illumina, San Diego, CA, USA) was used to construct the database; then the Agilent Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, USA) was used to perform quality control of the eligible libraries. Double-terminal sequencing was performed using the NovaSeq 6000 SP kit (500 cycles, Illumina, San Diego, CA, USA) [21].

2.6. Determination of Rumen Metabolites

After slow thawing at 4 °C, an appropriate amount of methanol/acetonitrile/aqueous solution (with acetonitrile and water in a =1:1 volume ratio) was added to the samples. The mixture was then subjected to vortex stirring and ultrasound treatment at a low temperature for 30 min. Subsequently, the samples were left to stand at −20 °C for 10 min and centrifuged at 4 °C for 20 min at a speed of 14,000× *g*. The supernatant was vacuum dried, and 100 μL of acetonitrile aqueous solution (with acetonitrile and water in a 1:1 volume ratio) was added for redissolving. After swirling, centrifugation was performed again at 14,000× *g* for 15 min at 4 °C. Finally, the supernatant was collected for further analysis.

The samples were separated using an HILIC column with the 1290 ultra-high performance liquid chromatography system (Agilent). The samples were automatically injected at 4 °C. To avoid fluctuations in the instrument detection signal, a random sequence method

was employed for continuous analysis of the samples, and quality control samples were inserted into the sample queue to monitor and evaluate the system stability and analyze the reliability of the experimental data. Primary and secondary spectral peaks were extracted using a mass spectrometer (AB Sciex, Framingham, MA, USA). The samples were separated using ultra-high-performance liquid chromatography (UHPLC) and detected using a TripleTOF-6600 mass spectrometer (AB Sciex, Framingham, MA, USA) with electrospray ionization (ESI).

2.7. Data Analysis

2.7.1. Rumen Fermentation Parameters

The general linear model of the IBM SPSS Statistics 23 software was used to analyze the relationship between two factors. The statistical analysis model (Yijk= μ + Fi + Vj + Fi × Vj + eijk) was used to analyze the dietary and oxalic acid supplemental doses at different hemicellulose levels and their interaction effects. Here, μ represents the population average, where Fi denotes the dietary effect of different hemicellulose levels (i = 1–2) and Vj is the supplemental dose of oxalic acid (j = 1–7). Fi × Vj represents the interaction effect between dietary hemicellulose level and oxalic acid dosage; eijk indicates the residual error. Duncan's multiple comparison method was used to test the significance of factors 1 and 2 and their interactions (factor 1 × factor 2). $p < 0.05$ was considered significant and $p < 0.01$ was considered an extremely significant difference.

2.7.2. Rumen Microbial Diversity

QIIME2 v2019.4 https://qiime2.org/ (accessed on 20 May 2023) and the R software version 3.2.0 (R Foundation for Statistical Computing, Vienna, Austria) were used to analyze the sequencing results. The demux plug-in was employed for the multiplex decomposition of the original sequence. The cutadapt plug-in was used for primer cutting. The DADA2 plug-in was used for quality filtering, denoising, splicing, and elimination of mosaics. The Alpha diversity index was calculated using the ASV table in QIIME2. The Jaccard index, Bray–Curtis index, and UniFrac distance were used to quantify beta diversity. Principal component analysis was used to visualize the samples. Linear discriminant analysis effect size (LEfSe) was used to measure the differences in microorganisms among the groups. Based on the MetaCyc https://metacyc.org/ (accessed on 20 May 2023) and KEGG (https://www.genome.jp/kegg/pathway.html, accessed on 22 May 2023) databases, functional prediction was performed using PICRUSt2 (https://huttenhower.sph.harvard.edu/picrust/, accessed on 22 May 2023).

2.7.3. Rumen Metabolites

ProteoWizard software (V3.0.8789)was employed to convert the original data into the mzXML format, which was used for the alignment and retention of peaks. Peak areas were extracted and corrected for time. The structures of the metabolites were initially identified from the data obtained by XCMS(V3.12.0), and the data were preprocessed. Finally, quality evaluation and analysis of the experimental data were performed. Lastly, Mothur software (mothur v.1.44.0) was used to calculate the metabolome data and Spearman correlation coefficients between microorganisms and bacteria. Based on the Spearman correlation coefficient matrix results, (the rho correlation coefficient is between −1 and 1; when −1 < rho < 0, the two are negatively correlated; when 0 < rho <1, the two are positively correlated. When rho = 0, there is no correlation between the two), and R software (V4.0.3)was used for heat mapping.

3. Results

3.1. Nutritional Digestibility In Vitro

After 6 h of in vitro fermentation, the IVDMD of the treatment groups supplemented with oxalic acid was significantly higher than that of the control group when the diet contained low hemicellulose (HM10.3%) levels ($p < 0.05$; Table 1). When the fermentation time was extended to 12 h, the IVDMD of the treatment groups supplemented with 2.5, 5, 10, and 20 mg/kg DM oxalic acid at the low hemicellulose (HM10.3%) level also surpassed that of the control group. However, IVDMD was lower in the groups treated with 40 and 80 mg/kg DM oxalic acid than that in the control group. In the treatment groups with the high hemicellulose (HM17%) diet, the IVDMD in the 5 mg/kg DM oxalic acid group was significantly greater than that in the control group ($p < 0.05$; Table 1).

Different levels of hemicellulose and oxalic acid significantly affected rumen IVCPD, IVEED, and IVNDFD in vitro, and the interaction between hemicellulose levels and oxalic acid dose also significantly affected these three parameters ($p < 0.05$). After 6 h of fermentation, the IVCPD values in the oxalic acid supplementation groups were greater than that of the control group under the HM10.3% diet. However, under the HM17% diet, the IVCPD values in the 20 and 80 mg/kg DM oxalic acid supplementation groups were significantly lower than that of the control group. The IVCPD values of the 2.5, 20, 40, and 80 mg/kg DM oxalic acid-supplementation groups were significantly lower than that of the control group under the HM17% diet ($p < 0.05$; Table 1). After 12 h of fermentation, the IVCPD of the 10 and 80 mg/kg DM oxalic acid supplementation groups was significantly greater than that of the control group under the HM10.3% diet ($p < 0.05$; Table 1).

At the HM17% level, the IVEED of the 80 mg/kg DM oxalic acid supplementation group was significantly greater than those of the 2.5, 10, 20, 40 mg/kg DM oxalic acid supplementation and the control groups ($p < 0.05$) after 6 h of fermentation. With an increase in fermentation time, the IVEED of all oxalic acid supplementation groups was significantly less than that of the control group at 12 h of fermentation with low hemicellulose levels ($p < 0.05$). The IVEED of the 2.5 and 10 mg/kg DM oxalic acid supplementation groups at the HM17% level was significantly greater than those of the other oxalic acid treatment groups and the control group ($p < 0.05$; Table 1).

At the HM10.3% level, after 6 h of fermentation, the IVNDFD in the groups supplemented with 2.5, 5, and 80 mg/kg DM oxalic acid was significantly greater than those of the other oxalic acid treatments and the control group ($p < 0.05$). The IVNDFD values of the groups supplemented with 2.5, 5, 10, and 20 mg/kg DM oxalic acid were significantly higher than those of the groups supplemented with 40 and 80 mg/kg DM oxalic acid, as well as the control group after 12 h of fermentation ($p < 0.05$). At the HM17% level, the group supplemented with 5 mg/kg DM oxalic acid had a higher IVNDFD value than the groups supplemented with 2.5, 10, 20, and 40 mg/kg DM oxalic acid, and the control ($p < 0.05$; Table 1).

Table 1. Effects of oxalic acid (OA) supplementation in different hemicellulose (HM) diets on in vitro dry matter digestibility (IVDMD), crude protein digestibility (IVCPD), crude fat digestibility (IVEED), and neutral detergent fiber digestibility (IVNDFD) in vitro. This table recorded in vitro fermentation for 6 h and 12 h after adding oxalic acid at different doses (0, 2.5, 5, 10, 20, 40, 80 mg/kg DM) under the conditions of low (HM10.3%) and high (HM17%) hemicellulosic diet, respectively.

		HM10.3%							HM17%								*p*-Value		
Item (%)	**Time (h)**	**0 mg/kg**	**2.5 mg/kg**	**5 mg/kg**	**10 mg/kg**	**20 mg/kg**	**40 mg/kg**	**80 mg/kg**	**0 mg/kg**	**2.5 mg/kg**	**5 mg/kg**	**10 mg/kg**	**20 mg/kg**	**40 mg/kg**	**80 mg/kg**	**SEM**	**HM**	**OA**	**HM × OA**
IVDMD	6	28.29	30.90	30.07	30.59	30.24	30.03	29.24	29.84	28.87	8.99	27.94	28.24	29.56	26.59	0.27	0.01	0.49	0.33
	12	35.87	35.88	36.63	36.69	36.94	35.78	35.60	34.28	33.46	34.84	33.96	33.72	34.04	33.91	0.28	<0.01	0.95	0.98
IVCPD	6	23.10 ^{c}	30.77 ^{a}	29.83 ^{ab}	29.41 ^{ab}	30.93 ^{a}	31.45 ^{a}	27.23 ^{b}	33.60 ^{a}	31.01 ^{ab}	31.97 ^{a}	31.08 ^{ab}	27.22 ^{bc}	32.37 ^{a}	25.08 ^{c}	0.34	<0.01	<0.01	<0.01
	12	38.67 ^{b}	38.39 ^{b}	38.28 ^{b}	43.89 ^{a}	40.72 ^{ab}	40.70 ^{ab}	45.08 ^{a}	40.47 ^{a}	36.75 ^{b}	40.44 ^{a}	37.72 ^{ab}	35.36 ^{b}	37.01 ^{b}	35.74 ^{b}	0.34	<0.01	0.01	<0.01
IVEED	6	18.63 ^{cd}	27.37 ^{a}	19.24 ^{c}	18.08 ^{d}	20.98 ^{b}	28.10 ^{a}	16.81 ^{e}	27.89 ^{c}	30.69 ^{b}	31.57 ^{ab}	23.36 ^{d}	21.59 ^{e}	30.87 ^{b}	31.96 ^{a}	0.47	<0.01	<0.01	<0.01
	12	29.05 ^{a}	23.25 ^{c}	23.65 ^{c}	25.63 ^{b}	26.50 ^{b}	18.50 ^{d}	26.43 ^{b}	29.10 ^{c}	31.37 ^{b}	16.84 ^{f}	37.82 ^{a}	29.33 ^{c}	24.86 ^{e}	27.48 ^{d}	0.44	<0.01	<0.01	<0.01
IVNDFD	6	12.24 ^{c}	15.43 ^{a}	15.31 ^{a}	13.42 ^{b}	12.62 ^{bc}	12.67 ^{bc}	14.53 ^{a}	7.53 ^{ab}	8.51 ^{a}	6.05 ^{ab}	6.47 ^{ab}	7.14 ^{ab}	7.99 ^{ab}	4.79 ^{b}	0.36	<0.01	<0.01	<0.01
	12	21.53 ^{d}	22.31 ^{c}	22.92 ^{c}	23.59 ^{b}	24.62 ^{a}	21.07 ^{d}	20.30 ^{e}	12.10 ^{bc}	12.59 ^{bc}	16.74 ^{a}	13.41 ^{bc}	11.25 ^{c}	11.09 ^{c}	14.71 ^{ab}	0.45	<0.01	<0.01	<0.01

^{a,b,c,d,e,f}, Different superscripts in the same row imply that their mean values are significantly different ($p < 0.05$), and the value decreases from a to f.

3.2. Rumen Fermentation Parameters

The effects of dietary hemicellulose levels and oxalic acid supplementation on rumen gas production and ammonia nitrogen concentration in vitro differed significantly between the groups ($p < 0.01$). Gas production over 6 h of in vitro fermentation in both hemicellulose groups supplemented with 40 mg/kg DM oxalic acid was higher than that in the other oxalic acid supplementation groups and the control ($p < 0.05$). After 12 h of fermentation, the gas production of the 40 mg/kg DM oxalic acid group was lower than that of all other oxalic acid dosage groups at both dietary hemicellulose levels ($p < 0.05$; Table 1). During in vitro fermentation for 6 h, at the HM10.3% level, the ammonia nitrogen concentration of the 5 mg/kg DM oxalic acid group was significantly higher than that of the 20 and 40 mg/kg DM oxalic acid and control groups ($p < 0.05$); at the HM17% level, the ammonia nitrogen concentrations of the 10 and 20 mg/kg DM oxalic acid supplementation groups were significantly higher than those of 2.5, 5, 40, and 80 mg/kg DM oxalic acid groups ($p < 0.05$; Table 2).

As the fermentation time increased, acid production gradually increased, and the pH of all groups continued to decrease. The effects of hemicellulose levels on rumen pH and total volatile fatty acid (TVFA) concentrations in vitro were extremely significant. The concentration of TVFA was significantly affected by the interaction between hemicellulose level and oxalic acid dosage ($p < 0.01$). After 6 h of in vitro fermentation, the pH values of all oxalic acid groups were lower than that of the control group at the HM10.3% level. The pH values of the 40 and 80 mg/kg DM oxalic acid groups were significantly lower than those of the other oxalic acid dosages and the control group ($p < 0.05$). At the HM17% level, the pH values of the 10 and 80 mg/kg DM oxalic acid groups decreased as time increased. When the fermentation time reached 12 h, there was no significant difference among the groups at the HM10.3% level ($p > 0.05$). At the HM17% level, the pH values of the 10 and 80 mg/kg DM oxalic acid groups decreased as time passed and were significantly lower than those of the other oxalic acid dosages and the control group ($p > 0.05$; Table 2).

At the HM10.3% level, the TVFA concentration in the 10 mg/kg DM oxalic acid group was significantly higher than that in the other oxalic acid groups and the control ($p < 0.05$). After 6 h of fermentation (Figure 1A), the TVFA concentration in the oxalic acid groups was significantly higher than that in the control group ($p < 0.05$). At the HM17% level, the TVFA concentration in the 10 and 20 mg/kg DM oxalic acid groups was significantly higher than in the other oxalic acid groups and the control group ($p < 0.05$). After 12 h of fermentation (Figure 1B), the TVFA concentration in the 80 mg/kg DM oxalic acid group was significantly higher than that in the other oxalic acid groups and the control ($p < 0.05$). Additionally, the TVFA concentration in the 2.5 mg/kg DM oxalic acid group also increased with time and was significantly higher than the control group ($p > 0.05$; Table 2).

Table 2. Effects of oxalic acid (OA) supplementation in different hemicellulose (HM) diets on rumen fermentation parameters in vitro. This table shows the determination results of ammonia nitrogen (NH_3-N) concentration, Gas PROD, pH, and total volatile fatty acid (TVFA) concentration during in vitro fermentation at 6 h and 12 h after adding different doses of oxalic acid to diets with different hemicellulose levels. The units of each parameter are mg/dL, mL, none (pH value), and mmol/L, respectively. The data processing and significance test methods were the same as in Table 1—that is, Duncan's multiple comparison method was used to judge the significance of differences, and standard error (SEM) was also listed on the right side of the table, which provided an important reference for evaluating data reliability and inter-group differences, and helped to further understand the effects of oxalic acid addition and hemicellulose level on rumen fermentation parameters.

		HM10.3%							HM17%								*p*-Value		
Item	**Time (h)**	**0 mg/kg**	**2.5 mg/kg**	**5 mg/kg**	**10 mg/kg**	**20 mg/kg**	**40 mg/kg**	**80 mg/kg**	**0 mg/kg**	**2.5 mg/kg**	**5 mg/kg**	**10 mg/kg**	**20 mg/kg**	**40 mg/kg**	**80 mg/kg**	**SEM**	**HM**	**OA**	**HM × OA**
NH_3-N	6	17.97 ^{d}	19.77 ^{abc}	21.08 ^{a}	20.32 ^{ab}	18.50 ^{cd}	18.91 ^{bcd}	20.20 ^{ab}	22.06 ^{ab}	20.47 ^{c}	19.60 ^{c}	23.46 ^{a}	22.52 ^{a}	19.96 ^{c}	20.94 ^{bc}	0.18	<0.01	<0.01	<0.01
(mg/dL)	12	28.70 ^{a}	26.05 ^{b}	28.23 ^{a}	22.87 ^{c}	23.12 ^{c}	24.30 ^{c}	23.80 ^{c}	25.04	25.85	25.33	25.38	25.09	25.63	25.63	0.19	0.672	<0.01	<0.01
Gas PROD	6	50.94 ^{cd}	57.26 ^{bc}	61.34 ^{b}	59.95 ^{b}	46.76 ^{d}	135.12 ^{a}	51.8 ^{cd}	65.2 ^{d}	67.45 ^{d}	64.45 ^{d}	79.36 ^{c}	101.25 ^{b}	129.86 ^{a}	108.92 ^{b}	4.48	<0.01	<0.01	<0.01
(mL)	12	100.05 ^{d}	153.35 ^{b}	170.94 ^{a}	149.92 ^{b}	113.46 ^{c}	108.31 ^{cd}	118.39 ^{c}	85.04 ^{c}	152.92 ^{a}	113.24 ^{b}	136.33 ^{a}	112.06 ^{b}	93.73 ^{c}	145.5 ^{a}	4.04	<0.01	<0.01	<0.01
pH	6	7.10 ^{a}	7.07 ^{ab}	7.06 ^{ab}	7.04 ^{ab}	7.04 ^{ab}	7.01 ^{b}	7.01 ^{b}	6.95	6.96	6.95	6.95	6.90	6.91	6.95	0.02	<0.01	0.89	0.99
value	12	6.88	6.83	6.80	6.80	6.81	6.81	6.77	6.73	6.75	6.71	6.69	6.71	6.72	6.69	0.02	<0.01	0.94	0.99
TVFA	6	43.45 ^{f}	69.77 ^{d}	64.53 ^{e}	94.61 ^{a}	76.85 ^{c}	80.75 ^{b}	78.03 ^{c}	78.38 ^{d}	76.79 ^{d}	84.81 ^{bc}	96.19 ^{a}	94.00 ^{a}	86.36 ^{b}	82.78 ^{c}	1.14	<0.01	<0.01	<0.01
(mmol/L)	12	118.36 ^{d}	135.83 ^{b}	138.92 ^{b}	144.61 ^{a}	115.59 ^{d}	127.38 ^{c}	117.32 ^{d}	107.10 ^{d}	114.62 ^{bc}	107.33 ^{d}	119.02 ^{b}	112.33 ^{cd}	110.32 ^{cd}	29.06 ^{a}	1.09	<0.01	<0.01	<0.01

^{a,b,c,d,e,f}, Different superscripts in the same row imply that their mean values are significantly different ($p < 0.05$), and the value decreases from a to f.

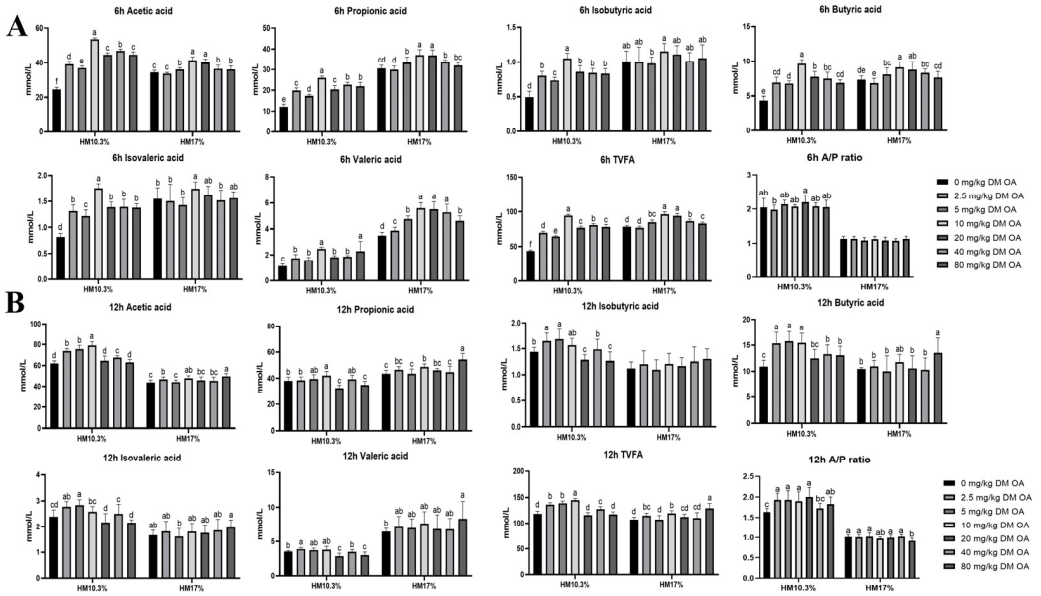

Figure 1. Effects of oxalic acid (OA) supplementation (0, 2.5, 5, 10, 20, 40, and 80 mg/kg DM) in low (HM10.3%) and high (HM17%) hemicellulose diets on acetic acid, propionic acid, butyric acid, isobutyric acid, isovaleric acid, valeric acid, total volatile fatty acids and the ratio of acetic acid to propionic acid production (mmol/L) in vitro for 6 h (**A**) and 12 h (**B**). Different superscripts in figure imply that their mean values are significantly different ($p < 0.05$).

In the hemicellulose diets supplemented with 10 mg/kg DM oxalic acid, the concentrations of acetic acid, propionic acid, butyric acid, and total acid were significantly higher than those in the other treatment and control groups ($p < 0.05$; Figure 1). Under the HM10.3% diet, oxalic acid supplementation promoted propionic acid fermentation, enhanced propionic acid production, and decreased ethylene to propylene ratio, which potentially improved rumen fermentation function and enhanced VFA composition potential.

3.3. Microbial Diversity In Vitro

3.3.1. Ruminal Bacterial Alpha Diversity Indexes

Pielou's evenness, Shannon, and Simpson indexes were significantly affected by the interactive effects of different hemicellulose levels in the diet and the addition of oxalic acid ($p < 0.01$; Figure 2C). Adding 10 mg/kg DM of oxalic acid to a diet with an HM10.3% had no significant effect on the alpha diversity index of rumen microbes ($p > 0.05$). Adding 10 mg/kg DM of oxalic acid to the diet with HM17% significantly reduced Pielou's evenness, Shannon, and Simpson indexes ($p < 0.01$). Adding no oxalic acid to the diet at both hemicellulose levels in both groups had no significant effect on these indexes. The HM17% group with 10 mg/kg DM of oxalic acid had significantly higher Pielou's evenness and Shannon index values than the HM10.3% group with added oxalic acid ($p < 0.05$). Adding 10 mg/kg DM of oxalic acid to both hemicellulose groups significantly reduced the Pielou's evenness, Shannon, and Simpson indexes of the HM17% group compared to that of the HM10.3% group ($p < 0.05$). In this study, the addition of oxalic acid at the high hemicellulose level reduced the diversity of rumen microbial communities.

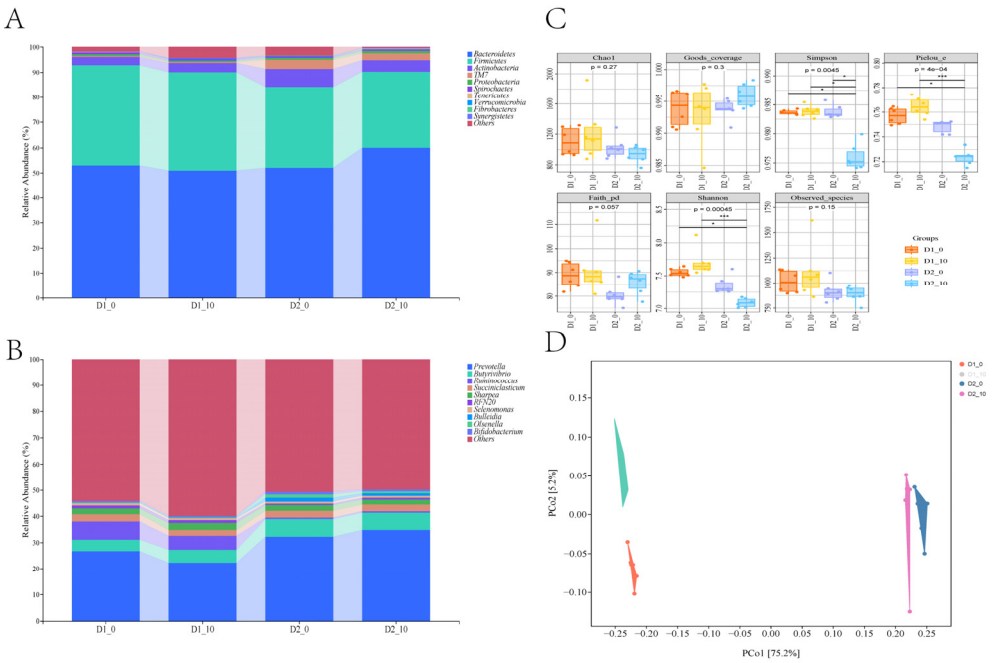

Figure 2. Effects of oxalic acid (OA) supplementation in different hemicellulose (HM) diets on rumen microorganisms in vitro. (**A**): Represents the relative abundance distribution of bacteria at the door level after in vitro fermentation, and each column height represents the relative proportion of different treatment groups to the door. Color distinguishes different doors. (**B**): Relative abundance of in vitro fermentation bacteria at the generic level. The width of each bar chart is the same, and the length indicates the relative content of different genera in different treatment groups. (**C**): The α diversity index of 12 h in vitro fermentation was given, including the Pielou evenness index, Shannon index, and Simpson index. In the diagram, the different shapes and colors of the points represent different treatment groups, and the positions of the points on the axis reflect the Alpha diversity index value of the corresponding treatment group. By comparing the point distribution between different groups, the difference in microbial community diversity under different treatment conditions can be directly judged. (**D**): Visualize beta diversity using methods such as principal component analysis (PCA). Each point in the diagram represents a different sample, and the distance between the points indicates the similarity in the composition of the microbial communities between the samples. The closer the distance, the more similar the microbial community structure between the samples and the greater the difference.

3.3.2. Ruminal Bacterial Phylum Composition

The top ten phyla in relative abundance were Bacteroidetes, Firmicutes, Actinobacteria, phylum TM7, Proteobacteria, Spirochaetes, Tenericutes, Verrucomicrobia, Fibrobacteres, and Synergistetes. Bacteroidetes, Firmicutes, and Actinobacteria were the dominant phyla, each with relative abundances greater than 1% (Figure 2A).

Adding 10 mg/kg DM of oxalic acid at the HM10.3% level significantly affected the relative abundances of *Bacteroidetes* and *Proteobacteria* during in vitro fermentation of rumen contents ($p < 0.05$). The addition of 10 mg/kg DM of oxalic acid at the high hemicellulose level significantly affected the relative abundance of *Bacteroidetes*, *Actinobacteria*, *Phylum TM7*, *Spirochaetes*, and *Tenericutes* during in vitro rumen content fermentation ($p < 0.05$). The addition of oxalic acid significantly affected the relative abundances of *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Phylum TM7*, *Proteobacteria*, *Spirochaetes*, *Tenericutes*, *Verrucomicrobia*, and *Fibrobacteres* during in vitro rumen fermentation at both hemicellulose levels ($p < 0.05$). The relative abundances of Bacteroidetes, Actinobacteria, and Phylum TM7 were significantly affected by the interaction between oxalic acid and different hemicellulose levels ($p < 0.01$). After 12 h of in vitro rumen fermentation, adding 10 mg/kg DM of oxalic

acid at the HM10.3% level significantly reduced the relative abundances of *Bacteroidetes* and *Proteobacteria* compared with those of the control group ($p < 0.05$). Adding 10 mg/kg DM of oxalic acid at the HM17% level significantly increased the relative abundances of *Bacteroidetes*, *Spirochaetes*, and *Tenericutes* compared to those of the control group. The relative abundances of *Firmicutes*, *Spirochaetes*, and *Verrucomicrobia* in the HM10.3% groups were significantly higher than those in the HM17.0% groups ($p < 0.05$), with the relative abundance of Firmicutes differing by up to 8.9%.

3.3.3. Rumen Bacterial Genus Composition

The top ten microbial genera were *Prevotella*, *Butyrivibrio*, *Ruminococcus*, *Succiniclasticum*, *Sharpea*, *RFN20*, *Selenomonas*, *Bulleidia*, *Olsenella*, and *Bifidobacterium* (Figure 2B). With relative abundances greater than 1%, the dominant bacterial genera also varied depending on the dietary level of hemicellulose and the amount of oxalic acid added. During the 12-h rumen fermentation outside the body, there were five dominant bacterial genera in the HM10.3% group with 10 mg/kg DM of oxalic acid added, namely, *Prevotella*, *Butyrivibrio*, *Ruminococcus*, *Succiniclasticum*, and *Sharpea*. Similarly, there were five dominant bacterial genera in the HM17% group with 10 mg/kg DM of oxalic acid added, namely *Prevotella*, *Butyrivibrio*, *Succiniclasticum*, *Sharpea*, and *Bulleidia*.

Adding 10 mg/kg DM of oxalic acid to the HM10.3% level significantly affected the relative abundances of *Prevotella*, *Ruminococcus*, and *Selenomonas* during in vitro rumen fermentation ($p < 0.05$). Adding 10 mg/kg DM of oxalic acid to the HM17% diet significantly affected the relative abundances of *Prevotella*, *Olsenella*, and *Bifidobacterium* during in vitro rumen fermentation ($p < 0.05$). The addition of 10 mg/kg DM of oxalic acid to both hemicellulose levels significantly affected the relative abundances of *Prevotella*, *Butyrivibrio*, *Ruminococcus*, *Sharpea*, *RFN20*, *Bulleidia*, *Olsenella*, and *Bifidobacterium* during in vitro rumen fermentation ($p < 0.05$). *Prevotella*, *Ruminococcus*, *Bulleidia*, and *Olsenella* were significantly affected by the interaction between oxalic acid addition and hemicellulose level ($p < 0.01$). Compared to the control group, adding 10 mg/kg DM of oxalic acid to the HM10.3% significantly affected the relevant parameters.

3.4. The Relationship Between Rumen Fermentation Parameters and Bacterial Genus Composition

By analyzing the correlation between the top ten genera and rumen fermentation parameters under different levels of hemicellulose, we examined whether oxalic acid promoted or inhibited the relationship between them. A 12-h in vitro simulation of rumen fermentation was conducted at the HM10.3% level in the diet (Figure 3A,B). In the control group, *Sharpea* showed a significant positive correlation with acetic acid and total acid levels ($p < 0.05$), and a moderate positive correlation with NH_3-N ($p < 0.05$). *RFN20* showed a significant positive correlation with NH_3-N ($p < 0.05$). *Olsenella* showed a significant positive correlation with acetic acid concentration ($p < 0.05$). In the group treated with 10 mg/kg DM of oxalic acid, *Butyrivibrio* showed a significant negative correlation with total acid ($p < 0.05$) and a complex correlation with IVNDFD ($p < 0.05$). *Sharpea* also showed a significant positive correlation with the IVNDFD ($p < 0.05$). *Ruminococcus* showed a significant negative correlation with propionic acid and total acid, and a complex correlation with the acid-to-propionate ratio ($p < 0.05$). In the diet with HM17% (Figure 3C,D), the control group showed a significant negative correlation between *Butyrivibrio* and methane production ($p < 0.05$) and a moderate negative correlation between *Sharpea* and methane production ($p < 0.05$). A significant negative correlation was found between *Selenomonas* and the acid-to-propionate ratio ($p < 0.05$), and a moderate positive correlation between *Olsenella* and propionic acid ($p < 0.05$). *Prevotella* showed a significant negative correlation with the isobutyric acid content. *Bifidobacterium* showed a significant positive correlation

with the total acid content ($p < 0.05$) in the control group. The correlation between genera and fermentation parameters was more complex in the groups treated with 10 mg/kg DM oxalic acid.

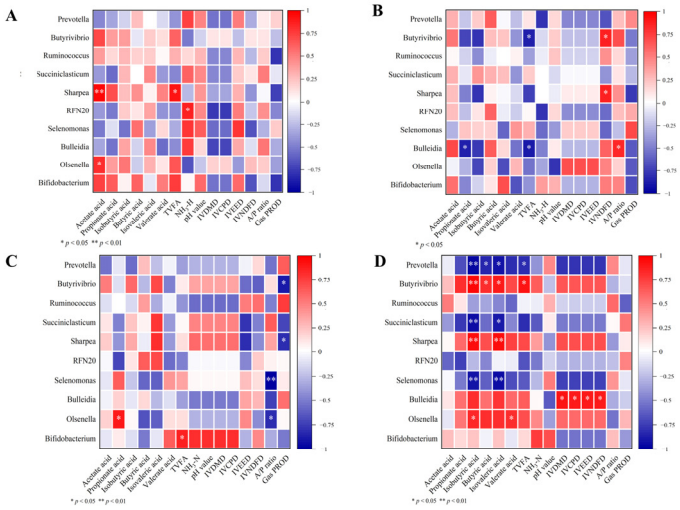

Figure 3. The correlation between rumen fermentation parameters and bacterial genus composition. (**A**,**B**): Correlation analysis results of rumen microorganisms and fermentation parameters under low hemicellulose (HM10.3%) level and oxalic acid supplemental level. In the figure, bacterial genera are taken as horizontal coordinates, fermentation parameters are taken as vertical coordinates, and the correlation between them is expressed in the form of scatter plots and straight lines. The thickness of the lines and the depth of the colors indicate the strength of the correlation. The value next to this line is Spearman correlation coefficient (**C**,**D**): Correlation analysis of rumen microorganisms and fermentation parameters when oxalic acid is added to a high hemicellulose (HM17%) level. The specific expression method and analytical significance are the same as those in (**A**,**B**).

3.5. Rumen Metabolites In Vitro

Based on the results of the fermentation parameters, the groups supplemented with 10 mg/kg DM in diets with different hemicellulose levels for 12 h were selected for rumen metabolite group analysis. A total of 795 metabolites were detected. Among these, organic acid heterocyclic compounds accounted for the highest proportion, accounting for 21.1% of total metabolites. The other metabolite types accounted for less: 20.9% were attributed to organic acids and their derivatives, 16.5% to phenyl compounds, 6.5% to organic nitrogen compounds, 6.5% to organic oxygen compounds, and 2.6% to nucleosides, nucleotides, and analogs. Lastly, the polyketones of phenylpropane accounted for 2.4%, and hydrocarbons accounted for 1.6% (Figure 4A).

A variety of differential metabolites (standard FC > 1, $p < 0.01$, VIP > 1.5) were screened after 12 h of in vitro fermentation. Compared to oxalic acid added to the HM10.3% diet, the differential metabolites (+)-.alpha.-tocopherol and Maltotriose were significantly down-regulated. Under the HM17% diet supplemented with oxalic acid, differential metabolites Trp-Cys-Lys, 13-keto-9z, and 11e-octadecadienoic acid were significantly upregulated, while 2-pyridinecarboxylic, glycocholic acid, and other substances were significantly down-regulated. Supplementation with 10 mg/kg DM oxalic acid in both hemicellulose diets was compared with the HM10.3% and 10 mg/kg DM oxalic acid group. The differential metabolites of Maltotriose, L-carnitine, Atrazine desisopropyl, and Ginkgolide B in the HM17% and 10 mg/kg DM oxalic acid group were detected, and other substances were significantly up-regulated (Figure 4B–E).

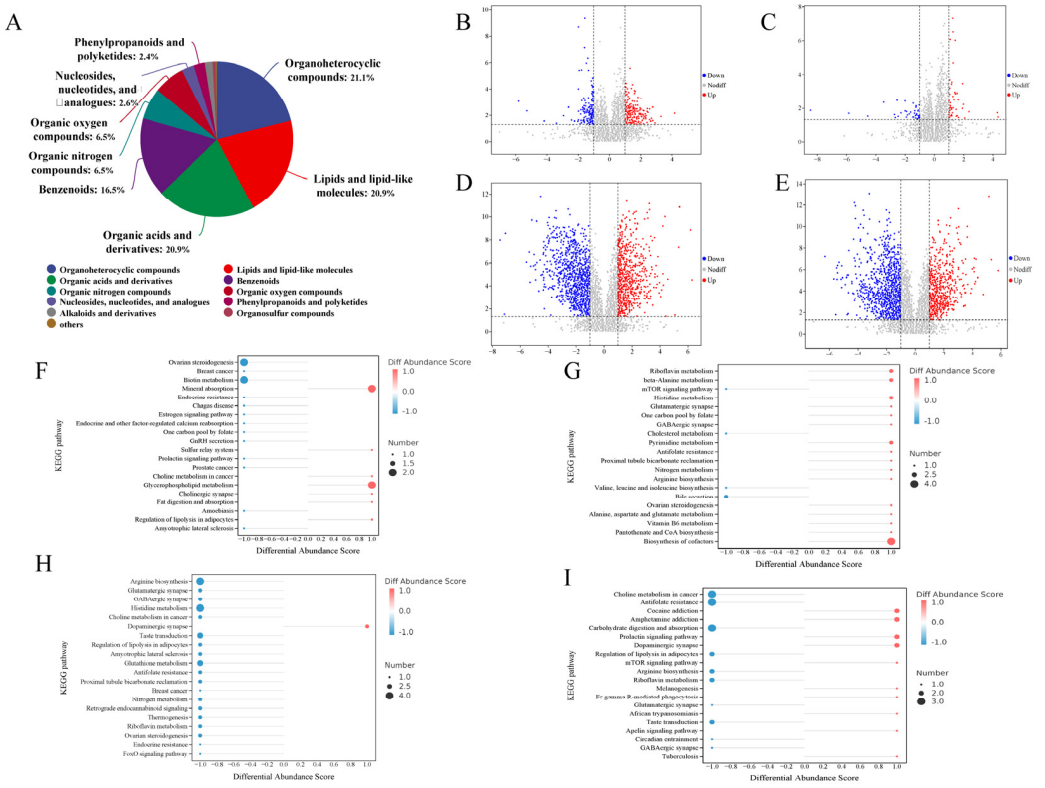

Figure 4. Effects of oxalic acid (OA) supplementation in different hemicellulose (HM) diets on rumen metabolites in vitro. (**A**) The statistical classification of metabolites in the rumen was presented. The detected metabolites were divided into organic acid heterocyclic compounds, organic acids and their derivatives, etc., and the size of each fan region indicated the proportion of metabolites in the total metabolites. (**B**,**F**): Results of differential metabolites and metabolic pathway analysis were presented when oxalic acid was added at a low HM level. The figure shows the relative content changes of differential metabolites in the form of bar charts. Up-regulated metabolites are represented by red bars, down-regulated metabolites are represented by blue bars, and the names and change multiples of metabolites are marked above the bar charts. At the same time, the main metabolic pathways involved in differential metabolites were shown through the metabolic pathway network diagram. Nodes represented metabolites or enzymes, lines represented metabolic reaction relationships, and the thickness of lines represented the relative size of the metabolic flow. (**C**,**G**): The analysis of differential metabolites and metabolic pathways when oxalic acid is added at a high HM level is shown. The specific expression method and analytical significance are the same as those in (**B**,**F**). The metabolic changes under different hemicellulose levels are compared. (**D**,**H**): The analysis results of different metabolites and metabolic pathways at high and low HM levels without oxalic acid were presented, providing a control for the study of the effects of oxalic acid addition or not on rumen metabolism, and further clarifying the role of oxalic acid in rumen metabolism. (**E**,**I**): The analysis of different metabolites and metabolic pathways when oxalic acid was added at both high and low HM levels was demonstrated.

3.6. Rumen Metabolic Pathway

According to the *p* and FDR values, several KEGG metabolic pathways were significantly enriched. After 12 h of in vitro fermentation, the differential metabolite enrichment pathways between the HM10.3% and 10 mg/kg DM group and the control group were analyzed. This showed that the differential metabolites were mainly involved in mineral absorption and glycerophospholipid metabolism. The metabolite enrichment pathways of the HM17% and 10 mg/kg DM oxalic acid group compared to the control group were mainly concentrated in riboflavin metabolism, beta-alanine metabolism, histidine metabolism, pyrimidine metabolism, and biosynthesis of cofactors (Figure 4F–I).

3.7. The Correlation Between Rumen Metabolites and Rumen Bacterial

Correlations between the top 50 metabolites and the different bacteria were analyzed. In the HM10.3% diet supplemented with 10 mg/kg DM oxalic acid, ruminal metabolites turkesterone, and salicylaldehyde were significantly positively correlated with *Prevotella*, *Ruminococcus,* and *Succinolytica* relative abundances ($p < 0.05$). When 10 mg/kg DM oxalic acid was added to the HM17% diet, rumen metabolites simazine hydroxy and pirimiphos-ethyl were significantly positively correlated with *Lactococcus lactis* and *Aminococcus aminoethyl* ($p < 0.05$). When the two diets were supplemented with 10 mg/kg DM oxalic acid, the vitamin B5 and pelanoic acid contents in the rumen metabolites of the HM17% diet were significantly positively correlated with Ruminococcus and RNF20 bacteria ($p < 0.05$; Figure 5A,B).

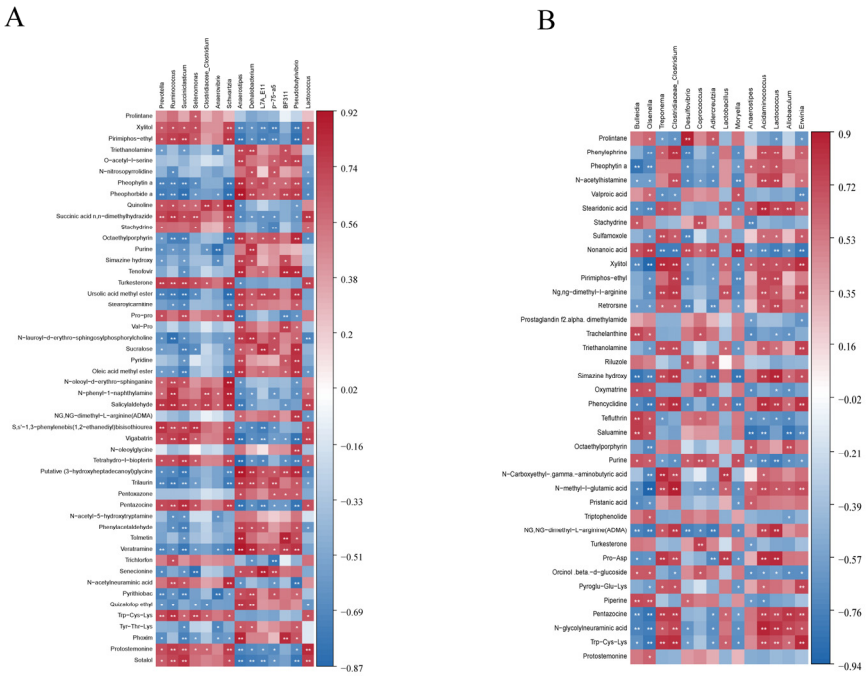

Figure 5. The correlation between rumen metabolites and bacterial genus composition (**A**): The correlation analysis results between rumen metabolites and bacterial genus composition were demonstrated when 10 mg/kg DM oxalic acid was added to a diet with low hemicellulose level. It is presented in the form of a heat map. The depth of color in the figure indicates the strength of the correlation; red indicates a positive correlation, blue indicates a negative correlation, and the darker the color, the stronger the correlation. (**B**): The correlation analysis between rumen metabolites and bacterial genera was presented when 10 mg/kg DM oxalic acid was added at a high hemicellulose level. The specific expression method and analytical significance were the same as in (**A**). ** Indicates that $p < 0.001$, and * indicates when $0.05 \geq p \geq 0.001$.

4. Discussion

Maintaining optimal rumen pH is crucial for rumen performance, as well as overall health and ruminant nutrition [22]. Decreased rumen pH and subacute rumen acidosis (SARA) are common challenges [23], especially in intensive sheep production systems. In this study, we found that oxalic acid supplementation with hemicellulose levels of different diets had little effect on rumen pH, which was consistent with the results of previous studies. For example, in the study of Belenguer [24], by injecting 0.6 mmol/kg of oxalic acid into rumen fistulas of sheep every day and monitoring rumen pH during several hours of feeding, it was found that the addition of oxalic acid had no significant effect on rumen pH, which was consistent with our experimental results. In addition, in

another study by Cao [7], when exploring the effect of oxalic acid on rumen fermentation in sheep, the pH will only change when the dosage of oxalic acid is 1% or 2% of the raw material, indicating that the effect of rumen pH is limited when the dosage of oxalic acid is small. These results imply that oxalic acid supplementation at different dietary hemicellulose levels does not adversely affect the rumen pH in sheep. This observation indicates that it can be used safely without disrupting rumen stability. The rumen of ruminants contains a complex microecosystem that serves as the primary site for nutrient digestion and absorption [25]. To assess feed degradation rates in the rumen, common indexes such as IVDMD, IVCPD, IVEED, and IVNDFD are often employed to evaluate the nutrient degradation rate in the rumen, providing insights into the efficiency of nutrient background and microbial activity in the rumen [26]. In vitro rumen fermentation can simulate some rumen processes, yet differs from in vivo conditions. Factors like feeding behavior, gastrointestinal motility, metabolic regulation, and organ interactions in vivo are challenging to replicate in vitro. Nonetheless, in vitro results offer valuable insights for in vivo studies. They help in screening potential combinations of oxalic acid and hemicellulose diet and understanding their impact on fermentation indicators. Subsequent in vivo experiments can then target these findings. In vivo, growth performance, feed conversion, and nutrient digestibility can be observed to evaluate the practical application of the oxalic acid–hemicellulose interaction. Comparing in vitro and in vivo outcomes also aids in understanding rumen fermentation regulation mechanisms, such as how oxalic acid doses affect hemicellulose degradation by rumen microbes [26–28].

While oxalic acid is not commonly used as a feed additive for ruminants, our study revealed a significant interaction between diets with varying levels of hemicellulose and oxalic acid supplementation. Specifically, oxalic acid supplementation had a more pronounced effect in diets with low hemicellulose levels (HM10.3%) compared to diets with high hemicellulose levels (HM17%). This observation suggests that supplementation of oxalic acid to a low-hemicellulose diet improves the breakdown of carbohydrates, proteins, fats, and neutral detergent fiber. It likely facilitates the colonization of relevant bacteria in the rumen, potentially improving nutrient degradation and microbial activity.

In ruminants, proteins are broken down into peptides and amino acids, which are further degraded into ammonia nitrogen, organic acids, and CO_2 [27]. Ammonia nitrogen, certain peptides, and free amino acids are crucial precursors for protein formation in microorganisms [28]. Consequently, ammonia nitrogen levels in the rumen serve as indicators of protein utilization in the diet. Ortega [29] observed that under normal conditions, rumen ammonia–nitrogen content remains stable; however, under certain conditions, it can adversely affect protein synthesis. Moreover, at higher nutrient levels, the rate of ruminal protein decomposition surpasses that of protein synthesis, leading to significant nitrogen loss. Belenguer et al. [24] demonstrated that the addition of 4.8 g/d of oxalic acid to rumen fistulas resulted in changes to both ammonia–nitrogen levels and rumen pH within a few hours of feeding. Furthermore, in Belenguer et al. [24], incorporation of oxalic acid into the diet reduced the mean value of ammonia nitrogen in rumen fistulas. Studies have shown that certain strains in the rumen, such as Bacillus and Clostridium, can produce a variety of proteases capable of specifically recognizing and cleaving peptide bonds in proteins. The Tan study [27] highlighted that proteases synthesized by rumen microbes are crucial for the initial digestion of proteins. Specifically, the subtilisin produced by Bacillus subtilis can efficiently hydrolyze feed proteins into smaller polypeptides. The polypeptide is subsequently hydrolyzed into amino acids by peptidases, which can be classified into endopeptidases and exopeptidases. Endopeptidases specifically target internal peptide bonds within the polypeptide chain, cleaving it into shorter peptide fragments. Exopeptidase hydrolyzes amino acids sequentially from the terminus of the peptide chain [30].

The addition of oxalic acid alters the amino acid metabolism pathway in certain rumen microorganisms. Bacteria capable of degrading oxalic acid, when utilizing it as a carbon or energy source, may activate a series of enzymes involved in amino acid metabolism. According to relevant studies, these bacteria derive energy through oxalic acid metabolism and drive the deamination of amino acids. Under the catalysis of amino acid deaminases, amino acids undergo deamination to produce ammonia nitrogen and corresponding keto acids [31]. For instance, alanine reacts with alanine deaminase to generate pyruvate and ammonia nitrogen. Pyruvate subsequently participates in microbial energy metabolism, providing energy for growth and reproduction, while ammonia nitrogen is released into the rumen environment, leading to changes in ruminal ammonia nitrogen levels [32]. Our study found that the addition of oxalic acid at a low hemicellulose level decreased the average rumen ammonia–nitrogen concentration. Conversely, the addition of appropriate oxalic acid at a high hemicellulose level increased the average rumen ammonia–nitrogen concentration. These changes in ammonia–nitrogen levels can be considered a phenotypic response to the dietary treatments, reflecting the adaptive mechanisms to varying oxalic acid and hemicellulose levels. We feel this phenotypic response could be attributed to the relatively small amount of oxalic acid used in this experiment. Furthermore, at high hemicellulose levels, oxalic acid supplementation may enhance the microbial abundance associated with protein degradation, thereby converting feed proteins into oligopeptides and amino acids, which are then degraded into ammonia nitrogen.

It is worth noting that gas production in the rumen is a reliable indicator of microbial activity and carbohydrate fermentation efficiency [33]. In our study, at low hemicellulose levels, the group supplemented with 5 mg/kg DM of oxalic acid showed the highest gas production. Conversely, at high hemicellulose levels, the groups supplemented with 10 and 20 mg/kg DM of oxalic acid exhibited the highest gas production. These results suggest that adding an optimal amount of oxalic acid to diets with varying hemicellulose levels regulates ruminal microbial activity and enhances carbohydrate metabolism.

Notably, 60% of ruminants' energy comes from VFAs, with over 95% being acetic acid, propionic acid, and butyric acid. Among these, propionic acid demonstrates the highest energy conversion rate, followed by acetic acid [24]. Acetic acid contributes to lactose formation, thereby contributing to an improved milk yield in sheep. Additionally, propionic acid serves as a precursor for gluconeogenesis, and higher concentrations of propionic acid are associated with increased fattening effects. In a study by Belenguer, daily injection of 0.6 mmol/kg body weight oxalic acid led to changes in the proportions of certain acids, including an increase in ethylene and propyl and a decrease in butyric acid and trace volatile fatty acids. Our study found that adding 10 mg/kg DM oxalic acid led to higher concentrations of acetic, propionic, butyric, and total acids at both high and low hemicellulosic levels. At low hemicellulose levels, oxalic acid supplementation hindered the transition to propionic acid fermentation, while at high hemicellulose levels, it facilitated propionic acid fermentation, resulting in a lower ethylene-to-propylene ratio. These findings suggest that an optimal amount of oxalic acid supplementation simulated the rumen microecosystem in vitro, and enhanced the acid-producing capacity of rumen bacteria positively impacting the VFA profile.

The rumen ecosystem is home to a diverse array of microorganisms, primarily bacteria [34]. Factors such as diet, additives, host species, and environmental conditions influence microbial diversity [35]. Alpha diversity indices, such as Chao1, Shannon, and Simpson diversity indices, are commonly used to assess species abundance within microbial communities [36]. Previous studies have shown that oxalic acid reduces the microbial diversity in sheep; however, with continued supplementation, the diversity gradually recovers [24]. It has been reported that the addition of oxalic acid to diets with varying

hemicellulose levels results in decreased microbial diversity, which is consistent with the findings of the present study. This may be attributed to the slow proliferation of oxalic acid-degrading bacteria, such as oxalicogenes and fecalicococcus, with their diversity gradually rebounding over time. In our experiments, the composition of Bacteroidetes varies across different parts of the rumen; however, *Bacteroidetes* and *Firmicutes* were the dominant phyla, as expected [37]. Bacteroidetes (40.95%) and *Firmicutes* (36.36%) are the prevalent groups in sheep rumen [38]. *Prevotella*, *Butyrivibrio*, and *Pseudobutyrivibrio* are bacterial genera that promote fiber fermentation and are key components of *Bacteroides* and *Firmicutes*. Our study found that supplementing with 10 mg/kg DM of oxalic acid at high hemicellulose levels (HM17%) significantly increased the abundance of *Bacteroidetes*, *Actinobacteria*, *TM7*, *Proteobacteria*, *Tenericutes*, and *Fibrobacteres* ($p < 0.05$). This observation suggests that oxalic acid supplementation at high dietary hemicellulose levels enhances the presence of beneficial bacteria involved in cellulose and hemicellulose fermentation. Additionally, supplementation with 10 mg/kg DM oxalic acid significantly affected the presence of *Prevotella*, *Butyrivibrio*, *Ruminococcus*, *Sharpea*, *RFN20*, *Bulleidia*, *Olsenella*, and *Bifidobacterium* ($p < 0.05$). Some studies have indicated that *Prevotella* promotes fiber degradation, making it an important genus for enhancing hemicellulose degradation [39].

The presence of oxalic acid can affect the rumen microecological environment, especially in relation to hemicellulose levels. Our correlation analysis revealed that after 12 h of in vitro rumen fermentation, Butyrivibrio in the group supplemented with 10 mg/kg DM of oxalic acid at high hemicellulose levels (HM17%) showed a significant positive correlation with isobutyric acid, butyric acid, isovaleric acid, and total acids ($p < 0.05$). Similarly, *Sharpea* exhibited a significant positive correlation with isobutyric acid and isovalent acid ($p < 0.05$). These results suggest that supplementing oxalic acid at high dietary hemicellulose levels enhances the potential of Butyrivibrio and Sharpea to produce more VFAs.

In the present study, we also identified 34 different bacterial genera between the two groups supplemented with 10 mg/kg DM oxalic acid. This result indicates that oxalic acid supplementation to diets with different hemicellulose levels influences the composition of rumen microflora during in vitro fermentation and may enhance its biological functions. Using non-targeted metabolomics, we found that oxalic acid supplementation had a regulatory effect on rumen metabolites, with organic acid heterocyclic compounds being the primary metabolites. At a low hemicellulose level, the differential metabolite pathway was primarily associated with mineral absorption and lipid metabolism. At high hemicellulose levels, the pathway focused on cofactor biosynthesis.

The metabolites of 0 mg/kg DM oxalic acid added to hemicellulose were primarily involved in choline metabolism, anti-folate resistance, and carbohydrate digestion. In the low hemicellulose level, the addition of 10 mg/kg DM oxalic acid notably increased alpha-tocopherol, a form of vitamin E known for its widespread use and high activity [40]. Studies have demonstrated that alpha-tocopherol can disrupt the transport of very low-density lipoprotein (VLDL) and reduce its secretion, thus preventing liver dysfunction [41].

In the low hemicellulose group with no oxalic acid (0 mg/kg DM) supplementation, there was a significant upregulation of L-glutamate, an amino acid crucial for cellular and systemic levels [42]. L-glutamate plays a key role as a source of energy and precursor for various biomolecules in the gut and liver. Interestingly, in the low hemicellulose group supplemented with 10 mg/kg DM oxalic acid, there was a significant upregulation of choline, which has been shown to promote lipid metabolism in dairy cows by affecting transcriptional pathways related to liver lipid metabolism [43]. Conversely, in the high hemicellulose level groups, the addition of 10 mg/kg DM oxalic acid significantly reduced glycinocholic acid, which has been linked to the expression of connective tissue growth factor in hepatocytes and activation of hepatic stellate cells [44]. Additionally, in the high

hemicellulose group with no oxalic acid (0 mg/kg DM) group and high hemicellulose group with 10 mg/kg DM oxalic acid groups, there was a significant upregulation of ginkgolide B, a potent platelet-activating factor (PAF) receptor antagonist [45]. This particular research project concentrated its efforts on examining the effects of oxalic acid supplementation on rumen fermentation processes, the diversity of microbial populations within the rumen, and the metabolic pathways that are influenced by this dietary addition. The study was specifically interested in how these factors are impacted when the diets contain varying levels of hemicellulose. The findings indicated that the supplementation of oxalic acid has the potential to enhance the production of volatile fatty acids (VFA), which are crucial for the energy metabolism of ruminants. However, the study also highlighted that the optimal dosage of oxalic acid required to achieve this boost in VFA production is not a one-size-fits-all solution; rather, it varies depending on the hemicellulose content present in the diet and the duration of fermentation. Through a systematic analysis of various combinations of these factors, the research provided a clearer understanding of the dose–response relationship.

Furthermore, the study revealed that oxalic acid supplementation has a significant impact on the relative abundance of different microorganisms within the rumen. The presence of varying levels of hemicellulose in the diet was found to modify the way oxalic acid influences these microbial communities. Utilizing metabolomic techniques, the research also sheds light on the metabolic pathways that are commonly affected by oxalic acid supplementation. Specifically, it identified changes in carbohydrate digestion processes and choline metabolism, which are essential for the overall health and productivity of ruminant animals. In summary, this study not only confirmed previously known effects of oxalic acid but also uncovered its unique role and the intricate interactions it has with varying levels of hemicellulose in the diet. These insights are valuable for optimizing dietary strategies for ruminant animals, ensuring that they receive the most benefits from their feed while maintaining a healthy and efficient rumen fermentation process.

In the dietary formulation for ruminants, it is essential to consider various factors to determine the dosage adaptation scheme for hemicellulose and oxalic acid. For a diet low in hemicellulose (HM10.3%), it is recommended to add 10 mg/kg DM of oxalic acid (OA), which can significantly increase the concentration of rumen volatile fatty acids, supplying energy for animals. For instance, in beef cattle fattening, this can accelerate the fattening speed and shorten the cycle. For a high hemicellulose (HM17%) diet, during 6-h fermentation, adding 10–20 mg/kg DM of OA is beneficial, while during 12-h fermentation, either 10 mg/kg DM or 80 mg/kg DM is preferable to meet the energy requirements of different growth stages or production states. Dairy cows during the period of peak lactation, that can improve milk production and quality. Additionally, the fermentation time also affects the formulation. In short-term fermentation scenarios, such as the early stage of rapid fattening of lambs, rumen fermentation can be rapidly optimized according to the aforementioned dosages. During long-term feeding, rumen fermentation parameters (such as volatile fatty acids and ammonia nitrogen concentrations) should be regularly monitored, and the oxalic acid dose should be flexibly adjusted according to the growth stage of the animal. In addition, milk composition and rumen indexes should be dynamically adjusted through detection at different lactation stages in cows. Moreover, different breeds of ruminants (such as yaks, which have a unique ability to digest high-fiber diets) and the feeding environment should also be considered. The oxalic acid dose can be appropriately increased for energy supply in cold regions or winter, and may be reduced in hot and humid environments. When the quality of roughage is high, the oxalic acid dose should also be fine-tuned to achieve the best rumen fermentation and nutrient utilization effects [46].

In the field of ruminant nutrition research, there are three key directions that merit further exploration. First, consider the long-term effects of oxalic acid supplementation. While the short-term effects are currently understood, it remains unclear whether long-term supplementation will cause irreversible changes in rumen microbial communities. This includes the potential gradual extinction of certain microbial populations or a permanent shift in their dominant status. To address this, long-term tracking experiments, metagenomics, transcriptomics, and other technologies are necessary. These methods can dynamically monitor changes in microbial community structure and functional gene expression to determine their impact on the long-term stability of the rumen microbial ecosystem. Second, there is a need to explore the potential effects of long-term oxalic acid intake on the overall health of ruminants, an area that is currently under-researched. Subsequently, we can focus on its effects on the immune system, reproductive performance, and bone health, and comprehensively evaluate its impact on animal health through continuous monitoring of immune indicators, reproductive cycles, offspring quality, and detection of mineral content and metabolic indicators in blood and bone. Third, given the widespread use of feed additives in ruminant farming, it is significant to study the synergistic effects of oxalic acid with other common additives, such as probiotics, prebiotics, and enzyme preparations. In the future, multi-factor experiments can be designed to explore the effects of combined use on rumen fermentation, animal growth performance, and health status. Clarifying the synergistic mechanism will help develop more efficient and comprehensive feed additive formulations and provide optimized nutrition control schemes for the breeding industry.

5. Conclusions

This study showed that the addition of oxalic acid to hemicellulosic diets increased rumen fermentation and affected microbial diversity and metabolism in vitro. These results were especially pronounced in the diet containing 17% hemicellulose. Supplementation with an appropriate amount of oxalic acid increased the breakdown of dry matter, neutral detergent fiber, and crude protein in the rumen. This supplementation significantly improved ruminal IVCPD, IVEED, ammonia nitrogen concentrations, and acetate, propionate, and TVFA production. These findings suggest that oxalic acid can play a significant role in fostering a favorable rumen environment, enhancing rumen fermentation efficiency, and facilitating hemicellulose digestion. However, to maximize these benefits, it is crucial to determine the optimal dosage of oxalic acid based on specific dietary conditions. Future studies should focus on identifying the ideal concentrations and exploring the long-term effects of oxalic acid supplementation on rumen health and animal performance.

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Abbreviations

The following abbreviations are used in this manuscript:

HM	Hemicellulose
OA	Oxalic acid
VFA	Volatile fatty acid
YC	Yeast culture
IVDMD	In vitro dry matter digestibility
IVCPD	In vitro crude protein digestibility
IVEED	In vitro ether extract digestibility
IVNDFD	In vitro neutral detergent fiber digestibility

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