

Article

Comparative Analysis of Wheat Hay and Silage in Methane Production, Fermentation Characteristics and Microbiota Using In Vitro Rumen Cultures

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Received: 12 October 2020; Accepted: 24 November 2020; Published: 27 November 2020



Abstract: This study determined the effects of wheat stage, preservation treatment, and harvest time on the fermentation characteristics, methane production, and bacterial diversity. In this study, processing wheat into hay can reduce methane production. The MWS7 (wheat harvested at 7:00 in milk stage and preserved as silage) group had a significantly lower CO₂ compared with the DWS15 (wheat harvested at 15:00 in dough stage and preserved as silage) group. Neutral detergent fiber degradation in the hay treatment harvesting at 7:00 was significantly higher than that in other treatments. The butyrate proportion in the DWH7 (wheat harvested at 7:00 in dough stage and preserved as hay) group was higher than that in the MWS7 group. Results from high-throughput sequencing showed that there were differences in the relative abundance of some minor rumen microbiota among the treatments. The MWS7 group had greater microbial diversity and the MWH7 group (wheat harvested at 7:00 in milk stage and preserved as hay) had higher species richness. In addition, the MWH7 group had a lower *Methanobrevibacter* abundance and methane production. Overall, the MWH7 group may have advantages of rumen fermentation and reduce methane production.

Keywords: whole-crop wheat; methane mitigation; rumen fermentation; high-throughput sequencing; microbial community

1. Introduction

Among the agricultural residues, wheat straw is the second largest biomass feedstock in the world, however, most of it has not been properly used and even been burned for a long time [1]. The burning of wheat straw leads to not only large amounts of resource waste but also severe environment pollution including carbon dioxide (CO₂), carbon monoxide (CO), methane (CH₄), N₂O, and fine particulate matter (PM_{2.5}) [2]. Therefore, finding an effective way to dispose wheat straw is of great significance concerning over the adverse impact of burning straw. Additionally, developments in plant breeding and agronomic techniques in the production of whole-crop wheat, together with its lower production

cost sometimes, have resulted in the increased use of this crop as feed for ruminants [3]. The whole-crop wheat, which consists of wheat straw and wheat grain, must be more nutritious than wheat straw for ruminants. Several studies have shown that fermented whole-crop wheat can increase dry matter intake and improve rumen fermentation such as rumen concentration of ammonium nitrogen $\text{NH}_3\text{-N}$, total volatile fatty acid (VFA) concentration, and the efficiency of microbial N synthesis [4,5]. However, there is little published information about the comparative analysis of wheat hay and wheat silage in fermentation characteristic using in vitro rumen cultures.

On the other hand, an environmental issue exists in the beef production systems, that is the production of enteric methane. Methane, which could be generated during the process of feed fermentation in the gastrointestinal tract of ruminants, is a forceful greenhouse gas, globally totals about 80 megatons annually [6]. Besides, rumen methane production represents 2 to 12% feed energy loss, depending on types of diets [7]. It has been reported that increasing the starch content of diet can benefit methane mitigation, which can fight greenhouse warming [8]. It was revealed that the increased ratios of grain to straw plus chaff of whole-crop wheat silage in the finishing diets of steers can reduce the enteric methane emission [9]. Therefore, the increased starch content of whole-crop wheat relative to wheat straw may create potential benefits not only for diminishing greenhouse gases, but also reducing feed energy loss.

The rumen is a complex microbial ecosystem and is inhabited by diverse microbiota including archaea, bacteria, fungi and protozoa, which play an important role in digesting and degrading feeds to microbial proteins and volatile fatty acids (VFAs) [10]. The study of ruminal microecology is complicated, owing to the daily variation in the environment, microenvironments within the rumen, the effects of animal physiology on the rumen, and the substrate degradation capability of ruminal microorganisms [11]. Different forages have a great impact on rumen metabolism, thus affecting the rumen microbiota [12]. For example, using 16S rRNA gene-cloning library technology, Niu et al. [13] reported that whole-crop wheat hay can be used to replace *Leymus chinensis* in the finishing diet of dairy bulls without adverse effect on the ruminal bacteria composition. Therefore, necessary study should be done to discover the microbiota variation of whole-crop wheat hay and silage in different harvest stage and time when fermenting in the rumen liquid. The previous study found that the nutritional value of the whole-crop wheat varied from different growth period [14]. Harvesting wheat crops in the afternoon can improve the ensiling characteristics compared to that in the morning, because treated with sunlight illumination, the wheat has a greater ruminal digestion and utilization of $\text{NH}_3\text{-N}$ by microbes [15].

To facilitate the research of the rumen microbial ecology, an in vitro system as a model was used to evaluate the ruminal microenvironment. In the study, two kinds of cellulosic biomass were harvested at different time (7:00 and 15:00) in different stage (milk stage and dough stage), preserved in different way (hay and silage), then subjected to anaerobic fermentation in the rumen liquid. The purpose of this research was to determine the differences in fermentation characteristics and microbiota of whole-crop wheat hay and silage using in vitro rumen cultures.

2. Materials and Methods

2.1. Experimental Design

The whole-crop wheat was harvested at 7:00 and 15:00 in Zhuozhou City, Hebei Province of China, respectively, with a cutting height of about 10 cm during the milky ripe stage. Subsequently, a portion of wheat was sun cured (hay) for 5 days and a portion was ensiled in the vacuum bag for forty days. The whole-crop wheat in the dough stage was processed in the same procedure, resulting in eight treatments: wheat harvested at 7:00 in milk stage and preserved as hay (MWH7) or silage (MWS7), wheat harvested at 7:00 in dough stage and preserved as hay (DWH7) or silage (DWS7), wheat harvested at 15:00 in milk stage and preserved as hay (MWH15) or silage (MWS15), wheat harvested at 15:00 in dough stage and preserved as hay (DWH15) or silage (DWS15). The chemical composition of the preserved forages is presented in Table 1.

Table 1. The chemical composition ¹ of different treatments ² (% dry matter—DM basis).

Item	Treatments								SEM ³	<i>p</i> -Value ^{4,5}		
	MWH7	MWS7	DWH7	DWS7	MWH15	MWS15	DWH15	DWS15		S	Tr	Ti
EE	1.58 ^b	2.51 ^{ab}	1.6 ^b	3.31 ^a	2.42 ^{ab}	2.82 ^{ab}	1.82 ^{ab}	1.81 ^{ab}	0.185	0.15	0.022	0.214
aNDF	47.88 ^b	47.01 ^{bc}	47.13 ^{bc}	43.97 ^c	46.52 ^{bc}	47.68 ^b	51.9 ^a	41.44 ^c	0.556	0.009	0.031	0.185
ADF	28.58	29.09	25.81	28.66	27.08	28.99	26.37	25.98	0.423	0.189	0.159	0.128
OM	91.42 ^b	90.71 ^d	92.18 ^a	90.7 ^d	92.14 ^a	90.89 ^{cd}	92.05 ^a	91.33 ^{bc}	0.132	0.036	0.043	0.033
CP	10.99 ^b	12.06 ^a	10.44 ^c	11.06 ^b	10.39 ^c	11.23 ^b	10.44 ^c	11.2 ^b	0.1126	0.019	0.089	0.015

¹ EE = ether extract; aNDF = neutral detergent fiber assayed with a heat stable amylase and expressed inclusive of residual ash; ADF = acid detergent fiber; OM = organic matter; CP = crude protein. ² MWH7 = wheat harvested at 7:00 in milk stage and preserved as hay; MWS7 = wheat harvested at 7:00 in milk stage and preserved as silage; DWH7 = wheat harvested at 7:00 in dough stage and preserved as hay; DWS7 = wheat harvested at 7:00 in dough stage and preserved as silage; MWH15 = wheat harvested at 15:00 in milk stage and preserved as hay; MWS15 = wheat harvested at 15:00 in milk stage and preserved as silage; DWH15 = wheat harvested at 15:00 in dough stage and preserved as hay; DWS15 = wheat harvested at 15:00 in dough stage and preserved as silage. ³ SEM: Standard error of the mean. ⁴ a–d: Means within the same row without the same letter superscripts are significantly different (Tukey's test; $p < 0.05$); S: stage; Tr: treatment; Ti: time; ⁵ *p*-value is the significance level based on actual statistics.

2.2. Inoculum and Incubation

The trial animals were cared and handled following the protocols approved by the Animal Welfare and Ethics Committee of China Agricultural University. Two cannulated Angus bulls (three years old) were used as the donors of rumen fluid and were fed a total mixed ration (TMR) that consist of (% of dry matter—DM basis) a concentrate mixture (30%), grass hay (20%), and corn silage (50%). The animals were fed twice daily at 05:00 and 17:00, respectively. Approximately 300 mL of rumen fluid were obtained from each cattle and incorporated into a 500 mL bottle, at about 3 h after morning feeding. The rumen fluid was filtered through four layers of gauze after mixing, then taken to the laboratory immediately. The fresh rumen fluid prepared was used as the inoculum of the *in vitro* cultures. The buffered medium for incubation was prepared according to the description of the previous study [16], which included a trace-element solution, a carbonic acid buffer solution, a phosphate buffer solution, a sodium sulfide reducing solution (Na_2S) and a resazurin indicator. All the feeds were smashed and sieved through 1.0-mm sieve as the substrate samples. Ground feed substrate (200 mg) was accurately weighed and put into each glass syringe (calibrated volume of 100 mL), which contained 30 mL of the rumen fluid-buffer mixture (ratio = 1:2). The rumen fluid-buffer mixture was then continuously gassed with CO_2 . There was no air in the syringes, whose heads were sealed. Subsequently, the syringes were incubated in a water bath with intermittent shaking at 39 °C for 24 h. Five syringes were used as the repetition of each treatment, and additional three ones without substrate were used as the blanks for incubation. Each sample measurement was repeated twice.

2.3. Sampling and Chemical Analyses

The substrates of different treatments were sampled and stored at −20 °C until analyzed for chemical composition. The dry matter (DM; methods 934.01) and ash (method 942.05) of substrate samples were determined according to AOAC [17]. The content of organic matter (OM) was calculated as the difference between 100 and the ash content. The crude protein (CP; method 990.03) was analyzed according to AOAC [17] by a Kjeldahl nitrogen testing instrument (Kjeltec™ 2100, Foss Technology Corp., Hoganas, Sweden). The ether extract (EE; method 920.39) was determined in line with the AOAC by an ANKOMXT15 Extractor (Ankom Technology Corp., Macedon, NY, USA). Analysis of the neutral detergent fiber (aNDF), and acid detergent fiber (ADF) were carried out using Ankom Fibre Analyser (Ankom Technology, Fairport, NY, USA) in accordance with the method described by Van Soest et al. [18], and α -amylase and sodium sulfite were applied in the procedure of aNDF.

At the end of incubation, the total gas production was recorded. Then gas was sampled from each syringe into a glass tube for determining the gas composition. The value of total gas production is that the total gas production (mL) divided by mass (g). The CH_4 and CO_2 of the gas samples were analyzed using the gas chromatography (Model TP-2060T, Beijing Tianpu Instrument, Beijing, China). The conditions for the analysis were TCD detector, TDX-01 column, size 1 m × 2 mm × 3 mm, column temperature 70 °C and detector temperature 100 °C. The carrying gas was argon with the flowing rate of 30 mL/min.

The pH of the incubation residues was measured immediately by a portable pH meter (HJ-90B, Aerospace Computer Corporation, Haidian District, Beijing, China). Next, three aliquots of 1-mL culture were obtained from each glass syringe into 2 mL centrifuge tube and stored in liquid N for microbial analysis. The remaining culture was mixed and strained with filter bags (Ankom Technology, Macedon, NY, USA) to measure the DM and aNDF degradability of the added substrates gravimetrically according to the method of Blümmel et al. [19].

Six aliquots of 1 mL culture filtrates, which was added 0.25 mL of metaphosphoric acid (25 g/100 mL), were sampled into centrifuge tubes to analyze $\text{NH}_3\text{-N}$ concentration and VFA respectively. The concentration of $\text{NH}_3\text{-N}$ in the culture medium was determined in accordance to Bremner and Keeney [20] by a spectrophotometer (UV-1700, Shimadzu Corporation, Kyoto, Japan). The VFA concentrations were measured by a high-performance gas chromatograph (GC-2014;

Shimadzu Company, Japan), which was equipped with a hydrogen flame detector and a Rtx-Wax capillary column (30 m × 0.25 mm ID × 0.25 µm, polyethyleneglycol; Restek, Evry, France).

2.4. DNA Extraction and 16S rRNA Pyrosequencing

The DNA was extracted from each culture sample using a bacterial DNA Kit (DP302, Tiangen Biotech CO., LTD, Beijing, China) in accordance with the manufacturer's instructions. DNA concentration and purity were evaluated using 1% agarose gels electrophoresis. According to the concentration, DNA was diluted to 1 ng/µL using sterile water. The V3-V4 region of the bacterial 16S rRNA genes were amplified from extracted DNA using the specific primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') with the barcode. PCRs were performed in 30 µL volumes PCR mixture consisted of 15 µL of Phusion® High-Fidelity PCR Master Mix (ThermoFisher, New England Biolabs, Ipswich, MA, USA), 0.2 µM primers, and 10 ng of DNA template. The amplicon mixture was performed as the following conditions: initial 98 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, annealing at 50 °C for 30 s, and elongating of 72 °C for 60 s with a final extension of 72 °C for 5 min. Operating electrophoresis on 2% agarose gel was to detect the PCR products, which then were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Subsequently, sequencing libraries were generated with the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's instructions and index codes were added. At last, high-throughput sequencing was performed on an Illumina HiSeq 2500 platform and 250 bp paired-end reads were generated (Novogene, Beijing, China).

2.5. Sequence Analyses

Paired-end reads were merged using FLASH (V1.2.7, <http://ccb.jhu.edu/software/FLASH/>) [21], a very accurate and fast analysis tool, to obtain raw tags. Then the clean tags were obtained by screening the raw tags using the Quantitative Insights Into Microbial Ecology (QIIME) V1.8 pipeline [22]. Then the effective tags finally obtained by removing the chimera sequences using the Uchime algorithm [23]. Sequences analyses were performed by Uparse software (Uparse v 7.0.1001, <http://drive5.com/uparse/>) [24] to be assigned to the same operational taxonomic units (OTUs) at an identity threshold of 97%. For each representative sequence, taxonomic information was annotated by using the Green Gene Database (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>) [25] based on the Ribosomal Database Project RDP classifier (Version 2.2, <http://sourceforge.net/projects/rdp-classifier/>) [26] algorithm. Alpha diversity was evaluated to analyze complexity of species diversity for a sample by calculating Good's coverage, ACE richness, Shannon diversity index, and Observed-species with QIIME (Version 1.7.0). Beta diversity analysis was used to evaluate differences of samples in species complexity with QIIME software (Version 1.7.0). Principal Coordinate Analysis (PCoA) was performed to get principal coordinates and visualize from complex, multidimensional data using the QIIME software package, thereby comparing the microbial communities among all samples.

After assembling and filtering, the raw sequencing data were submitted to the NCBI Sequence Read Archive (SRA), and the SRA records will be accessible with the following link (<https://www.ncbi.nlm.nih.gov/sra/PRJNA540111>) under the accession number PRJNA540111.

2.6. Statistical Analysis

The data were analyzed using the PROC MIXED of SAS version 9.0 software (SAS Institute Inc., Cary, NC, USA) in a 2 × 2 × 2 factorial design. The normality and homogeneity of the variance were checked using proc UNIVARIATE. Significance among the treatments evaluated by Duncan's multiple range tests was declared at $p < 0.05$, whereas $0.05 < p < 0.10$ were considered as a trend.

3. Results

3.1. Differences in Feed Degradability and Rumen Fermentation

The effects of different treatments on substrate digestibility and fermentation by ruminal cultures are shown in Table 2. The pH in the DWH7 group was higher than those in the MWS15, MWS15, and DWH15 groups ($p < 0.05$). Treatment and time had a significant effect on the Gas production ($p < 0.05$). The Gas production in the silage treatment groups harvested at 15:00 (MWS15, DWS15) were significantly higher than those in the hay treatment groups harvested at 7:00 (MWH7, DWH7) ($p < 0.05$). Stage, treatment, and time had significant effect on the DM degradation ($p < 0.05$). The DM degradation in the MWS15 group was higher than that in other groups ($p < 0.05$). Treatment and time had significant effect on the aNDF degradation ($p < 0.05$). The aNDF degradation in the DWH7 group was higher than that in other groups ($p < 0.05$). Treatment affected the methane production significantly ($p < 0.05$). The methane production in the wheat silage treatments (MWS7, DWS7, MWS15, DWS15) was higher than that in the wheat hay treatments (MWH7, DWH7, MWH15, DWH15) ($p < 0.05$). Time affected the CO₂ production significantly ($p < 0.05$). The CO₂ production in the MWS7 group was lower than that in the DWS15 group ($p < 0.05$).

3.2. Differences in Rumen VFA Profile

The effects of different treatments on VFA profile are shown in Table 2. Stage, treatments, and time had no effects on the total VFA concentration and the molar proportion of acetate, propionate, isobutyrate and valerate ($p > 0.05$). The acetate to propionate (A:P) ratio in the MWS7 and DWH15 groups were lower than that in the DWS15 group ($p < 0.05$). Stage had a significant effect on the butyrate concentration ($p < 0.05$). The DWH7 group had a higher butyrate concentration compared with the MWS7 group ($p < 0.05$). The isovalerate concentration was affected by the stage and treatment ($p < 0.05$). The concentration of isovalerate in the DWS7 group was significantly higher than that in other groups ($p < 0.05$).

3.3. Changes in the Microbiota Community during Anaerobic Digestion

Analysis of the alpha diversity of the bacterial community among the treatments is presented in the Figure 1. In the present study, overall Good's coverage was above 98% (Figure 1a), shown that most of the microbial phylotypes were explored at a 0.97 similarity. The Shannon index (Figure 1b), which represents community diversity of the samples, was trending higher in the MWS7 group than that in the DWH7 ($p = 0.066$) and MWH7 ($p = 0.0817$) groups; ACE (Figure 1c) and observed species (Figure 1d) are mainly applied to evaluate the richness of the microbial community. ACE in the DWH7 group was lower than that in the MWH7 group ($p = 0.0342$). ACE in the MWH7 and MWS7 groups were trending higher than that in the MWS15 group ($p = 0.0548$, $p = 0.0962$, respectively). Observed species-level OTUs in the MWH7 and MWS7 groups were trending higher than that in the DWH7 group ($p = 0.0584$, $p = 0.0731$, respectively). Observed species-level OTUs in the MWH7 and MWS7 groups were trending higher than that in the MWS15 group ($p = 0.0731$, $p = 0.0910$, respectively). These different evaluation methods showed that the diversity of microbial community was more abundant in the MWS7 group than that in the DWH7 and MWH7 groups, while the species richness of the MWH7 and MWS7 groups were higher than those of the DWH7 and MWS15 groups.

Table 2. Effects of different treatments on vitro fermentation ^{1,2}.

Item	Treatments ³								SEM	<i>p</i> -Value ⁴		
	MWH7	MWS7	DWH7	DWS7	MWH15	MWS15	DWH15	DWS15		S	Tr	Ti
pH	6.78 ^{ab}	6.78 ^{ab}	6.83 ^a	6.78 ^{ab}	6.77 ^b	6.67 ^c	6.79 ^{ab}	6.76 ^b	0.010	0.006	0.003	0.004
Gas (mL/g)	257.8 ^c	266.1 ^{bc}	262.2 ^c	276.1 ^a	272.4 ^{ab}	280.9 ^a	276.7 ^a	278.4 ^a	0.407	0.077	0.002	<0.001
Ammonia (mmol/L)	11.1	10.6	11.5	11.4	10.4	11.1	11.6	10.5	0.225	0.373	0.599	0.615
DM degradation (%)	37.6 ^d	41.5 ^b	32.5 ^e	40.4 ^{bc}	38.2 ^{cd}	44.2 ^a	39.1 ^{bcd}	38.9 ^{bcd}	0.705	0.001	<0.001	0.003
aNDF degradation (%)	43.2 ^b	42.3 ^{bc}	46.9 ^a	39.6 ^{cd}	40.7 ^{bc}	39.4 ^{cd}	36.7 ^d	40.1 ^c	0.658	0.359	0.036	<0.001
Methane (%)	19.8 ^b	19.6 ^b	19.1 ^b	22.1 ^a	19.9 ^{ab}	21.0 ^{ab}	19.6 ^b	21.0 ^{ab}	0.285	0.453	0.015	0.669
CO ₂ (%)	71.3 ^{ab}	67.3 ^b	67.9 ^{ab}	71.0 ^{ab}	70.5 ^{ab}	71.8 ^{ab}	72.6 ^{ab}	73.5 ^a	0.666	0.428	0.808	0.042
VFA (mM)												
TVFA	49.2	47.5	51.0	53.3	49.6	48.3	46.6	51.5	0.830	0.272	0.547	0.466
Acetate(A)	34.2	32.5	34.9	36.8	34.3	33.3	31.8	35.5	0.607	0.359	0.559	0.483
Propionate(P)	8.26	8.81	8.78	8.97	8.44	8.40	8.17	8.69	0.129	0.534	0.289	0.326
A: P ratio	4.14 ^a	3.68 ^c	3.97 ^{ab}	4.11 ^a	4.06 ^{ab}	3.96 ^{ab}	3.89 ^b	4.08 ^a	0.034	0.228	0.175	0.643
Isobutyrate	0.56	0.60	0.60	0.64	0.59	0.61	0.55	0.60	0.009	0.522	0.056	0.522
Butyrate	4.51 ^{ab}	3.8 ^c	4.92 ^a	4.75 ^{ab}	4.54 ^{ab}	4.2 ^{bc}	4.46 ^{ab}	4.74 ^{ab}	0.089	0.004	0.096	0.956
Isovalerate	1.11 ^c	1.2 ^{bc}	1.21 ^{bc}	1.44 ^a	1.16 ^{bc}	1.22 ^{bc}	1.07 ^c	1.29 ^b	0.027	0.047	0.001	0.141
Valerate	0.61	0.63	0.65	0.67	0.59	0.61	0.56	0.71	0.014	0.135	0.075	0.339

¹ a–f: Means within the same row without the same letter superscripts are significantly different (Tukey's test; $p < 0.05$); S: stage; Tr: treatment; Ti: time; ² SEM: Standard error of the mean.

³ MWH7 = wheat harvested at 7:00 in milk stage and preserved as hay; MWS7 = wheat harvested at 7:00 in milk stage and preserved as silage; DWH7 = wheat harvested at 7:00 in dough stage and preserved as hay; DWS7 = wheat harvested at 7:00 in dough stage and preserved as silage; MWH15 = wheat harvested at 15:00 in milk stage and preserved as hay; MWS15 = wheat harvested at 15:00 in milk stage and preserved as silage; DWH15 = wheat harvested at 15:00 in dough stage and preserved as hay; DWS15 = wheat harvested at 15:00 in dough stage and preserved as silage. ⁴ *p*-value is the significance level based on actual statistics.

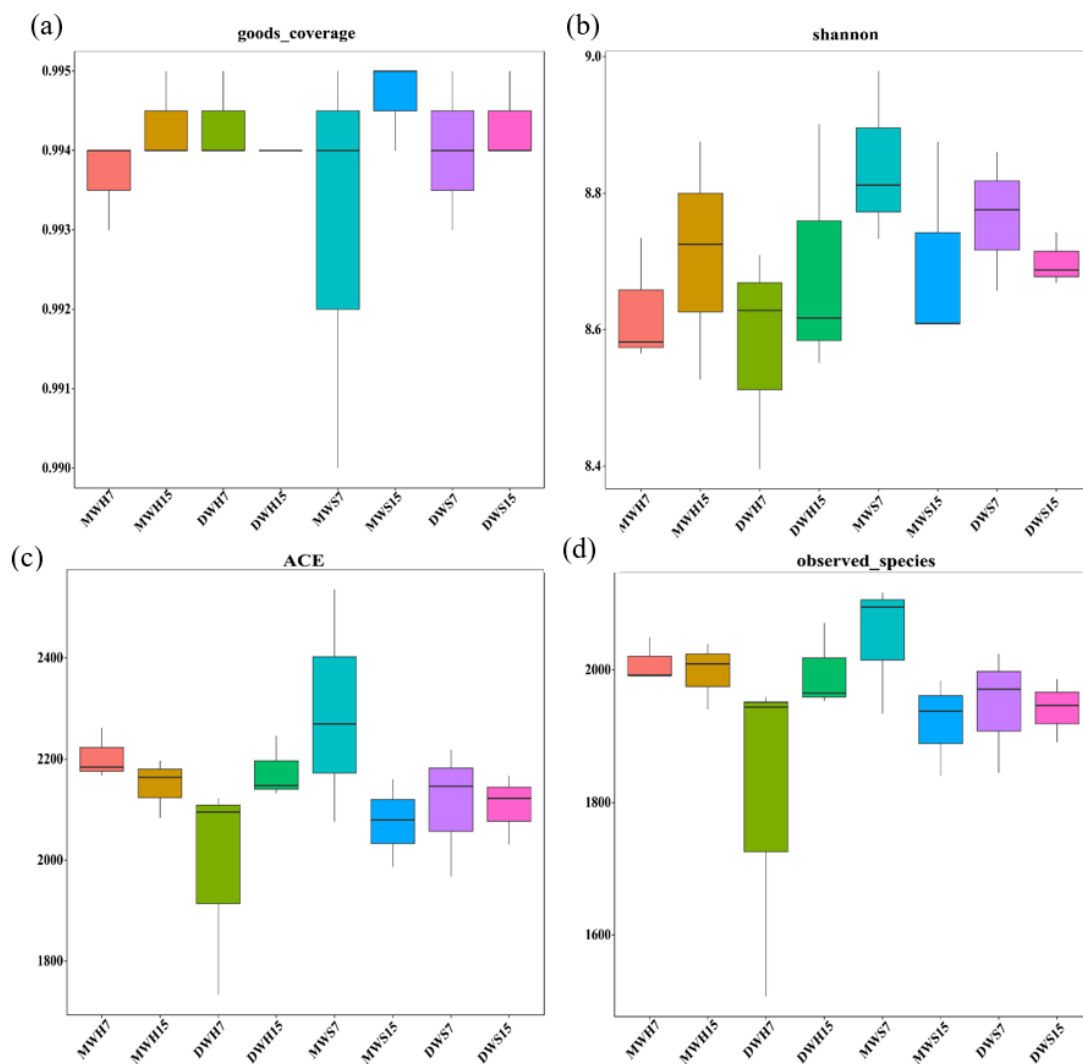


Figure 1. Estimators of the alpha diversity of bacterial community in the samples. (a) Good's coverage; (b) Shannon index; (c) ACE; (d) observed species. MWH7 = wheat harvested at 7:00 in milk stage and preserved as hay; MWS7 = wheat harvested at 7:00 in milk stage and preserved as silage; DWH7 = wheat harvested at 7:00 in dough stage and preserved as hay; DWS7 = wheat harvested at 7:00 in dough stage and preserved as silage; MWH15 = wheat harvested at 15:00 in milk stage and preserved as hay; MWS15 = wheat harvested at 15:00 in milk stage and preserved as silage; DWH15 = wheat harvested at 15:00 in dough stage and preserved as hay; DWS15 = wheat harvested at 15:00 in dough stage and preserved as silage.

The beta diversity analysis, which is shown by principal coordinate analysis (PCoA) in Figure 2, was conducted to further explore the diversity of microbial communities among different treatments. The PCoA plots revealed that the bacteria communities differed significantly among some treatments using PC1 and PC2 (40.85% and 21.92%, respectively, of the explained variance). The bacterial community at the phylum levels is presented in Figure 3a. In all samples, the predominant phyla were Firmicutes (48.96%) and Bacteroidetes (42.22%), followed by Proteobacteria (2.39%), Euryarchaeota (2.24%), and Actinobacteria (0.93%). Minor phyla included Tenericutes (0.74%), Saccharibacteria (0.68%), Candidate_division_SR1 (0.54%), Spirochaetae (0.43%), and Fibrobacteres (0.31%). The other phyla accounted for 0.56% of the bacteria. Figure 3b shows the differences of the phylum Saccharibacteria among the treatments. The abundance of Saccharibacteria in the DWH7 group was higher than that in the MWS7 and MWS15 groups. The abundance of Saccharibacteria in the DWS15 group was higher than that in the DWH15, MWS15 and MWS7 groups. Figure 3c shows the differences of the phylum

Fibrobacteres among the treatments. The abundance of Fibrobacteres in the MWS7 group was higher than that in the MWH7 group.

The bacterial communities at the genus levels are presented in Figure 4a. The composition of the main bacteria anaerobic fermentation of different treatments was similar. The most abundant genus was *Rikenellaceae_RC9_gut_group* (20.21%), followed by *Prevotella* (8.43%), *Christensenellaceae_R-7_group* (7.41%), *Ruminococcaceae_NK4A214_group* (5.05%), *Methanobrevibacter* (2.14%), *Ruminococcaceae_UCG-010* (2.63%), *Ruminococcaceae_UCG-014* (2.58%), *Ruminiclostridium_5* (2.46%), *Eubacterium_coprostanoligenes_group* (2.09%), and *Pseudomonas* (0.17%). Other known genera took up 46.82% of the total bacterial community. Figure 4b shows the differences of the genus *Ruminiclostridium_5* among the treatments. The relative abundance of *Ruminiclostridium_5* in the MWS7 group was higher than that in the MWH15 and DWS15 groups. Figure 4c shows the differences of the genus *Methanobrevibacter* among the treatments.

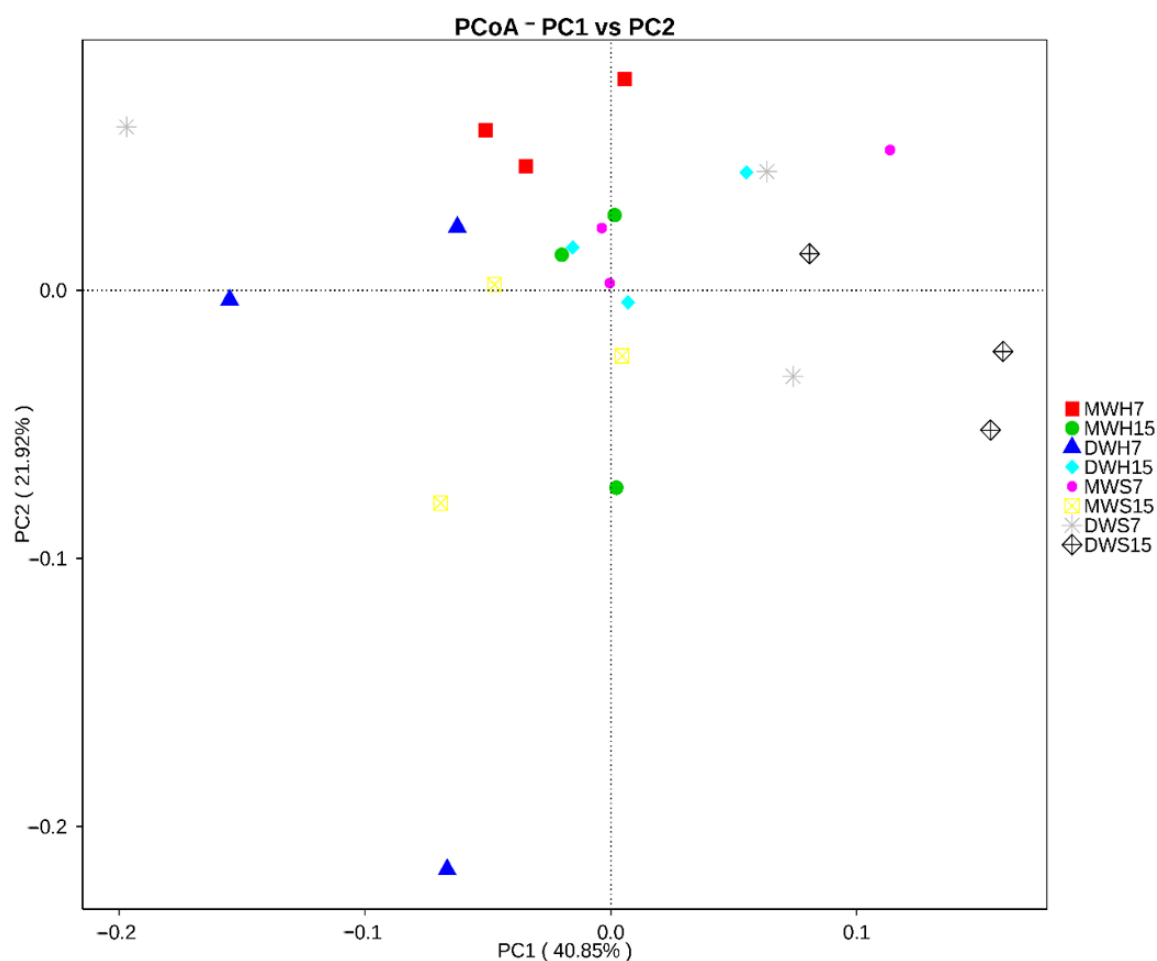


Figure 2. Principal coordinate analysis (PCoA) of microbial community structure in anaerobic digesters fed with different types of wheat, with shapes and colors representing different treatments. MWH7 = wheat harvested at 7:00 in milk stage and preserved as hay; MWS7 = wheat harvested at 7:00 in milk stage and preserved as silage; DWH7 = wheat harvested at 7:00 in dough stage and preserved as hay; DWS7 = wheat harvested at 7:00 in dough stage and preserved as silage; MWH15 = wheat harvested at 15:00 in milk stage and preserved as hay; MWS15 = wheat harvested at 15:00 in milk stage and preserved as silage; DWH15 = wheat harvested at 15:00 in dough stage and preserved as hay; DWS15 = wheat harvested at 15:00 in dough stage and preserved as silage.

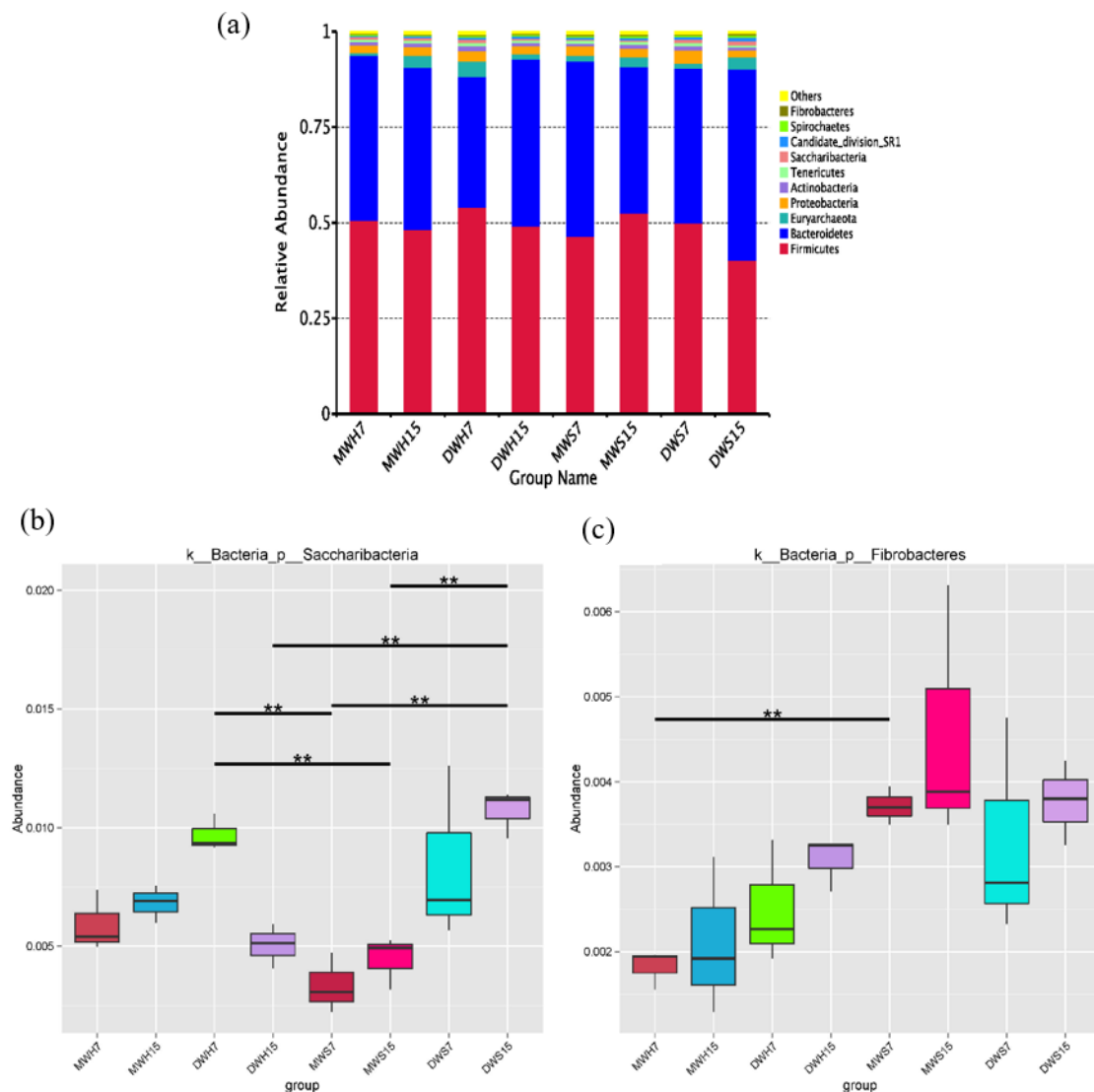


Figure 3. Taxonomic profiles at the phylum level in anaerobic fermentation of different treatments from 16S rRNA gene sequencing. **(a)** The relative abundance of the top 10 phyla of rumen microbial community among treatments; **(b)** The differences of the phylum Saccharibacteria among the treatments; **(c)** The differences of the phylum Fibrobacteres among the treatments. MWH7 = wheat harvested at 7:00 in milk stage and preserved as hay; MWS7 = wheat harvested at 7:00 in milk stage and preserved as silage; DWH7 = wheat harvested at 7:00 in dough stage and preserved as hay; DWS7 = wheat harvested at 7:00 in dough stage and preserved as silage; MWH15 = wheat harvested at 15:00 in milk stage and preserved as hay; MWS15 = wheat harvested at 15:00 in milk stage and preserved as silage; DWH15 = wheat harvested at 15:00 in dough stage and preserved as hay; DWS15 = wheat harvested at 15:00 in dough stage and preserved as silage. ** $p < 0.01$.

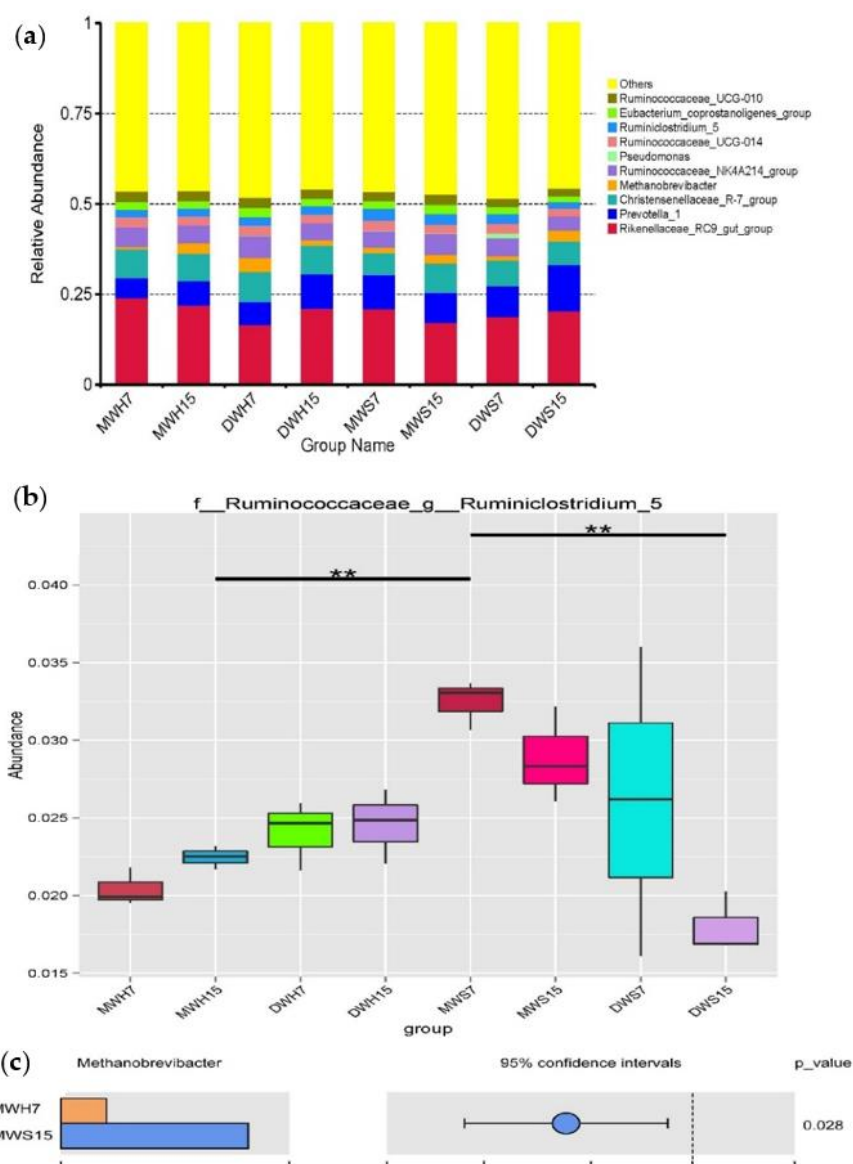


Figure 4. Bacterial community barplot analysis at the genus level in anaerobic fermentation of different treatments. **(a)** The relative abundance of the top 10 genera of rumen microbial community among treatments; **(b)** The differences of the genus *Ruminiclostridium_5* among the treatments; **(c)** The differences of the genus *Methanobrevibacter* between the groups. MWH7 = wheat harvested at 7:00 in milk stage and preserved as hay; MWS7 = wheat harvested at 7:00 in milk stage and preserved as silage; DWH7 = wheat harvested at 7:00 in dough stage and preserved as hay; DWS7 = wheat harvested at 7:00 in dough stage and preserved as silage; MWH15 = wheat harvested at 15:00 in milk stage and preserved as hay; MWS15 = wheat harvested at 15:00 in milk stage and preserved as silage; DWH15 = wheat harvested at 15:00 in dough stage and preserved as hay; DWS15 = wheat harvested at 15:00 in dough stage and preserved as silage. ** $p < 0.01$.

4. Discussion

The process of fermentation by ruminal cultures largely depends on available substrate. The pH of substrate fermentation is an important index to reflect whether rumen environment is healthy. In the present study, the pH values (6.4–6.8) were within the range of normality [27], which indicated that the rumen internal environment was static to some extent among different treatments. This likely due to the significant interaction effect on the pH of stage, treatment, and time. Moreover, the higher culture pH in the DWH7 group than in the MWS15 may be a result of the numerically higher ammonia concentration

in the DWH7 group than in the MWS15 group [28]. The gas production in vitro is a valuable index that describes the fermentability of ruminant feedstuffs and estimates DM degradation [29,30]. The greater gas production in the wheat harvested at 15:00 (MWH15, MWS15, DWH15, DWS15) may be because that the enzyme activity evaluated or some harmful bacteria were killed through the strong sunshine at noon, thereby increasing the digestibility of OM in the rumen fermentation. The higher DM degradation in the MWS15 group probably resulted from the greater microbial and enzymatic activity [31]. The higher aNDF degradation in the DWH7 group may be attributed to the more fibrolytic bacteria attached to the surface of the feed after the wheat was harvested at 7:00 in the dough stage and dried in the sun. In the process of rumen fermentation, there were more fibrolytic bacteria to be fond of digesting the wheat in the DWH7 group.

Methane inhibition in the MWH7 group may be explained by the decreased availability of hydrogen for methanogenesis in the anaerobic fermentation of wheat hay [28]. The abundance of *Methanobrevibacter* decreased significantly in the MWH7 group, while the methanogens have a symbiotic relationship with the protozoa in the rumen, which play a very important role in the generation of methane. The protozoa can produce a large number of hydrogen molecules in its own metabolic process, which can be used by methanogens to synthesize methane [32]. Therefore, we concluded that utilizing wheat hay in the ruminants not only benefits the global environment [33], but also reduces dietary gross energy loss [33]. CO₂ is one of the main gas composition in the anaerobic fermentation of rumen fluid, the higher CO₂ production in the wheat harvested at 15:00 probably because of the higher gas production in those groups, which resulted from the higher DM degradation [34]. Besides, CO₂ also is one of the main greenhouse gases in the atmosphere [35]. Therefore, the wheat group harvested at 7:00 (MWH7, MWS7, DWH7, DWS7) is beneficial for the global environment when fermenting in the rumen.

Substrate composition affects the VFA concentration in the fermentation processes [36]. Consequently, the unaffected concentration of total VFA, acetate, and propionate, indicating that the fermentation pattern is similar, which could be explained by that all the substrate in the fermentation were wheat among different treatments. Although no significant differences occurred in acetate or propionate production in the in vitro study, there were lower acetate but higher propionate level just in the value in MWS7 and DWH15 groups. Therefore, the observed values of A:P ratio in the MWS7 and DWH15 groups were lower, indicating that there were more easy-to-ferment carbohydrates in these two substrates, thereby producing more propionate and reducing the A:P ratio. After the absorption of reticulorumen wall, the majority of butyrate can be transformed into beta-hydroxy acid, which is used for the energy supply of the body tissues. Butyrate is very important in the rumen of young ruminants, it can not only promote the proliferation and differentiation of rumen epithelial cells, but also improve the intestinal peristalsis and sensitivity [37]. In this experiment, the content of butyrate in the DWH7 group was significantly higher than that in the MWS7 group. This may be due to the rapid propagation of the microorganisms (such as *Butyrivibrio fibrisolvens*) that release butyrate during the fermentation process, which illustrates that there existed superimposed effect among these factors. As previously reported by Andries et al. [38], the majority of ruminal cellulolytic microbes utilize the branched-SCFA (ex. isovalerate) to produce branched-chain amino acids for the sake of proliferating and improving their functions and enzyme activity. Therefore, the higher concentration of isovalerate in the DWS7 group could account for the lower utilization by bacteria and the greater degree of protein metabolism.

In the present study, the most abundant phyla were Firmicutes, Bacteroidetes, and Proteobacteria, which are the major bacterial phyla taking an important part in anaerobic fermentation of rumen [13,39,40]. The phylum Firmicutes can produce all kinds of lipases, proteases, cellulases and other extracellular enzymes, which enables hydrolysis of complex macromolecules such as fats, proteins, amino acids, hemicellulose, cellulose, and sugars [41]. Bacteroidetes are mainly associated with the hydrolysis of complex macromolecular OM, such as the degradation of carbohydrates into monosaccharides, and subsequent hydrolysis of these macromolecules into small molecules of acetic acid, lactic acid, and succinic acid [42]. Spirochaetes was a kind of ubiquitous bacteria, which was explored in anaerobic

digesters [43]. This phylum is able to ferment glucose in the anaerobic fermentation [44] and may be conducive to the acidogenesis through the fermentation of sugars generated from the hydrolyzation of the wheat grass [45].

More recently, the complete genomes of Saccharibacteria, obtained through metagenomics, suggested that some members have an obligate fermentative metabolism, fermenting glucose and other sugars, while producing lactate [46]. Fibrobacteres are cellulolytic bacteria in the anaerobic fermentation of rumen [45], the abundance of Fibrobacteres in the MWS7 group was higher than that in the MWH7 group. However, the difference in the aNDF degradation between MWS7 and MWH7 groups was not observed. This may be because that the Fibrobacteres sequences took up an average of only 0.31% of the total bacterial community, thereby the effect of Fibrobacteres on the aNDF digestibility was subtle among treatments.

We notice the highest abundance genus was *Rikenellaceae_RC9* bacteria, this result was in accordance with the research reported by Zened et al. [45]. The *Rikenellaceae_RC9* has not been researched extensively in the anaerobic fermentation of rumen liquid, however, the previous study reported by Pitta et al. [47] had already revealed that this genus and other cellulose-decomposing bacteria gather together, indicating that this genus is associated with the degradation of structural carbohydrates. *Methanobrevibacter* belongs to the phylum Euryarchaeota, which can use hydrogen or formate as substrates to produce CH₄ in the rumen [48]. Therefore, the lower abundance of *Methanobrevibacter* in the MWH7 group may account for the lower CH₄ production in the MWH7 group indicating that it is beneficial for the inhibition of methane in the environment to utilize wheat harvested at 7:00 in milk stage and preserved as hay in the feed of ruminants.

During anaerobic fermentation, *Prevotella* has the rate-limiting activity of the dipeptidyl peptidase type IV, which takes responsibility for the hydrolysis of oligopeptides [49]. Therefore, its function is related to the protein metabolism and ammonia production, especially the breakdown of oligopeptides [50]. In this study, treatments did not affect the relevant abundance of *Prevotella*, which could account for the undifferentiated ammonia production. *Ruminococcaceae_NK4A214* group, *Ruminococcaceae_UCG-010*, *Ruminococcaceae_UCG-014* and *Ruminiclostridium_5* all belong to Ruminococcaceae, a family has the ability to hydrolyze and ferment carbohydrates, might take an important part in the digestive process of wheat grass [51,52].

5. Conclusions

This study compared the methane production, fermentation characteristics and microbiota of whole-crop wheat hay and silage using in vitro rumen cultures and 16S rRNA gene amplicon sequencing technology. On the whole the gas production in the wheat groups harvested at 15:00 was higher than that harvested at 7:00 in this study. The hay treatment can reduce methane production in fermentation, especially the DWH7 group. The aNDF degradation was higher in the DWH7 group than that in other groups. The MWS7 group had greater microbial diversity and the MWH7 and MWS7 groups had higher species richness. There were differences in the relative abundance of some minor rumen microbiota rather than the dominant bacteria at the phylum and genus levels among treatments. This study demonstrated that the wheat hay treatment harvested at 7:00 in the milk stage and dough stage can be more effective and practical in energy saving and pollution abatement, and the combination of agriculture and animal husbandry.

Author Contributions: Conceptualization, W.N., B.C., and H.S.; formal analysis, W.N. and H.W.; investigation, Y.H., Q.Q., and T.S.; project administration, B.C. and H.S.; writing—original draft preparation, W.N.; writing—review and editing, H.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Demonstration Project of Exploitation and Utilization of High-Quality Silage and Roughage Resources (16200157), National Key R&D Program of China (2018YFD05018000), Key Technology R&D Program of Ningxia (2017BY078), and China Agriculture Research System (CARS-37).

Conflicts of Interest: We declare the submitted work was not carried out in the presence of any personal, professional or financial relationships that could potentially be construed as a conflict of interest.

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