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Persistent action of cow rumen microorganisms in enhancing biodegradation of wheat straw by rumen fermentation



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HIGHLIGHTS

G R A P H I C A L A B S T R A C T

- High WS decomposition was achieved by the persistent action of cow rumen microflora.
- Degradation characteristics of raw WS subject to rumen fermentation were analysed.
- Rich core lignocellulolytic enzymes were secreted by the rumen bacteria and fungi.
- Firmicutes and Basidiomycota were the dominant lignocellulolytic bacteria and fungi.
- Degradation mechanisms for the *in vitro* rumen fermentation of WS were elucidated.

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ABSTRACT

Rumen fermentation is known to be effective for lignocellulosic-wastes biodegradation to certain extent but it is still unclear if there exists a termination of the microorganisms' action to further degrade the bio-refractory fractions. In order to illuminate the related microbiological characteristics, experiments were conducted in a prolonged duration of rumen fermentation of mechanically ruptured wheat straw, with inoculation of cow rumen microorganisms in vitro. Although the organic wastes could not be biodegraded quickly, continuous conversion of the lignocellulosic contents to volatile fatty acids and biogas proceeded in the duration of more than three months, resulting in 96–97% cellulose and hemicellulose decomposition, and 42% lignin decomposition. X-ray diffraction and Fourier transform infrared spectroscopy further demonstrated the characteristics of lignocellulosic structure decomposition. Under the actions of cow rumen microorganisms, stable pH was maintained in the fermentation liquid, along with a steady NH4-N, volatile fatty acids accumulation, and a large buffering ability. It was identified by enzyme analysis and Illumina MiSeq sequencing that the rich core lignocellulolytic enzymes secreted by the abundant and diverse rumen bacteria and fungi contributed to the persistent degradation of lignocellulosic wastes. Members of the Clostridiales order and Basidiomycota phylum were found to be the dominant lignocellulolytic bacteria and fungi, respectively. It could thus be inferred that the main lignocellulose degradation processes were a series of catalytic reactions under the actions of lignocellulolytic enzymes secreted from bacteria and fungi. The dominant hydrogenotrophic methanogens (Methanomassiliicoccus, Methanobrevibacter, Methanosphaera, and Methanoculleus) in the rumen could also assist CH₄ production if the rumen fermentation was followed with anaerobic digestion.

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1. Introduction

The global rise in energy consumption, the predicted increase in energy demands in the near future, the depletion of low-extraction cost fossil fuel reserves, and climate change are the driving forces behind searching for renewable and eco-friendly energy systems based on renewable lignocellulosic biomass. Agricultural waste is the most popular and abundantly available lignocellulosic biomass with the potential to be utilized for energy recovery (He et al., 2019). Wheat straw (WS) is the largest agricultural lignocellulosic waste, with 154-185 million tonnes being produced per year (Abbasi and Abbasi, 2010). Different approaches have been applied to convert the lignocellulosic waste to energy products, including anaerobic digestion, fermentation, combustion, pyrolysis, and gasification (Hosseini Koupaie et al., 2019). Lignocellulose materials consist of cellulose, hemicellulose, and lignin, which together form a rigid and complex structure that increases the difficulty of degrading lignocellulosic waste. Lignocellulosic material often reguires aggressive pretreatment (such as physical, chemical, biological, and multiple or combinatorial pretreatments) to improve fermentation production efficiencies and enhance biomass digestibility. In comparison with the conventional pretreatment methods, biological pretreatment could be attractive, as it uses natural microorganisms or their enzymes under mild conditions (Hu et al., 2012). The major advantages of biological pretreatment (such as fungal, microbial consortium, enzymatic, ensilage, and microaeration pretreatments) are low energy consumption, simple operating conditions and equipment, no or minimum inhibitor formation, low downstream processing costs, and no requirement for recycling chemicals after pretreatment.

Despite the biologically recalcitrant nature of lignocellulosic biomass, it can be efficiently digested by rumen microorganisms in natural ecosystems (Sauer et al., 2012; Vida and Tedesco, 2017). The rumen in ruminants is a natural lignocellulosicdegrading system that is inhabited by a complex microbial population including bacteria, fungi, archaea, and protozoa, which all need enzyme components with high enzyme activity to effectively digest lignocellulosic waste to microbial proteins, volatile fatty acids (VFAs) and gases (Campanaro et al., 2017; Hess et al., 2011). Furthermore, VFAs are the major products in such a system and could be further converted to other energy products, including electricity, H₂, and bioplastics. Rumen microorganisms have been successfully employed to digest a variety of lignocellulosic biomass, including agricultural residues and aquatic plants (Hu and Yu, 2005; Yue et al., 2007a, 2007b). Many studies have indicated that the rumen microorganisms exhibited higher ability and activity for the degradation of lignocellulosic wastes than other common anaerobic microbes, but it is still unclear if there exists a termination of the microorganisms' action to further degrade the bio-refractory fractions.

To mimic the natural rumen ecosystems, there is considerable interest in reaching a clearer understanding of the diversity of biological mechanisms employed during lignocellulose degradation. Hu et al. (2008) reported that approximately 25.5% of the lignin in WS was removed within 13 days of anaerobic fermentation by rumen microorganisms after dewaxing with toluene/ethanol. The anaerobic rumen microorganisms digestibility of lignocellulosic wastes was enhanced by microwave irradiation because the structure of wax and lignin covering the cattail surface was broken down (Hu et al., 2012). Additionally, a previous study reported that ammonification caused a significant improvement in the digestibility of corn straw by cow rumen microorganisms (Jin et al., 2014). However, microwave irradiation pretreatment consumes energy, and chemical pretreatment may produce inhibitors that disturb the structure and stability of the ruminal community. Changing environments, dietary ingredients, and nutrient levels will lead to a change in the community composition and function of the microbiome (Cobellis et al., 2016; Sauer et al., 2012). To date, the real fermentation characteristics and mechanisms of the ruminal community in natural rumen systems digesting untreated lignocellulose waste are also still unclear, and determining them is the vital step to develop an artificial rumen system for lignocellulose waste conversion.

Ruminants do not produce the enzymes needed to degrade most types of complex lignocellulolytic biomass, and the rumen provides an environment for a rich and dense consortium of anaerobic microbes that fulfil this metabolic role (Henderson et al., 2016). Anaerobic fungi play an important role in fermenting lignocellulosic biomass into sugars in the rumen and represent a very promising enzyme resource for the conversion of lignocellulosic biomass into biofuels. Due to bacteria and fungi capable of secreting extracellular enzymes, the rumen ecosystem represents a virtually untapped resource of novel enzymes with tremendous potential for industrial applications (Yue et al., 2013). The lignocellulolytic enzyme is composed of lignin-degrading enzymes (lignin peroxidase, LiP; manganese peroxidase, MnPs; laccase, Lac), hemicellulose-degrading enzymes (xylanase; carboxymethyl cellulose, CMCase; xylitolase; xylan esterase), and cellulose-degrading enzymes (β -glucosidase, BG; endoglucanase, EG; cellobiose hydrolase, CBH) (Dashtban et al., 2009; Xing et al., 2020). Ligninolytic enzymes may remove amounts of lignin similar to those obtained from a fungal pretreatment (Zabed et al., 2019). Bacteria and fungi are mainly involved in lignocellulose degradation, and archaea are related to CH₄ formation. To acquire more detailed information about the rumen ecosystem, nextgeneration sequencing technology has the potential to provide vital insights into complex rumen microbial communities. A core microbiome of seven abundant genus-level groups (Prevotella, Butyrivibrio, and Ruminococcus, as well as unclassified Lachnospiraceae, Ruminococcaceae, Bacteroidales, and Clostridiales) was defined for 67% of the Global Rumen Census sequences (Henderson et al., 2016; Moraïs and Mizrahi, 2019; Seshadri et al., 2018). The major metabolic cascades of the rumen microbiome were identified in the Global Rumen Census project using information from metabolic studies and analysis of the reference genomes (Moraïs and Mizrahi, 2019; Seshadri et al., 2018). However, little effort has been made to study the changes in microbial community and enzyme contents during rumen fermentation of raw lignocellulose, which is an invaluable complement to what has already been learned about lignocellulose degradation in the rumen. The main objective of this study was to investigate the anaerobic biodegradation characteristics of lignocellulosic wastes with WS as a representative substrate in prolonged duration of rumen fermentation with inoculation of cow rumen microorganisms in vitro. Then, the enzymatic activity and microorganisms during rumen fermentation were analysed to elucidate the mechanisms for such an improvement of anaerobic digestibility by cow rumen microbial communities.

2. Materials and methods

2.1. Cow rumen sampling and wheat straw

Samples of rumen contents comprising fluids and solids were taken from the fresh stomachs of a mature cow from a local slaughterhouse in Xi'an, China, and brought to the laboratory in a sealed bottle. The cow rumen contents were mixed before experiment to avoid artificially introducing a source of heterogeneity, and then squeezed through an 18 mesh screen to remove large particles (> 1.0 mm) and flushed with nitrogen gas (N₂) to provide anaerobic conditions after they were loaded and sealed. WS was obtained from a rural area near Xi'an, China. To prepare a homogeneous and reduced-size sample, the WS was cut short and then triturated by a high-speed universal smashing machine (FW100, Tianjin Taisite Instrument Co., Ltd., China) into particles. The volatile solids (VS) contents of above WS clipping were 0.830 g g^{-1} dry substrate. The physicochemical characteristics of the cow rumen fluid used in this study were determined, and they are presented in Table S1.

2.2. Experimental design

A batch assay was conducted to assess the effect of cow rumen microorganisms on the characteristics of WS in rumen fermentation. The experiment was divided into two groups (namely, blank group and experimental group). The blank group (including twelve 120 mL serum bottles) involved rumen fermentation only with cow rumen fluid, as shown in Table S1. The experimental group (including twelve 120 mL serum bottles) was rumen fermentation with cow rumen fluid and a WS additive. To avoid the generation of VFAs in large amounts in such a short time, and the accumulation of VFAs induced acidosis of microorganisms, the addition of the WS and cow rumen fluid occurred at a percentage of 3% (w/v) was relatively suited for the balance of the VFAs production and microorganism growth according preliminary experimental results (data not shown). The tests were performed in duplicate in a water-heating incubator for agitation at 150 rpm and 39 \pm 1 °C for 93 days in order to investigate the persistent action of rumen microorganisms in enhancing lignocellulosic-wastes biodegradation during the rumen fermentation in vitro. Samples of each group were collected at predetermined intervals after 10, 20, 33, 43, 53, and 93 days to identify the persistent action of cow rumen microorganisms in thorough biodegradation of WS. Each time triplicate samples were collected for the analyses. The collected samples in centrifuge tubes were used for chemical analysis. Meanwhile, the biogas was measured during the whole fermentation. On the other hand, the lignocellulose-degrading enzyme contents in rumen fermentation and the microbial community of the two groups were sampled and investigated, and X-ray diffraction (XRD) and Fourier transform-infrared (FTIR) spectroscopy were applied.

2.3. Lignocellulose-degrading enzyme content

Samples were centrifuged at 12,000g for 15 min, and then the supernatant was filtered using a syringe membrane with a 0.45 µm pore size. The sample was diluted with deionized water to obtain absorbance readings in a linear measurement range, and these readings were converted to activity in U/mL. The contents of lignocellulose-degrading enzymes were measured by enzyme-linked immunosorbent assay (ELISA) kits (MSKBIO, Wuhan, China). Absorbance was measured by a multiscan spectrophotometer (Varioskan[™] LUX, Thermo Fisher, Finland) at 450 nm.

2.4. Microbial community analysis

Samples were collected from the initial cow rumen fluid (Blank0) and the two groups on day 93 (Blank93 and WS93, respectively) to characterize the diversity of microbial communities *via* high throughput sequencing technology. DNA was extracted with the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA) according to the manufacturer's instructions. A polymerase chain reaction (PCR) targeting 16S rRNA genes was performed using the forward primer 341F (5'-CCTACGGG NGGCWGCAG-3') and the reverse primer 805R (5'-GACT ACHVGGGTATCTAATCC-3') for bacteria and primers 349F (5'-GYGCASCAGKCGMGAAW-3') and 806R (5'-GGACTACVSGGGTAT CTAAT-3') for archaea. PCR targeting the 18S rRNA gene was performed using the primers Fung (5'-ATTCCCCGTTACCCGTTG-3') and NS1 (5'-GTAGTCATATGCTTGTCTC-3') for eukaryota. After being purified and quantified, the PCR products of the V3-V4 region of the 16S rRNA gene and the NS1-fung region of the 18S rRNA gene were sequenced using the Illumina MiSeq sequencer (Sangon Biotech Shanghai Co., Ltd., China). The obtained sequence fragments were assembled using Flash software. Rarefaction curves and the Shannon diversity index, Chao1 species richness estimator and coverage index were calculated by mothur to identify the species diversity for each sample (Zhang et al., 2017). UCHIME was then used to remove chimaeric sequences, and sequences with >97% similarity were clustered to form operation taxonomic units (OTUs). The ribosomal database project was used for alignment at a confidence threshold of 80% in order to

2.5. Analytical methods

taxonomical classification (Wang et al., 2007).

The content of soluble chemical oxygen demand (SCOD), total chemical oxygen demand (TCOD), total solids (TS), VS, alkalinity, protein, carbohydrate, and NH_{4}^{+} -N were analysed based on standard methods (APHA, 2005). The pH was measured with a portable pH meter (Horiba, Kyoto, Japan). The biogas production, composition of the various biogases (CH₄, CO₂, N₂ and H₂), was measured using a PerkinElmer clarus 680 gas chromatograph equipped with a thermal conductivity detector (TCD) and a 2 m carbon molecular sieve TDX-01 column. The temperatures of the column and the detector were maintained at 140 °C and 150 °C, respectively. VFA levels (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid) were assayed using a Shimadzu GC14B gas chromatograph equipped with a flame ionization detector (FID) using nitrogen as the carrier gas and a DB-FFAP capillary column. The oven temperature was increased from 100 °C to 250 °C. The temperatures of the injector and detector were both set at 250 °C. Analysis of cellulose, hemicellulose, and lignin was conducted using an HPLC system (LC-20A; Shimadzu, Japan) with an Aminex HPX-87H column (300 mm \times 7.8 mm) (Bio-Rad, USA) and a refractive index detector (RID-10A; Shimadzu, Japan) according to the method developed by Sluiter et al. (2008). The crystallinity of raw materials and residues were studied using XRD. The crystallinity index (CrI) is calculated from the ratio between the areas of all crystalline peaks (101, 10ī, 021, 002 and 040) and the total area based on the corresponding XRD patterns (Park et al., 2010). Chemical bond changes were studied by FTIR spectrometer (IR Prestige-21, Shimadzu Corporation, Japan) at $4000-400 \text{ cm}^{-1}$.

2.6. Kinetic modelling

2.6.1. First-order kinetic model

During the batch experiments, experimental data were simulated using a first-order kinetic model (Gunaseelan, 2004). The first-order kinetic model shown as Eq. (1) was used to describe the hydrolysis constant.

$$P = P_0 \cdot [1 - \exp(-kt)] \tag{1}$$

where *P* is biogas production (mL), P_0 is biogas production potential (mL), and *k* is hydrolysis constant (d⁻¹).

2.6.2. First-order exponential decay model

The degradation rate (b_{AN}) of lignocellulose biomass during the rumen fermentation was determined by fitting an exponential function to the measured lignocellulose biomass as a function of time (Eq. (2)) (Xing et al., 2016).

$$Y = A_0 exp(-b_{AN} \cdot t)$$
⁽²⁾

where Y is the remaining lignocellulose biomass (i.e., cellulose,

Y١

hemicellulose, and lignin) percentage of the starting value (%), A_0 is a constant, b_{AN} is the degradation rate (d⁻¹), and *t* is the rumen fermentation time (d). From the exponential degradation Eq. (2), we can define

the degradation efficiency of lignocellulose biomass (Y', %) as

$$= 100 - Y.$$



Fig. 1. Changes and degradation efficiency of cellulose (a and a'), hemicellulose (b and b'), and lignin (c and c'), and biogas (including CO₂, H₂, and CH₄) productions (d and d') of the blank and experimental groups. The dashed line represents the degradation kinetic regression curves.

(3)

2.7. Statistical analysis

All the tests were conducted in triplicate. An analysis of variance was used to assess the significance of results, and p < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Degradation characteristics of lignocellulose in rumen fermentation

3.1.1. Degradation performance of cellulose, hemicellulose, and lignin

The cellulose, hemicellulose, and lignin contents in the blank and experimental groups were measured during the whole rumen fermentation, and the average values of cellulose, hemicellulose, and lignin contents used to calculate the corresponding degradation efficiencies as shown in Fig. 1. At Day 33, the degradation efficiencies of cellulose, hemicellulose, and lignin for the WS substrate were 68.4%, 51.8%, and 25.5%, respectively. At Day 93, the digestion efficiencies of cellulose, hemicellulose, and lignin were 97.6%, 95.8%, and 42.4%, respectively, indicating the continued action of cow rumen microorganisms for achieving almost complete decomposition of cellulose and hemicellulose, and a high lignin decomposition as well. Meanwhile, the first-order exponential decay model regressions agreed well with the degradation efficiency change of cellulose, hemicellulose, and lignin during the whole rumen fermentation ($R^2 = 0.9966, 0.9757, and 0.9634$), and the degradation rate (b_{AN}) of cellulose, hemicellulose, and lignin was 0.0370, 0.0229 and 0.0061 d⁻¹, respectively. These results indicated that the WS without physicochemical pretreatment could not be biodegraded quickly in the rumen fermentation. As shown in Table 1, the overall lignocellulose degradation efficiency has a closely relationship with the substrate type, pretreatment approach, rumen sources, substrate concentration, operated temperature, and fermentation time, and so on. In comparison with previous studies, a fibre content and mass balance analysis showed that lignin in the solid fibre could only be reduced by 5-31% (Hu et al., 2008; Hu and Yu, 2005; Yue et al., 2007a; Zhang et al., 2016). Thus, the efficiency of digesting lignocellulose biomass in this study can be further improved by extending the fermentation time. The lignocellulose biomass, especially for the cellulose, and hemicellulose, were degraded quickly during the initial period, which was consistent with the biogas production as shown in Fig. 1d'. The reason for this phenomenon might be that the part activity of the rumen microflora may be inhibited gradually with accumulation of VFAs or other lignocellulose degradation product. Thus, to maintain the high efficiency of the rumen microflora in vitro, the absorption and transformation of VFAs should be mimicked in artificial rumen systems. The b_{AN} of

Table 1

Comparisons of degradatio	n efficiency and	VFAs yield of lignocellulo	se with rumen fermentation.
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Comparisons of degradation efficiency and VFAs yield of lignocellulose with rumen fermentation.											
Substrate	Pretreatment	Rumen Substrat	Substrate	Operated	Fermentation	Degradation efficiency (%)			VFAs yield	References	
	approach	sources	concentration	temperature	time	Lignin	Cellulose	Hemicellulose			
Corn stover	Mill, $pH > 6.0$	Goat rumen	$10 \text{ g VS } \text{L}^{-1}$	25–40 °C	10 day	31.2%	-	-	0.59-0.71 g g ⁻¹ VS	Hu and Yu (2005)	
Cattail	Mill, $pH = 6.7$	Goat rumen	$4.1 \mathrm{~g~VS~L^{-1}}$	$40 \pm 1 \ ^\circ C$	6 day	-	-	-	$0.406 \text{ g g}^{-1} \text{ VS}$	Hu et al. (2006)	
Cattail	Mill, pH==6.7-7.2	Goat rumen	5.3 g VS L^{-1}	40 ± 1 °C	5 day	-	-	-	$0.443 \text{ g g}^{-1} \text{VS}$	Yue et al. (2007a, 2007b)	
Canna	Mill	Goat rumen	6.9 g VS L^{-1}	40 °C	5 day	5.2%	59.0%	72.5%	0.362 g g ⁻¹ VS _{degraded}	Yue et al. (2007a, 2007b)	
Wheat straw	Dewaxed with toluene/ethanol	Goat rumen	-	39 ± 1 °C	13 day	25.5 ± 2.0%	44.0 ± 3.3%	$43.2 \pm 2.4\%$	-	Hu et al. (2008)	
Rice straw	Mill	Cattle rumen	2.5% w/v	39 °C	5 day	20.6%	47.8%	58.9%	$0.36 \text{ g s}^{-1} \text{ VS}$	Zhang et al. (2016)	
Corn stover	Mill	Cattle rumen	3% w/v	39 °C	3 day	-	-	-	~0.336 g g ⁻¹ VS	Li et al. (2017)	
Wheat straw	Mill	Cow rumen	3% w/v	39 °C	33 day	25.5%	51.8%	68.4%	0.453 g COD g ⁻¹ VS	This study	
Wheat straw	Mill	Cow rumen	3% w/v	39 °C	93 day	42.4%	97.6%	95.8%	0.484 g COD g^{-1} VS	This study	

cellulose and hemicellulose in the blank was 0.0216 and 0.0173 d^{-1} (Fig. 1), which lower than that of the experimental group as mentioned above. However, the b_{AN} of lignin in the blank was 0.0106 d⁻¹, which faster than that of the experimental group (0.0061 d^{-1}) . The difference between lignin and cellulose/hemicellulose may be due to the change of substrate in the blank and experimental groups. Furthermore, a lower lignin degradation rate and higher lignin contents were achieved compared with these of cellulose and hemicellulose during the whole rumen fermentation, indicating that lignin degradation was the limiting step for the hydrolysis process. Lignin-degrading bacteria (actinomycetes, α -proteobacteria, and γ -proteobacteria) and fungi (such as white-rot, brown-rot, and soft-rot fungi) can completely degrade lignin (Bugg et al., 2011). Generally, biological pretreatment is commonly performed by employing pure cultures of bacteria and fungi. Fungal pretreatment, especially pretreatment with white-rot fungi, is predominantly used in biological pretreatment of biomass due to its high efficiency and downstream yields. According to recent reports, delignification of lignocellulosic biomass by white-rot fungi may vary between 3% and 72% (Zabed et al., 2019). For example, approximately 3% of lignin in WS was effectively delignified by a white-rot fungal strain (Ceriporiopsis subvermispora) after 18 days of pretreatment (Wan and Li, 2011), while a fungal pretreatment (*Trametes hirsuta*) of corn stovers resulted as much as 72% removal of lignin after 42 days (Sun et al., 2011). As shown in Table 1, the lignin degradation efficiency of 25.5-42.4% was achieved after prolong the rumen fermentation time from 33 to 93 days. Therefore, the high degradation efficiencies of cellulose, hemicellulose, and lignin in this study suggested that the ruminant microflora can increase the biodegradability of WS through prolong the rumen fermentation time.

As shown in Fig. 1d', CO₂, H₂, and CH₄ were the main components of the biogas during the whole rumen fermentation, and the corresponding contents were 97.4 \pm 1.8%, 0.4 \pm 0.1%, and 2.2 \pm 1.8%. Fungal groups can produce a large amount of H₂ and CO₂ while decomposing cellulose. However, the formation of other metabolites such as acetate, butyrate etc. hinders anaerobic fermentative biohydrogen production (Srivastava et al., 2019), which may be a reason for the low practical H₂ yield. Meanwhile, the low pH in rumen fluid of approximately 5.45 (Table S1) was beyond the methanogenic bacterial optimum pH range of 6.8-7.2, which was also consistent with the low richness and diversity indexes of archaea shown in Section 3.3. Zou et al. (2011) reported that CO₂ was the main component of the gaseous phase in the rumen. Therefore, CO₂ production may be used as an indicator to evaluate the degradation efficiency of lignocellulose biomass in rumen fermentation. The experimental group had higher biogas production than the blank (Fig. 1), suggesting that WS was hydrolysed during the rumen

fermentation. As shown in Fig. 1, 90% of the biogas was collected from the rumen fermentation of the blank and experimental groups after approximately 26 and 40 days, respectively. The first-order kinetic model regressions agreed well with the change in biogas content during the whole rumen fermentation ($R^2 = 0.9839$ and 0.9884), and the hydrolysis constant (k) of the blank and experimental groups were 0.1193 and 0.0576 d⁻¹, respectively. These results were in accordance with the digestion rate of lignocellulose biomass as shown in Fig. 1, indicating the degradation rate has a positively correlation with the hydrolysis constant.

In particular, the major roadblocks to the biological treatment of lignocellulosic biomass are the requirement of a pretreatment time relatively longer than the time needed for other contemporary thermochemical approaches (Zabed et al., 2019). To reduce the pretreatment time, the microbial consortium system offers some other advantages over single microbial pretreatments, such as increased adaptability, improved hydrolysis efficiency and productivity, pH control, and increased substrate utilization. Carrillo-Reves et al. (2016) reported that rumen microflora might be used as microbial consortia for biomass pretreatment. A high lignocellulosic degradation efficiency was achieved with the pretreatment time extension in this study. As mentioned above, the degradation time can be further decreased through immediate VFA conversion. On the other hand, combining these methods with other pretreatment methods before rumen fermentation can further shorten the fermentation time. For example, the fragments of the WS were dewaxed with toluene/ethanol in a Soxhlet apparatus for 10 h, and then the degradation efficiencies of lignin, cellulose, and hemicellulose of WS fermented in rumen fluid were 25.5 \pm 2.0%, 44.0 \pm 3.3%, and 43.2 \pm 2.4% after a 13 day fermentation (Hu et al., 2008). Hu et al. (2012) reported that microwave irradiation pretreatment could be used for enhancing anaerobic digestibility of lignocellulosic wastes by rumen microorganisms. Therefore, inoculation with rumen fluid, addition of a VFA absorption system, and combinations with other pretreatment approaches can be used to further enhance the degradation rate in in vitro rumen fermentations by rumen microorganisms, which needs be further confirmed and studied in the future.

3.1.2. XRD and FTIR

To elucidate the mechanisms for WS degradation in cow rumen fluid in vitro, both XRD and FTIR were used to evaluate the chemical structure changes of the raw WS (WS0) and cow rumen residual lignocellulose. As shown in Fig. 2, the XRD pattern of lignocellulose derived from the blank and experimental groups showed several characteristic diffraction peaks of crystalline cellulose at approximately 17.3°, 21.8°, and 34.6°. The identified characteristic peaks of cellulose were consistent with those of previous reports (Zhang et al., 2018). After 33 days of rumen fermentation, the peak intensities of Blank33 and WS33 samples at 2θ values of approximately 17.3°, 21.8°, and 34.6° decreased significantly compared to those of BlankO and WSO (p < 0.05, Fig. 2). The CrI clearly indicated the demolition of the crystal structure of cellulose (Wu et al., 2010). Any reduction in the crystallinity of cellulose would be associated with increases in cell wall fragility and susceptibility to attack by cellulolytic microorganisms (Hu et al., 2012). The CrI values of approximately 60.8% and 82.7% for BlankO and WSO were decreased to 36.7% and 69.1%, after 33 days of rumen fermentation (Fig. S1). Comparing the blank group (rumen residues alone) and the WS experiment, a higher relative reduction of CrI value occurred to the blank group, which mainly due to the structure of cellulose in the blank group was modified during the *in vivo* rumen fermentation period. Furthermore, the corresponding CrI of Blank33 and WS33 decreased slightly from 36.7% and 69.1% to 34.4% and 68.9%, respectively, by extending the fermentation time by approximately 60 days, which was in accordance with the change trends in the cellulose degradation efficiency (Fig. 1). Obviously, several characteristic diffraction peaks at 2θ values of approximately 28.2°, 40.4°, and 50.0° were detected in WS33 and Blank33 and not detected in WS0 and Blank0. Furthermore, the peak intensities at 2θ values of approximately 28.2°, 40.4°, and 50.0° in WS33 were higher than those in Blank33, and all of these intensities further decreased after 60 days of rumen fermentation (Fig. 2). These results



Fig. 2. X-ray diffraction (XRD) patterns (a and a') and FTIR spectra (b and b') of the blank and experimental groups at different fermentation times. Blank0: sampled from the initial cow rumen fluid; WS0: sampled from the raw WS; Blank33 and Blank93: sampled from the blank group after 33 and 93 days, respectively; WS33 and WS93: sampled from the experimental group with WS after 33 and 93 days, respectively.

indicated that WS was degraded by the action of cow rumen microorganisms *in vitro*.

The structural changes in cellulose, hemicellulose, and lignin could be extensively identified by using characteristic absorbance bands in FTIR spectra (Hu et al., 2008). The FTIR spectra of WS and cow rumen residual lignocellulose during the whole rumen fermentation are presented in Fig. 2. Several FTIR peaks of cellulose (1375 and 897 cm^{-1}), hemicellulose (1736 cm^{-1}), and lignin (1513 and 1252 cm^{-1}) were only shown in the experimental group but not the blank groups, which mainly due to the raw WS additive increased the content of lignocellulose contents in the experimental group. The corresponding assignments are listed in Table 2. The relative absorbances of the characteristic bands of lignin at 1513, 1252 and 1158 cm⁻¹ slightly decreased, and that at 1416 cm⁻¹ increased and then decreased with fermentation, which indicated the modified and degradation of lignin in WS. From the blank results (Fig. 2b), the relative absorbances of the characteristic bands of cellulose at 1644 and 1048 cm⁻¹ decreased and that at 2920 $\rm cm^{-1}$ increased, which suggested that the amount of absorbed water, represented by this bending vibrational band, decreased, which would be consistent with the degradation of cellulose shown in Fig. 1. For the experimental group (Fig. 2b'), the relative absorbances of the characteristic bands of cellulose at 2920, 1375, 1051, and 897 cm^{-1} decreased, and that at 1651 cm^{-1} increased first and then decreased with increasing fermentation time. Moreover, the characteristic peak of WS at 667 cm^{-1} also decreased gradually, which indicated that the intermolecular hydrogen bonds in crystalline cellulose were broken by cow rumen microorganisms. Hydrogen bonds between different layers of the polysaccharides contribute to the resistance of crystalline cellulose to degradation (Rubin, 2008). These results indicated that the degradation of cellulose and the decrease in crystalline cellulose were consistent with the decrease in CrI as mentioned above. Additionally, the relative absorbance of the characteristic peak of hemicellulose at 1736 cm^{-1} decreased, which was consistent with the decrease in hemicellulose content after 93 days of rumen fermentation in vitro (Fig. 1). In addition, the relative absorbance of the characteristic bands at 1560 cm⁻¹ occurred in the rumen fermentation, which may be related to several characteristic diffraction peaks at 2θ values of approximately 28.2°, 40.4°, and 50.0°, as shown in Fig. 2. Further studies to define these changes by using NMR methods and molecular vibrational spectroscopy imaging technology will help to explore the lignocellulose degradation mechanism in rumen fermentation using rumen microorganisms in vitro.

3.2. Degradation products of lignocellulose in rumen fermentation

3.2.1. SCOD and VFAs

The high hydrolysis and acidification efficiency for lignocellulosic substrates is the most attractive advantage of using rumen microorganisms (Yue et al., 2013). The main intermediates produced in the aqueous phase such as SCOD, protein, NH_4^+ -N, carbohydrate, and VFAs were investigated as shown in Fig. 3. At the beginning of the rumen

Table 2

Characteristic neaks of cellulose hemicellulose and lignin in FTIR analy	Sis
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Characteristic peak
Intermolecular H-bonds
C-H deformation in cellulose
C-O-C pyranose ring skeletal vibrations in cellulose
Aromatic C—H in-plane deformation, guaiacyl in lignin
Guaiacyl ring breathing with C=O stretching in lignin
C-H stretching in crystalline cellulose
Aromatic skeletal vibrations in lignin
Absorbed water bending in cellulose
C=O in hemicellulose
C-H in cellulose
O-H vibration and intramolecular H-bonds
N-H bend

fermentation *in vitro*, WS0 was estimated to be similar to Blank0. The SCOD concentration of the blank and experimental groups increased greatly and then decreased slightly, which was consistent with the change tendency of protein and NH⁴₄-N but contrasted with carbohydrate content changes. The SCOD, protein, and carbohydrate levels of the experimental group in WS33 were higher than those of the initial cow rumen fluid (Fig. 3a), which indicated that the WS in rumen fermentation with rumen microorganisms was degraded into SCOD, including VFAs and carbohydrates. When the rumen fermentation time extended from 33 to 93 days, the low degradation rate of lignocellulose with limited SCOD production cannot satisfy the endogenous metabolic consumption of microorganisms, as shown in Fig. 3a. Therefore, the fermentation time must be further reduced based on the acceptable SCOD yield and degradation efficiency of lignocellulose.

VFAs are important intermediates produced in large amounts during ruminal fermentation and are also major nutrient sources for the host animal, contributing significantly to ruminant productivity (Henderson et al., 2016). During artificial applications, VFAs could be used to produce high-value-added products such as CH₄, H₂, bioplastics, and electricity and to enhance denitrification as a carbon source and in other ways (Sivagurunathan et al., 2017). During the rumen fermentation in this study, the main VFA products generated were butyric and valeric acids, which were accompanied by a small amount of acetic, propionic, isobutyric, and isovaleric acids at pH 5.45 (Fig. 3b). This result was not consistent with those of Hu et al. (2004), who reported that acetic acid was the major aqueous product at pH values <5.5. In this study, high VFA yields > 0.45 g COD g⁻¹ VS with high cellulose, hemicellulose, and lignin degradation efficiencies were achieved at pH 5.45; these values were higher than most of those for lignocellulose biomass fermentation with rumen inocula (Hu et al., 2008; Hu and Yu, 2005; Yue et al., 2007a; Zhang et al., 2016). Those results confirmed that the WS was effectively converted into VFAs by cow rumen microorganisms at the low pH of 5.45 without chemical addition and other chemical pretreatment.

The pH affects the growth of microorganisms and the secretion of enzymes. The optimal pH of most lignocellulose-degrading enzymes was approximately 4.5-6.0 (Lange et al., 2019). Hu et al. (2006) found that a pH of 6.9 was optimal for the acidogenesis of cattails by rumen cultures. Therefore, maintaining the optimal pH in rumen fermentation was beneficial in achieving a high degradation efficiency of lignocellulose biomass. In general, elevated levels of NH₄⁺-N can increase the pH of the rumen fluid. As shown in Fig. 3a, both the NH₄⁺-N contents in the blank and experimental groups increased, which is conducive to increasing the pH in the cow rumen. On the other hand, the alkalinity concentrations in the two groups also increased, from the initial value of 12.3 to 22.5 and 26.25 g CaCO₃/L after 33 days fermentation (Fig. 3b), respectively, which indicated that the produced alkalinity maintained the pH under the accumulation of VFAs. With the fermentation time extending from 33 to 93 days, the VFA yield of WS was further improved from 0.453 to 0.484 g COD g^{-1} VS without the pH decreasing, and the alkalinity was decreased to near the original level, which suggested that the huge natural buffering ability of cow rumen fluid is important for a high degradation efficiency.

3.2.2. Lignocellulose-degrading enzymes

The enzymes secreted by ruminal bacteria and fungi play an important role during rumen fermentation (Sauer et al., 2012), and enzyme activities were measured at predetermined intervals (Fig. 4). The predominant enzymes secreted by the cow rumen microorganisms were EG, CMCase, xylitolase, xylan esterase, and Lac along with a small amount of xylanase, BG, LiP, MnP, and CBH. The initial concentrations of cellulolytic enzymes (134.4 U/mL), hemicellulolytic enzymes (125.3 U/mL), and ligninolytic enzymes (0.3 U/mL) in rumen fluid were positively correlated with the degradation efficiency of cellulose (97.6%), hemicellulose (95.8%), and lignin (42.4%) at the end of the cow rumen fermentation with WS as shown in Fig. 1. In the early



Fig. 3. Changes in (a) SCOD, protein, NH⁴₄-N, carbohydrate, and (b) VFA concentrations, alkalinity, and the pH of the blank and experimental groups at different fermentation times.

stage of the rumen fermentation (0–33 days), the lignocellulosedegrading enzyme levels in Blank33 had no obvious changes. However, the EG and CMCase contents in WS33 were decreased, which may be due to the rumen microorganisms being quickly adsorbed by the WS as reviewed by Miron et al. (2001). On the other hand, the changes in microbial status could alter the microbial enzyme activities. Therefore, the slightly increased Lac content in WS33 was attributed to the increased abundance and activity of ligninolytic microorganisms as shown in Section 3.3. After 93 days of fermentation, the EG and CMCase activities in WS93 were decreased and then restored to their original levels (Fig. 4). Moreover, the CMCase activity in the blank group was increased during the whole fermentation process. These results suggested that some rumen strains still could grow in prolonged duration of rumen fermentation *in vitro*, secrete lignocellulose-degrading enzymes such as CMCase, EG, and Lac, and continue to achieve a high degradation efficiency of cellulose, hemicellulose, and lignin.

3.3. Analysis of the microbial community

3.3.1. Species diversity and richness

To develop an in-depth understanding of microbial community changes with WS substrate in cow rumen fluid *in vitro*, the richness and diversity indexes of bacterial, fungal and archaeal communities after 93 days of rumen fermentation are shown in Table 3. The coverage



Fig. 4. Changes in the lignocellulolytic enzymes activity of the blank and experimental groups over different fermentation times.

Table 3						
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Richness and diversity indexes of bacterial, fungal and archaeal communities.

	Specimens	Sequence number	OTUs	Shannon	ACE	Chao1	Coverage	Simpson
Bacteria	Blank0	35121	1296	4.859	1564	1511	0.9910	0.0348
	Blank93	43580	998	3.626	1407	1343	0.9921	0.1367
	WS93	57609	911	3.472	1674	1445	0.9936	0.0914
Fungi	Blank0	42048	184	2.850	204	203	0.9994	0.1331
	Blank93	65343	163	2.246	292	214	0.9993	0.2484
	WS93	53810	132	2.026	211	180	0.9993	0.2476
Archaea	Blank0	74887	73	1.825	199	124	0.9995	0.2155
	Blank93	35902	80	1.232	272	191	0.9989	0.5256
	WS93	44223	199	1.898	491	334	0.9979	0.2478

for bacteria, archaea, and fungi was >0.99, indicating that most sequences were detected by high-throughput sequencing. The abundance of bacteria in the cow rumen fluid (84.0%) was larger than that of archaea (1.04%), fungi (0.04%), protozoa, and viruses through metagenomics sequencing (data not shown), indicating that bacteria predominated the rumen and were accompanied by a variety of archaea and fungi, which was consistent with results by Henderson et al. (2016). For the rumen bacteria, the number of operation taxonomic units (OTUs) and the Shannon and Chao1 indexes in WS93 and Blank93 were decreased compared to those of BlankO, and higher ACE/Chao1 indexes were found in WS93 than in Blank93 (Table 3). These results suggested that the diversity and richness of rumen bacteria were decreased in rumen fermentation in vitro with or without WS, and that the abundances of some special rumen bacteria such as Lachnospiraceae_NK3A20_group, p-1088-a5_gut_group, Caproiciproducens, Pirellula, and unclassified_Clostridiaceae were enhanced with WS substrate by compared to the blank group (Fig. 5a). Meanwhile, the OTUs and Shannon index for the rumen fungi were also decreased in the two groups after 93 days of rumen fermentation in vitro, and a lower diversity and abundance (lower Shannon and ACE/Chao1 indexes) was found in WS93 than in Blank93. Obviously, the diversity and abundance of bacteria and fungi were decreased under the mono-substrate (i.e., WS) supplied in experimental group and depleted of rumen residues in blank group. On the other hand, contrasting observations were found for the rumen archaea, which increased in abundance in WS93 and Blank93 under sufficient VFAs during the 93-day incubation as shown in Fig. 3b. These results indicated that the change of microbial flora in rumen fluid with the WS substrate was larger than that in rumen fluid without the WS substrate.

3.3.2. Bacterial community

As shown in Fig. 5, the taxonomy of the OTUs of Blank0, Blank93, and WS93 was classified, and genera with a relative abundance (RA) higher than 1% were defined as the dominant genera. Bioinformatic analysis revealed that the bacterial sequences were predominantly affiliated with three phyla (Proteobacteria, Bacteroidetes, and Firmicutes) in all the samples; these phyla were similar to those described in an earlier meta-analysis of rumen microbial communities worldwide (Henderson et al., 2016; Seshadri et al., 2018). After 93 days of rumen fermentation in vitro, the RA of the Proteobacteria and Bacteroidetes phyla decreased from 38.73% and 33.52% to 5.50% (3.60%) and 6.56% (2.70%) in Blank93 (WS93), respectively. However, the RA of the phylum Firmicutes increased from 20.57% to 60.28% (69.64%) in Blank93 (WS93). Compared with their RA values in Blank93, a lower RA of the phyla Proteobacteria and Bacteroidetes and a higher RA of the order Clostridiales were achieved in WS93, indicating that Clostridiales species could adapt to the WS rumen fermentation conditions and gradually become the predominant lignocellulolytic bacteria for the degradation of WS substrate in the rumen in vitro along with the extension of fermentation time.

As shown in Fig. 5a, the dominant bacterial genera belonging to the phyla Proteobacteria (38.73%) and Bacteroidetes (33.52%) in Blank0 were *Acinetobacter* (17.06%), *Pseudomonas* (5.80%), *Janthinobacterium*

(5.58%), and Prevotella (8.11%), which have the ability to breakdown protein to amino acids and ammonia (Moraïs and Mizrahi, 2019). This result was consistent with the increase in ammonia concentration from a low NH_4^+ -N value in Blank0 (Fig. 3a). The low NH_4^+ -N value in Blank0 was mainly owing to the fact that the ammonia can be immediately transformed into microbial protein and digested by animal during the rumen fermentation in vivo (Moraïs and Mizrahi, 2019). Furthermore, the genus Pseudomonas also degrades cellulose and lignin to produce hexose sugars, methanol, and methylamines (Seshadri et al., 2018). The genera Prevotella (8.11%), Dysgonomonas (2.70%), and Myroides (2.15%) belong to the phylum Bacteroidetes and have the ability to secrete hemicellulases to produce acetate, butyrate and propionate, as shown in Fig. 3b. For the Firmicutes phylum in BlankO, the high RA of Succiniclasticum (2.43%) was favourable for the generation of succinate from pyruvate to produce more propionate. Low RA of Clostridium_sensu_stricto (0.62%) in Blank0 limited the valerate concentration. After 93 days of rumen fermentation, the RA of the phyla Firmicutes (Succiniclasticum, unclassified_Clostridiaceae_1, and unclassified_Family_XI), Bacteria (unclassified_Bifidobacteriaceae, DNF00809, and Olsenella), and Planctomycetes (Pirellula and p-1088a5_gut_group) in Blank93 were improved. Meanwhile, the RA reduction in Proteobacteria (Acinetobacter, Janthinobacterium, and Pseudomonas) and Bacteroidetes (Prevotella, Dysgonomonas, and Myroides), and the gradual depletion of protein in the blank group indicated that the protein-degrading bacteria no longer belonged to dominant flora, which was consistent with the changes in ammonia concentration (Fig. 3a). The Pseudomonas genera, which were decreased in a low RA (2.26%) from 5.80%, secreted a small amount of ligninolytic enzymes (LiP, Lac, and MnP), which made the lignin removal efficiency not as high as cellulose and hemicellulose removal efficiency (Figs. 1 and 5).

As shown in Fig. 5a, the members of the Clostridiales order (Lachnospiraceae NK3A20 group, Caproiciproducens, unclassified_Clostridiaceae_1, and unclassified_Family_XI, etc.) were more abundant in WS93 compared to Blank93. The order Clostridiales secretes cellulase and hemicellulose to produce acetate and valerate (Seshadri et al., 2018). However, the RA of Succiniclasticum decreased slightly in WS93 compared with Blank93, which has the ability to produce propionate, as mentioned above. Thus, more acetate, butyrate, and valerate were produced in WS93 compared with Blank93, as shown in Fig. 3b. Furthermore, the RA of the Proteobacteria (Pseudomonas), Bacteroidetes (Prevotella), and Synergistetes (Pyramidobacter) phyla were further decreased slightly in WS93 compared with Blank93, which may be due to the gradual depletion of nutrients in the substrate and the accumulation of VFAs and ammonia inhibiting the growth, hydrolysis and activity of those bacteria.

3.3.3. Fungal community

As shown in Fig. 5b, the fungal sequences were predominantly affiliated with the phyla Ascomycota, Basidiomycota, and Neocallimastigomycota in all the samples, which consistent with the previous findings that they are the most abundant phyla in the rumen of dairy cows (Kumar et al., 2015). The RA of the phylum Neocallimastigomycota decreased from 30.57% to 0.01%, and that of



Fig. 5. Taxonomic classification, relative abundances (RA) and the RA increment values (Δ RA) of the dominant bacterial (a), fungal (b), and archaeal groups (c) between Blank0, Blank93, and WS93.

the phyla Ascomycota and Basidiomycota increased and decreased by 35.8% and 3.32%, respectively, in Blank93 compared with Blank0. The phylum Ascomycota is the largest group in the fungal kingdom, and its members mainly act as degraders of lignin in nutrient cycling (Beimforde et al., 2014). This may be the reason for the high lignin removal rate in the blank shown in Fig. 1. These changes in RA of above phyla implied that the Ascomycota phylum was gradually become the predominant lignocellulolytic fungi for the degradation of cow rumen residual lignocellulose along with the extension of fermentation time. Meanwhile, the RA of the Ascomycota and Basidiomycota phyla decreased and increased by 4.3% and 32.14% in WS93 compared with Blank93, respectively. The Basidiomycota are important contributors to ecosystem functioning at multiple levels and are the major degraders of lignin (Taylor et al., 2015). These results indicated that members of the Basidiomycota phylum were the predominant lignocellulolytic fungi for degradation of WS substrate in the rumen in vitro.

As shown in Fig. 5b, the dominant fungal genera in Blank0 were Pecoramyces (30.57%), Fusarium (12.96%), Valsa (5.93%), Didymella (8.90%), Cladosporium (5.34%), Aspergillus (4.54%), Wickerhamiella (3.38%), unclassified Boletales (6.39%), and unclassified Eukaryota (3.37%). These results suggest that the anaerobic rumen fungi of cow are largely occupied by a series of genera, which differs from those previously described for rumen (Kittelmann et al., 2012; Mura et al., 2019). After 93 days of rumen fermentation, the RA of Fusarium, Valsa, Diaporthe, Microascus, Corynespora, and Microsporum, which belong to the phylum Ascomycota, in Blank93 improved (Fig. 5b). The RA of the genus Wallemia (within Basidiomycota) was distinctly increased in WS93 compared with Blank93, and it, Fusarium and Valsa became the dominant fungi, as shown in Fig. 5b. Additionally, lignocellulolytic fungi produce a variety of lignocellulolytic enzymes that are responsible for the biodegradation of lignocellulosic agriculture waste in nature (Dashtban et al., 2009). However, to clarify analysis, lignocellulolytic enzymes produced with different rumen fungi should be further studied in the future.

3.3.4. Archaeal community

Nearly all archaea were identified as methanogens known to be residents of the rumen (Fig. 5c), and their RA values were comparable to those of previous studies (Janssen and Kirs, 2008). Alignment of the archaeal ratios showed that the archaea of all the rumen samples (BlankO, Blank93 and WS93) fell into the phylum Euryarchaeota (> 99.95%), showing that rumen archaea are much less diverse than rumen bacteria. Henderson et al. (2016) has reported that the dominant archaeal groups are remarkably similar in all regions of the world. In the present study, the majority of sequence reads were assigned to Methanomassiliicoccus, Methanobrevibacter, Methanosphaera, Methanoculleus, and Methanothrix, and the RA of the hydrogenotrophic methanogens (Methanomassiliicoccus, Methanobrevibacter, Methanosphaera, and Methanoculleus) were higher than those of acetoclastic methanogens (Methanothrix). Many researchers have indicated that acetoclastic methanogens are more sensitive than hydrogenotrophic methanogens to ammonium inhibition (Yang et al., 2019). Thus, the acetate-utilizing methanogens were inhibited with a high NH⁺₄-N concentration, as shown in Fig. 3a. After 93 days of rumen fermentation, the RA of the Methanosphaera genus was increased from 1.6% to 79.1% in Blank93, and this genus then became the dominant archaeal genus. Meanwhile, the abundance of the genus Methanobrevibacter in WS93 also increased, from 29.5% to 72.4% (Fig. 5c). The similar metabolic cascades of the dominant archaeal groups suggested that it possible to mitigate CH₄ emissions from ruminants, with the aim of reducing CH₄ emissions (Lan and Yang, 2019; Pérez-Barbería, 2017). From the perspective of an industrial application, however, there is no need to control the dominant hydrogenotrophic methanogens in the artificial rumen system. In contrast, these hydrogenotrophic methanogens should be enriched in anaerobic digestion to convert VFAs to CH₄.

3.4. Degradation processes of lignocellulose in rumen fermentation

To develop an in-depth understanding of the mechanisms of lignocellulose degradation in rumen fermentation, the WS degradation processes were derived from measurements of end product fluxes in combination with the main metabolic pathways of the rumen microorganisms reviews of the published literature (Russell and Rychlik, 2001; Seshadri et al., 2018). The hydrolysis of macromolecules (lignocellulose and protein) in rumen was the initial and limiting stage in achieving a high degradation efficiency in rumen fermentation by the microbial communities that grew and produced VFAs. As shown in Fig. 6, the protein in rumen was mainly degraded by the genera Acinetobacter, Pseudomonas, Janthinobacterium, and Prevotella, and then amino acids and ammonia were transformed into microbial protein and digested by the ruminants. The major degradation processes of lignocellulose breakdown by bacteria (the genera Clostridium_sensu_stricto, Pseudomonas, and Prevotella) and fungi (the genus Pecoramyces and phyla Ascomycota and Basidiomycota) is the production of cellulolytic enzymes, hemicellulolytic enzymes, and ligninolytic enzymes that catalyse various biochemical reactions for lignocellulose breakdown. On the other hand, since most rumen cellulose-degrading bacteria are non-motile, a tunnelling mechanism might be performed by some fungi and protozoa (Hu et al., 2008). The fermentation products (amino acid, pentose, and hexose sugar) from primary degraders can be converted to pyruvate as reported by previous studies (Russell and Rychlik, 2001; Seshadri et al., 2018). During the fermentation processes, the pyruvate was further degraded and converted to CO₂, H₂, and VFAs (formate, acetate, butyrate, propionate, and valerate). The RA values of the functional bacteria are closely related to the specific fermentation end product concentrations. The corresponding functional bacteria in each metabolic pathway for VFAs were distinguished, as shown in Fig. 6. As discussed in Section 3.3, the changes in the microbial community during rumen fermentation in Blank93 and WS93 were in accordance with the final fermentation products. However, through the current data or bioinformatics approaches, we still can't determine a certain enzyme was expressed by which microbe because of the ruminal niches, enzymes, and reaction pathways of the most ruminal bacteria and fungi are still unclear. Therefore, further research avenues focused on a comprehensive analysis of the physiology and epigenetics underlying lignocellulose-degrading enzyme production and metabolism pathways of ruminal bacteria and fungi are warranted. For the electron sink stage, the methanogens using CO₂, H₂, formate, methanol, methylamines, and acetate to synthetize CH₄ were well known from previous studies (Moraïs and Mizrahi, 2019; Seshadri et al., 2018). Therefore, a clear understanding of the complex lignocellulosic fermentation pathways of the rumen microorganisms includes the potential for the mass production of rumen to test and inoculate commercial anaerobic digesters for the most efficient utilization of lignocellulosic feedstocks.

4. Conclusions

The rumen is a natural ecosystem with the capacity to efficiently digest lignocellulosic biomass to VFAs. To convert the lignocellulosic waste to energy products and alleviate energy consumption, the biodegradation characteristics and products of WS in the prolonged duration of rumen fermentation with inoculation of cow rumen microorganisms *in vitro* were investigated in this study. The low degradation rate of cellulose, hemicellulose, and lignin was 0.0370, 0.0229 and 0.0061 d⁻¹, respectively, indicating that the WS without physicochemical pretreatment could not be biodegraded quickly. However, continuous conversion of WS to VFA and biogas proceeded smoothly in the prolonged duration of 93 days. A high degradation efficiency of cellulose (97.6%), hemicellulose (95.8%), and lignin (42.4%) and a high VFA yield (0.484 g COD g⁻¹ VS) were achieved by the persistent action of cow rumen microorganisms, which was in accordance with the changes in biogas and XRD and FTIR spectra. A large natural buffering



Fig. 6. Lignocellulosic metabolic pathways of the rumen microorganisms during rumen fermentation in this study.

ability, highly abundant and diverse rumen bacteria, a variety of fungi, and the presence of lignocellulose-degrading enzymes in cow rumen fluid were the main reasons for the persistent degradation of lignocellulosic wastes. Moreover, the diversity of the microbial flora in rumen fluid with WS substrate was determined to be higher than that in the cow rumen fluid without WS through high-throughput sequencing analysis. The Clostridiales order and Basidiomycota phylum were gradually became the dominant lignocellulolytic bacteria and fungi for the degradation of raw WS during the 93-day incubation. Furthermore, the WS degradation processes were derived from catalysed by cellulolytic, hemicellulolytic, and ligninolytic enzymes secreted from bacteria (the genera Clostridium_sensu_stricto, Pseudomonas, and Prevotella) and fungi (the genus Pecoramyces and the phyla Ascomycota and Basidiomycota). Additionally, the dominant hydrogenotrophic methanogens (Methanomassiliicoccus, Methanobrevibacter, Methanosphaera, and Methanoculleus) in rumen could also assist CH₄ production if the rumen fermentation was followed with anaerobic digestion.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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