RESEARCH ARTICLE

Mannan oligosaccharides enhance rumen cellulose degradation by modulating microbial composition and glycoside hydrolase expression

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The ruminal microbial structure is one of the important factors affecting cellulose degradation. The mannan oligosaccharides (MOS) have the potential to regulate the structure of rumen flora and are worth further exploring the molecular mechanisms by which MOS influence microbial community structure and the expression of genes related to cellulose degradation. This study systematically evaluated the effects of MOS regulation on fermentation parameters, the structure of microbial communities, and the potential functions of cellulose degrading microbiota isolated from the rumen of dairy cows. Microcrystalline cellulose was utilized as the sole carbon source over five subcultures to select and enrich an efficient cellulose degrading microbiota from the rumen. The effects of the addition of MOS on the microbial and its functions were investigated by analyzing fermentation parameters and metagenomic sequences. The result showed that MOS supplementation significantly enhanced the relative abundance of *Fibrobacter succinogenes* and *Megasphaera elsdenii* ($P < 0.05$), upregulated the expression of essential glycoside hydrolases (GH6, GH5_29, and GH25) ($P < 0.05$), increased the volatile fatty acid production and cellulase activity ($P < 0.05$). Additionally, the energy metabolism pathways were significantly enriched ($P < 0.05$). These findings provided valuable insights into the molecular mechanisms of MOS regulated cellulose degradation, thereby optimizing rumen function to improve ruminant productivity.

Keywords: ruminal microbial structure; mannan oligosaccharides; glycoside hydrolases; passage cultured.

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Introduction

Ruminants can use a unique rumen microbial ecosystem to specialize in the bioconversion of recalcitrant, complex carbohydrates into energy [1]. Previous studies have exercised that the ruminal microbial structure is one of the important factors affecting cellulose degradation [2]. Notably, the ruminal microbial structure was optimized through the regulation of exogenous additives to improve feed degradation efficiency [3]. There is meaningful to broaden knowledge of rumen microbial interactions and enzyme mechanisms that are employed within the rumen microbiome for efficiently optimization of feed conversion and ultimately the productivity of the host animal.

Mannan oligosaccharides (MOS) are functional oligosaccharides that have garnered significant

interest in recent years within ruminant nutrition research [4]. Studies have shown that dietary supplementation of MOS can improve cellulose digestibility by increasing the population of cellulolytic bacteria in the rumen. Zheng *et al*. reported that supplementing sheep diets with 1.6% MOS significantly improved carbohydrate digestibility [5]. Similarly, the addition of 0.6 g of MOS to beef cattle diets increased the abundance of *Ruminococcus albus* 7 and *Ruminococcus flavefaciens* FD-1, which both were the key species involved in cellulose degradation [6]. Although MOS has positive effects on the cellulose degradation of rumen cellulose degrading bacteria, the underlying regulatory mechanisms remain unclear. Cellulolytic bacteria can utilize various oligosaccharides as carbon sources with each oligosaccharide exerting distinct effects on bacterial growth and metabolism including the inhibition or induction of glycoside hydrolase production. Xylo oligosaccharides have been confirmed to enhance the expression of GH10 genes, which are associated with xylan degradation in the rumen [7].

Based on these findings, we hypothesized that MOS might regulate the composition of cellulolytic bacteria within the rumen, thereby modulating the expression of key glycoside hydrolase genes and promoting efficient fiber degradation. The effects of MOS on the fermentation functionality of rumen cellulolytic bacteria were systematically evaluated in this study using subculturing method that leveraged microbial adaptability to selectively enrich and isolate cellulolytic bacterial populations [8]. Microcrystalline cellulose was utilized as the sole carbon source over five subcultures to select and enrich an efficient cellulose degrading microbiota from the rumen. The effects of MOS addition on fermentation parameters, microbial structure, and gene expression were also assessed through metagenomic sequencing and enzyme activity analysis. This study provided valuable insights into the regulatory role of MOS in promoting the abundance of cellulolytic bacteria and upregulating essential glycoside hydrolase enzyme genes, thereby enhancing the fiber degradation capacity of the rumen microbiota. This study advanced current understanding of rumen microbial interactions and enzyme mechanisms, providing a scientific basis for the use of MOS in optimizing rumen function and improving feed efficiency in ruminants.

Materials and methods

Sample collection

Three female dairy cows aged 3 – 4 years old from Pingjibao Dairy Farm No. 3, Yinchuan, Ningxia, China were involved in this research. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Ningxia University (Yinchuan, Ningxia, China) (Approval No. NXU-2024-145). Rumen fluid samples were collected using a stainless-steel stomach tube prior to the morning feeding, shaken vigorously, filtered through four layers of cheesecloth to remove large particulates, and then immediately stored at 37°C incubator to maintain anaerobic conditions until further processing.

Microcrystalline cellulose medium preparation

The microcrystalline cellulose medium was prepared based on the recipe of Liu *et al*. with some modifications [9]. The medium contained 10 g of microcrystalline cellulose (Meilun Biotechnology Co., Ltd., Dalian, Liaoning, China), 5.0 g of $NaHCO_3$, 1.0 g of peptone, 1.0 g of yeast extract, 170 mL of cell-free rumen fluid, 0.495 g of KH_2SO_4, 0.495 g of $(NH_4)_2SO_4$, 0.99 g of NaCl, 0.066 g of $CaCl_2{\cdot}2H_2O$, 0.0957 g of $MgSO_4{\cdot}7H_2O$, 0.6534 g of $K_2HPO_4{\cdot}3H_2O$, and 1 mL of 0.1% resazurin in 1 L volume. The cell-free rumen fluid was prepared by centrifuging fresh rumen fluid at 2,743 ×g, 4°C, for 15 minutes followed by the second centrifugation of collected supernatant at 15,710 ×g, 4°C, for 30 minutes before stored at -20°C. CO_2 was continuously passed through the medium until its color faded. 1.5 g/L cysteine hydrochloride (Macklin Biochemical Co., Ltd., Shanghai, China) was added, and CO_2 was passed until the medium turned pale yellow. The

medium was then aliquoted into 20 mL serum bottles with 10 mL per bottle and sterilized at 121℃ for 15 minutes.

Cultivation of cellulose degrading microbiota

The cellulose degrading microbiota was cultivated following the method of Shakarami *et al*. [10]. Briefly, 0.1 mL of the rumen fluid was inoculated into 10 mL of the microcrystalline cellulose medium and incubated in HZQ-X300 Shaking Incubator (Yiheng Scientific Instruments Co., Ltd., Shanghai, China) at 39℃ and 120 rpm. After each 72 hours of incubation, 0.1 mL of the inoculum was transferred into fresh medium to initiate the next passage, while the remaining inoculum was frozen at -20℃ for volatile fatty acid (VFA) analysis and pH measurement. The subculturing process was repeated five times (P1-P5) with six replicates in each passage, resulting in a total of 30 samples for further analysis.

MOS treatment

Four MOS treatment groups including control (CMO), low-dose (LMO), medium-dose (MMO), and high-dose (HMO) groups supplemented with 0%, 0.5%, 1.5%, and 3% mannan oligosaccharides (Yingxin Laboratory, Shanghai, China), respectively, were included in this study with six replicates in each group. The four groups of media were inoculated with the cellulose degrading microbiota at a ratio of 1:100 at the beginning of the experiment. The inoculated medium was cultured at 39℃, 120 rpm, for 48 h. The fermentation was terminated by placing the fermentation broth in ice water [10] and stored at -20℃ for the analysis of volatile fatty acids and cellulase activity following pH measurement using a portable pH meter (Dongguan Wanchuang Electronic Products Co., Ltd., Dongguan, Guangdong, China).

Analysis of VFA and cellulose degrading enzyme activity

The VFA concentrations were analyzed using Shimadzu 2030 Gas Chromatography System (Shimadzu, Kyoto, Japan) equipped with a AE-FFAP 30 m × 0.25 mm × 0.33 µm fused silica column (Atech Technologies, Brunswick, OH, USA) [11]. Frozen stored rumen fluid was thawed and immediately analyzed for the activity of avicelase using avicel as the substrates. The enzyme assay was incubated at 39℃, pH 7.0, for 30 min. The amounts of released reducing sugars were quantified using the dinitro alicyclic acid colorimetry method at 540 nm [12]. The activity of β-glucosidase was measured using an Enzyme Linked Immunosorbent Assay (ELISA) kit (Jingmei Technologies Co. Ltd., Guangzhou, Guangdong, China) following the manufacturer's instructions. The absorbances were measured using a 3100 Multifunctional Microplate Reader (Boyan Biotechnology Co., Ltd., Nanjing, Jiangsu, China).

Metagenomic sequencing and analysis

Based on the results of the MOS treatment, two groups with the largest differences were selected for metagenomic analysis with three replicates in each group. Each extracted microbial DNA sample was fragmented to an average length of approximately 300 bp using the Covaris M220 system (Gene Company Limited, Hong Kong, China). The TruSeq™ DNA Sample Prep Kit (Illumina, San Diego, CA, USA) was employed to prepare individual sequencing libraries. Metagenomic sequencing was performed using the whole genome shotgun (WGS) strategy on the Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) utilizing the NovaSeq Reagent Kit following the established protocols provided by Illumina (www.illumina.com). The raw sequencing reads were deposited into the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) Sequence Read Archive (SRA) database (Accession No. PRJNA1163521). Open reading frames (ORFs) were predicted from each contig using MetaGene (http://metagene.cb.k.u-tokyo.ac.jp/) [13]. All ORFs with a sequence identity of ≥ 95% over at least 90% of their length were clustered by employing CD-HIT (http://www.bioinformatics.org/cd-hit) [14]. The longest sequence within each cluster was chosen as the representative to build a non-redundant gene catalog. Quality-filtered sequence reads were

Table 1. *In vitro* rumen fermentation parameters after serial passaging.

Items	Groups					SEM	*P* value
	P1	P2	P3	P4	P5		
pH	6.22[b]	6.55[a]	6.46[a]	6.35[a, b]	6.18[b]	0.05	0.020
TVFA, mM	28.65[a, b]	25.44[d]	25.82[c, d]	27.26[b, c]	29.61[a]	0.39	＜0.001
VFA proportion (mol/100 mol)							
Acetate	68.50[b, c]	67.03[c]	70.25[a, b]	69.07[a, b, c]	71.53[a]	0.47	0.019
Propionate	16.05[a]	15.73[a, b]	16.35[a]	13.83[b, c]	13.33[c]	0.40	0.035
Butyrate	7.48	7.49	7.71	7.58	7.57	0.01	0.70
A:P ratio	4.33[b]	4.35[b]	4.27[b]	5.03[a]	5.33[a]	0.16	0.029

Notes: TVFA: total volatile fatty acids. P1: first passage. P2: second passage. P3: third passage. P4: fourth passage. P5: fifth passage. The different superscripts (a, b, c, d) in a row designated a significant difference ($P < 0.05$).

aligned to these representative sequences at 95% identity using SOAP aligner (http://soap.genomics.org.cn/) [15], and gene abundance in each sample was measured in transcripts per million. Representative sequences from the non-redundant gene catalog were aligned to the NCBI NR database using BLASTP (Version 2.2.28+) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine taxonomic classification. For functional annotation, the Kyoto Encyclopedia of Genes and Genomes (KEGG) was utilized by running BLASTP (Version 2.2.28+) against the KEGG database (http://www.genome.jp/keeg/) [16]. Carbohydrate active enzyme annotations were predicted *via* hmmscan (http://hmmer.janelia.org/search/hmmscan) against CAZy database (version 5) (http://www.cazy.org/). A maximum e-value cutoff of 1e-5 was applied for all annotations.

Statistical analysis

All data were expressed as mean ± SD. Rumen fermentation parameters, Fibrolytic enzyme activities, carbohydrate active enzyme genes, and alpha diversity were assessed using one-way ANOVA followed by Duncan's test. Differences in microbial species were evaluated using the Kruskal-Wallis H test with post hoc comparisons conducted through the Tukey-Kramer method. The alpha diversity indices including ACE, Chao1, Shannon, and Simpson indices were calculated using the "qiime2-q2-diversity" command in QIIME2 (https://qiime2.org/). Beta diversity differences between lineages were assessed with unweighted UniFrac and Bray–Curtis dissimilarity and visualized through principal coordinates analysis (PCoA) plots using QIIME2 [17]. Linear discriminant analysis effect size (LEfSe) was used to analyze differential KEGG pathways. The results were visualized using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Spearman correlations between rumen microbial species and metabolites were computed using the "psych" package in R (https://www.r-project.org/) with the results presented as heat maps using the "pheatmap" package. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed with GraphPad Prism 7.0 and SPSS 19.0 (IBM, Armonk, NY, USA) for Windows.

Results

In vitro rumen fermentation parameters and fibrolytic enzyme activities

The pH values of passage 2 (P2) and passage 3 (P3) groups was significantly higher than that of passage 1 (P1) and passage 5 (P5) groups ($P < 0.05$). The concentrations of TVFA in P2 and P3 groups were significantly lower than that of P1 and P5 groups ($P < 0.05$). The molar proportion of acetate, as well as the acetate-to-propionate (A:P) ratio in P5 group were higher than that in P1 and P2 groups ($P < 0.05$), while the molar proportion of propionate exhibited an inverse

Table 2. *In vitro* rumen fermentation parameters and fibrolytic enzyme activities under mannan oligosaccharides (MOS) supplementation.

Items	Groups				SEM	*P* value
	CMO	LMO	MMO	HMO		
pH	6.23[a]	6.05[b]	5.80[b, c]	5.62[c]	0.02	＜0.001
TVFA, mM	29.35[b]	31.67[a]	31.92[a]	32.34[a]	0.28	＜0.001
VFA proportion (mol/100 mol)						
Acetate	73.17	73.33	72.16	72.83	0.04	0.723
Propionate	13.87	13.47	14.83	14.33	0.40	0.691
Butyrate	7.97	8.20	8.00	7.83	0.10	0.693
A:P ratio	5.37	5.52	4.98	5.21	0.18	0.754
Fibrolytic enzyme Activities (mU)						
Avicelase	178.23[b]	249.16[a]	214.30[a, b]	201.53[b]	9.75	0.039
β-glucosidase	231.56	269.55	264.58	246.03	9.73	0.440

Notes: TVFA: total volatile fatty acids. CMO: 0% MOS. LMO: 0.5% MOS. MMO: 1.5% MOS. HMO: 3% MOS. Different superscripts (a, b, c, d) in a row designated a significant difference ($P < 0.05$).

trend ($P < 0.05$). No significant differences were observed in the butyrate molar proportion among all five groups (Table 1). The pH values of LMO, MMO, and HMO groups were significantly lower than CMO group, while the TVFA in LMO, MMO, and HMO groups were significantly higher than CMO group ($P < 0.05$). The microbiome of LMO group exhibited the highest avicelase activity compared to the other groups ($P < 0.05$) with the enzyme activity being defined as the amount of enzyme used to release 1 nmol of reducing sugar as one milliunit (mU) (Table 2). No significant differences were found in the molar proportion of acetate, propionate, and butyrate, or in the activity of β-glucosidase among all five groups.

Rumen microbiota structure and composition

The CMO and LMO groups were selected for metagenomic analysis comparison due to LMO's significantly lower pH, higher TVFA levels, and the highest avicelase activity. A total of over 428.6 million raw reads with more than 71.3 million reads per sample were obtained. There were no differences observed in the ACE and Chao 1, Shannon, and Simpson (Figure 1A), or PCoA analysis (Figure 1B). The results found that *Firmicutes*, *Proteobacteria*, *Bacteroidota*, *Synergistetes*, *Fibrobacteres*, *Fusobacteria*, and *Actinobacteria* were the most dominant phyla identified across all samples with *Firmicutes* (23.47%), *Proteobacteria* (43.87%), and *Bacteroidota* (20.64%) as the most abundant species (Figure 1C). The relative abundance of *Firmicutes* and *Fibrobacteres* in LMO group were higher than CMO group ($P < 0.05$). At the genus level, the relative abundance of *Megasphaera*, *Streptococcus*, *Fibrobacter*, and *Sodaliphilus* in LMO group were higher than CMO group ($P < 0.05$), while the opposite was observed for *Bacteroides*, *Escherichia*, *Fusobacterium*, *Pyramidobacter*, *Prevotella*, and *Klebsiella* ($P < 0.05$) (Figure 1D). At the species level, the relative abundance of *Megasphaera elsdenii*, *Fibrobacter succinogenes*, *Streptococcus equinus*, *Sodaliphilus pleomorphus*, *Bacteroides ovatus*, and *Megasphaera hexanoica* in LMO group were higher than CMO group ($P < 0.05$), while *Bacteroides heparinolyticus*, *Bacteroides thetaiotaomicron*, *Escherichia coli*, and others in CMO group were higher than LMO group (Figure 1E) ($P < 0.05$). Additionally, *Fibrobacter succinogenes* and *Streptococcus equinus* were positively correlated with both the concentration of TVFA and avicelase activity ($P < 0.05$) (Figure 1F). *Megasphaera elsdenii* was positively correlated with avicelase activity ($P < 0.05$).

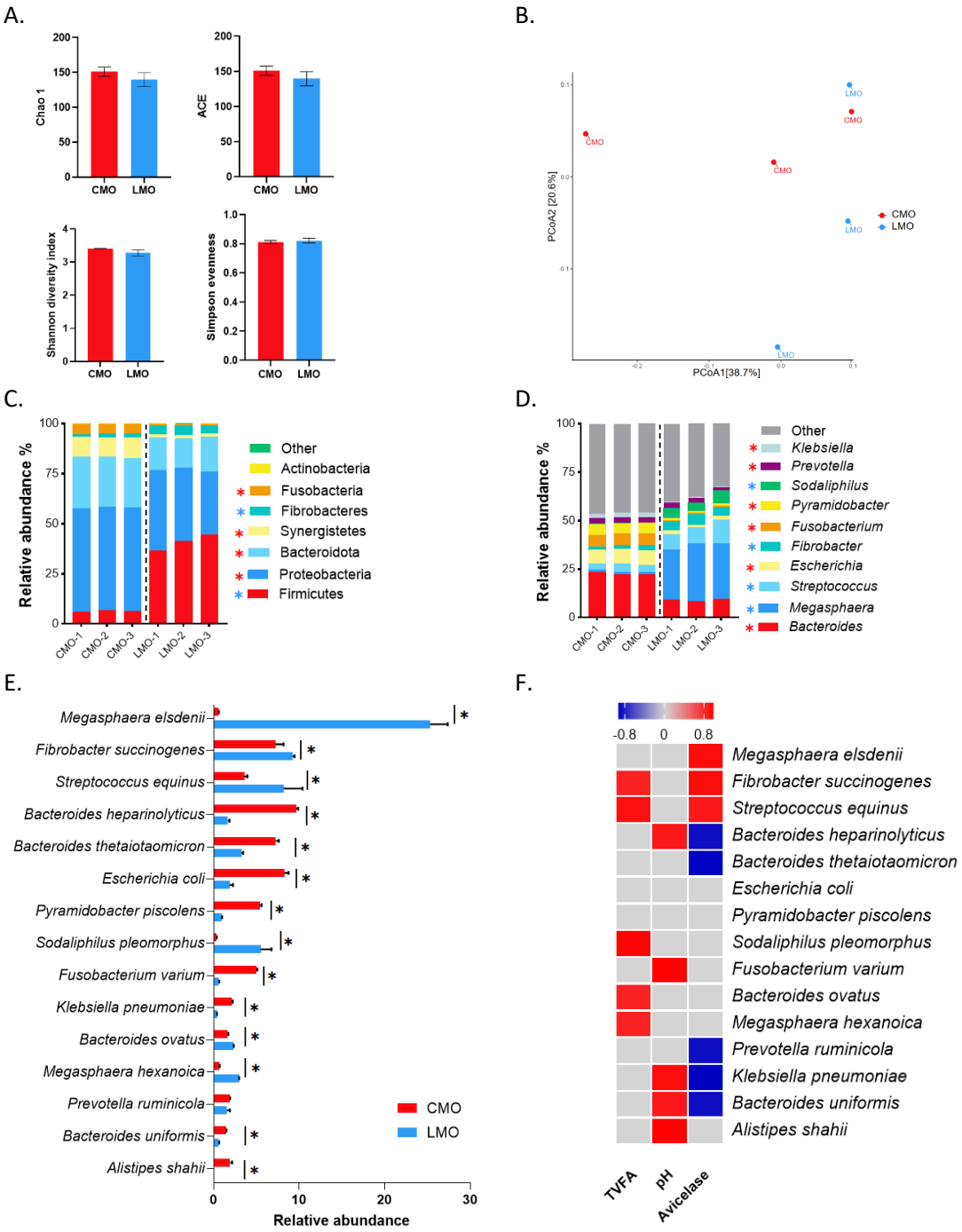

Figure 1. Microbiota structure and composition. **A.** Alpha diversity measurements of the microbiota as determined by metagenomics. **B.** PCoA profile of ruminal bacterial community based on Bray-Curtis dissimilarity determined by metagenomics. **C.** Major phyla with a relative abundance > 1% in all the samples. **D.** Major genera with a relative abundance > 1% in all samples. **E.** Microbial species identified in the rumen metagenome that significantly differed in relative abundance between the two groups (Phyla and genera marked with red and blue, respectively. * indicated significantly more predominant in CMO and LMO groups ($P < 0.05$), respectively. **F.** TVFA, pH, and avicelase activity.

Carbohydrate active enzymes and KEGG pathway

The analysis of glycoside hydrolase families revealed that, among the top 20 differentially abundant glycoside hydrolases, the abundance of

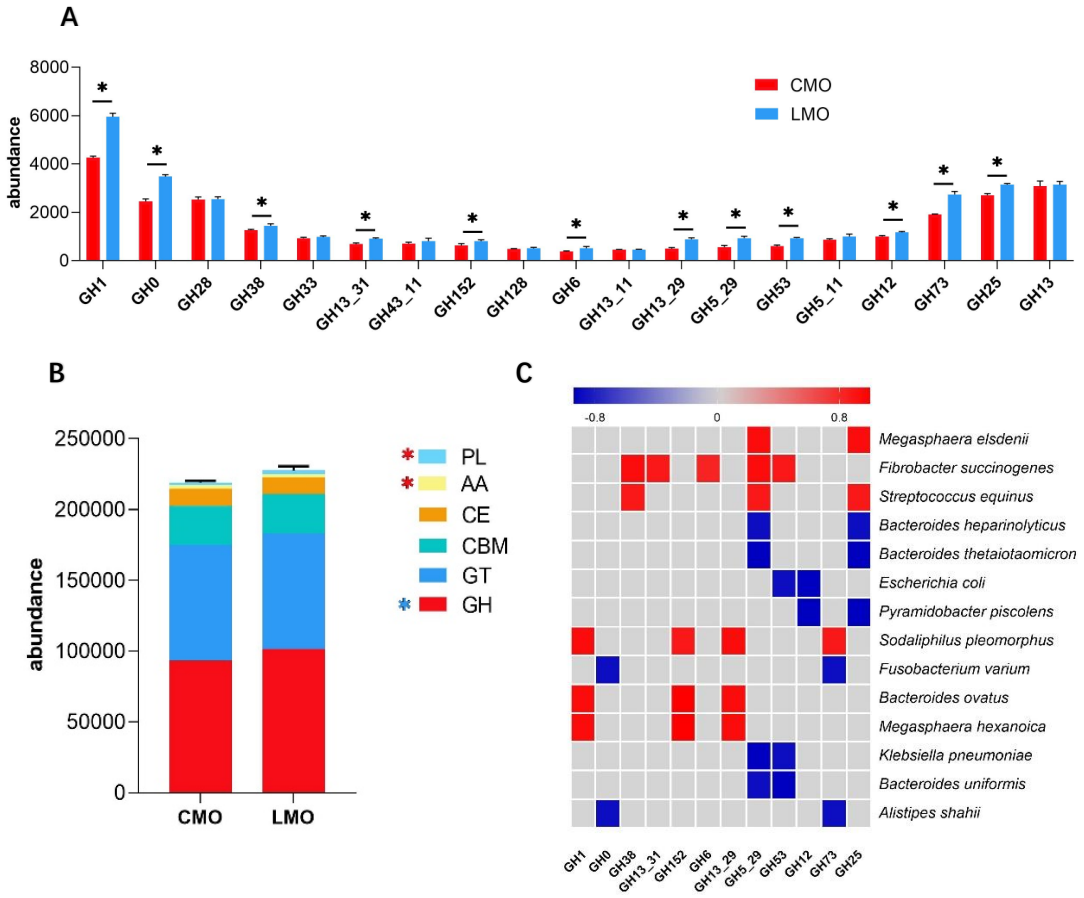

Figure 2. Abundance of carbohydrate active enzyme genes. **A.** Abundance of the top 20 glycoside hydrolases (GH) families. **B.** Abundance of carbohydrate active enzyme genes. **C.** Relationships between the top 20 GH families and bacterial community at species level with red and blue indicating positive and negative correction, respectively. * indicated $P < 0.05$. The red and blue asterisks indicated significantly more predominant in the CMO and LMO groups, respectively.

GH1, GH0, GH38, GH13_31, GH152, GH6, GH13_29, GH5_29, GH53, GH12, GH73, and GH25 in LMO group were significantly higher than CMO group ($P < 0.05$) (Figure 2A). The abundance of glycoside hydrolases (GHs), polysaccharide lyases (PL), and auxiliary activities (AA) in LMO group were significantly higher than that in CMO group ($P < 0.05$) (Figure 2B). In addition, *Megaspharea elsdenii* showed a positive correlation with the abundance of GH5_29 and GH25 ($P < 0.05$), while *Fibrobacter succinogenes* was positively correlated with the abundance of GH38, GH13_31, GH6, GH5_29, and GH53 ($P < 0.05$). *Streptococcus equinus* exhibited a positive correlation with the abundance of GH38, GH5_29, and GH25 ($P < 0.05$). Similarly, *Sodaliphilus pleomorphus* exhibited a positive correlation with the abundance of GH1, GH152, GH13_29, and GH73 ($P < 0.05$) (Figure 2C).

Differential modules based on Lefse analysis showed that enriched pathways in LMO group included cell motility, replication and repair, immune system, membrane transport, energy metabolism, environmental adaptation, and folding sorting and degradation (Figure 3A). Seven metabolic pathways were enriched in LMO group at level 3 compared with CMO group, which included bacterial chemotaxis, flagellar assembly, phospho transferase system (PTS), mismatch repair, homologous recombination, valine, leucine, isoleucine biosynthesis, and thiamine metabolism (Figure 3B).

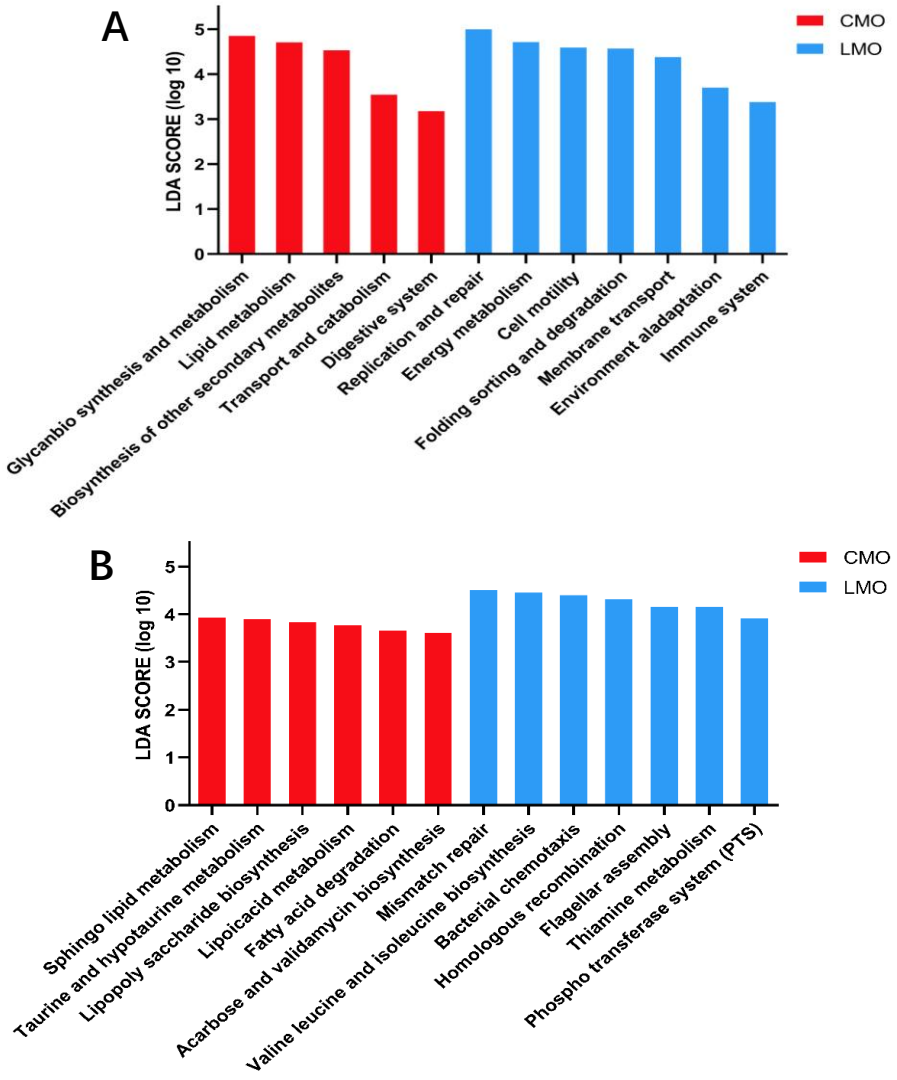

Figure 3. Analysis of KEGG pathways. **A.** Differential KEGG pathways between the two groups at KEGG level 2. **B.** Differential KEGG pathways between the two groups at KEGG level 3. LDA: linear discriminant analysis.

Discussion

Passage culture or serial subculturing is an effective microbiological technique for enriching and maintaining microbial communities [18]. One notable application of passage culture is in enhancing biogas production from lignocellulosic biomass, where selected microbial consortia exhibit significantly improved degradation capabilities, resulting in a 30-40% improvement in methane production [19]. In this research, continuous subculturing over five passages significantly reduced the pH value of the fermentation broth and increased the concentrations of TVFA, acetate concentration, and acetate-to-propionate ratio. These results indicated that subculturing promoted cellulose degradation by rumen microorganisms and shifted the fermentation pattern towards an acetate-dominant mode. MOS are functional carbohydrates that have demonstrated potential in enhancing rumen microbial community structure and metabolic activities [20]. In this study, the addition of 0.5% MOS significantly increased VFA production and microcrystalline cellulase activity, which consistent with findings

of numerous studies that showed the positive impact of MOS on rumen microbial fermentation capacity, particularly cellulose degradation [21]. Studies have shown that Tibetan ruminants, that are well adapted to digest low quality feeds, exhibit higher levels of cellulose degrading bacteria, particularly *Fibrobacter succinogenes*, compared to lowland ruminants [17]. MOS selectively promotes the growth of cellulose degrading bacteria, which is critical for ruminants in harsh environments, allowing them to digest and ferment large quantities of low quality roughage to produce VFA, supporting host maintenance, reproduction, and growth.

The rumen microbiota is primarily responsible for energy acquisition in ruminants. Numerous studies have shown that differences in rumen microbiota can alter energy efficiency [22]. Among the 16 phyla identified by macrogenomic analysis in this study, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were the most dominant microorganisms, considered crucial for meeting the energy requirements of ruminants. The relative abundance of *Firmicutes* increased significantly with higher energy levels in the diet [23]. Similarly, the cecal microbiota of obese individuals and mice contained more *Firmicutes* and fewer *Bacteroidetes* and other phyla [24], consistent with the positive correlation between the relative abundance of *Firmicutes* and dietary energy concentration [25]. The results of this study showed that addition of 0.5% MOS significantly increased the relative abundance of *Firmicutes* in the fermentation broth and enriched energy metabolism pathways, indicating the positive effect of MOS on promoting energy metabolism in rumen microorganisms. Xue *et al*. demonstrated that the relative abundance of *Fibrobacter succinogenes* in the rumen of high-yielding dairy cows was significantly greater than that in low-yielding cows [26]. Transcriptome sequencing revealed that *Fibrobacter* expressed most of cellulase and hemicellulose transcripts [27]. In this research, 0.5% MOS promoted the abundance of *Fibrobacter succinogenes*, which might improve roughage digestibility in dairy cows and increase productivity.

This study found that MOS significantly promoted the gene abundance of GH6 in the rumen microbiome. The GH6 family includes both endoglucanases and exoglucanases, which typically possess a conserved β-helix domain [28]. This structure enables GH6 enzymes to bind tightly to cellulose molecules and catalyze hydrolysis [29]. GH6 enzymes can act synergistically with other glycoside hydrolases [30], such as GH5 and GH9, and usually contain a carbohydrate-binding module (CBM) [31], which allows them to degrade highly recalcitrant crystalline cellulose, playing a critical role in cellulose degradation [32]. MOS may serve as substrates or inducers, activating the expression of genes related to the degradation of cellulose and other polysaccharides, thereby promoting the increased abundance of GH6 genes [33]. Additionally, the gene abundance of other GH families, GH1, GH0, GH38, GH3_31, GH152, GH13_29, GH5_29, GH53, and GH12, was significantly upregulated with the addition of 0.5% MOS. The GH1 family glycoside hydrolases including β-glucosidase and β-galactosidase hydrolyze various plant cell wall components, promoting cellulose degradation and volatile fatty acid production in the rumen, particularly under high fiber diets [33]. GH0 is a relatively new family of glycoside hydrolases, whose function is not yet fully defined but is involved in the initial degradation and modification of complex polysaccharides [34]. The GH38 family, primarily represented by α-mannosidase, hydrolyzes mannose-glycoside bonds, which plays a crucial role in the degradation of plant derived mannans in ruminants [35]. The GH3_31 family includes β-glucosidase and xylanase, which break down long chain cellulose or hemicellulose into soluble oligosaccharides or monosaccharides, providing readily absorbable carbon sources for ruminants [36]. Although research on GH152 is limited, it is known to be involved in the degradation of specific carbohydrates such as alginates [37]. GH13_29 is part of the starch hydrolase family and helps rumen microorganisms quickly

degrade soluble starch, balancing rumen pH, and improving fiber digestion. The GH5_29 family contains endoglucanases that hydrolyze the internal β-1,4 bonds of cellulose, producing cello oligosaccharides and glucose, which play an essential role in plant cell wall degradation [38]. GH53 is primarily composed of galactosidase, which specifically degrades β-1,4-galacturonosyl linkages in hemicellulose, enhancing the digestibility of fiber based feeds [39]. GH12 is involved in cellulose and hemicellulose degradation, working synergistically with other cellulose degrading enzymes to form stable enzyme complexes, significantly improving fiber degradation efficiency [40]. Additionally, *Fibrobacter succinogenes*, the most important cellulose degrading bacterium in this study, was significantly positively correlated with the gene abundance of GH38, GH13_31, GH6, GH5_29, and GH53. MOS enhanced the abundance of *Fibrobacter succinogenes*, thus increasing the gene abundance of GH families related to fiber degradation, thereby enhancing the fiber degradation capacity of the rumen microbiota. Further, MOS also promoted the abundance of GH73 and GH25 genes. The primary members of GH73 and GH25 are lysozymes, which degrade peptidoglycan in the cell walls of Gram-positive bacteria, inhibit pathogenic bacteria growth, and enhance the stability and functional diversity of the rumen microbiome [41]. GH73 coded lysozyme can enhance immune function by clearing necrotic cells and tissue debris, playing a crucial role in rumen health [42]. The abundance of GH73 gene in this study showed a significant positive correlation with *Sodaliphilus pleomorphus*, while GH25 gene demonstrated positive correlation with *Megasphaera elsdenii* and *Streptococcus equinus*. *Sodaliphilus pleomorphus* is a newly identified anaerobic bacterium originally isolated from pig feces, which inhibits pathogens like *E. coli* by secreting organic acids and competing for substrates [43]. *Megasphaera elsdenii* is an important lactate utilizing bacterium in the rumen, effectively alleviating ruminal acidosis caused by high concentrate grain diets [44]. *Streptococcus equinus* is a lactic acid producing bacterium, but unlike other lactic acid producers, it can coexist with *Megasphaera elsdenii*, reducing lactic acid accumulation in the rumen through metabolic cooperation [45]. In this research, MOS significantly promoted the growth of *Megasphaera elsdenii* with its relative abundance increasing nearly 30-fold after 0.5% MOS supplementation. Similar phenomena were observed when MOS was added to sheep diets [46]. MOS regulates gut microbial balance by promoting the growth of *Megasphaera elsdenii* and other bacteria, and by increasing the gene abundance of glycoside hydrolases such as GH73 and GH25, which act on bacterial cell wall polysaccharides, thus enhancing cellulose degradation capacity in the rumen.

This study revealed a distinct variation in the composition and functions of rumen cellulolytic bacteria following MOS supplementation. MOS substantially enhanced rumen function through enriching the key cellulose degrading bacteria such as *Fibrobacter succinogenes*. Additionally, the upregulation of glycoside hydrolase genes involved in cellulose degradation (GH6, GH38, and GH13_31) and the regulation of gut microbial balance (GH73 and GH25) further optimized ruminal energy metabolism. These effects collectively facilitated enhanced cellulose degradation by rumen microorganisms, leading to a significant increase of volatile fatty acid production. This study provided key insights into the molecular mechanisms through which MOS modulated rumen microbial communities, while optimizing cellulose utilization and improving overall rumen function.

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References

1. Osorio-Doblado AM, Feldmann KP, Lourenco JM, Stewart RL, Smith WB, Tedeschi LO, *et al*. 2023. Forages and pastures symposium: Forage biodegradation: Advances in ruminal microbial ecology. J Anim Sci. 101(89):213-226.
2. Thacharodi A, Hassan S, Ahmed ZHT, Singh P, Maqbool M, Meenatchi R, *et al*. 2024. The ruminant gut microbiome *vs* enteric methane emission: The essential microbes may help to mitigate the global methane crisis. Environ Res. 261(56):119-234.
3. Hassan FU, Arshad MA, Ebeid HM, Rehman MS, Khan MS, Shahid S, *et al*. 2020. Phytogenic additives can modulate rumen microbiome to mediate fermentation kinetics and methanogenesis through exploiting diet-microbe interaction. Front Vet Sci. 7(5):575-589.
4. Jana UK, Suryawanshi RK, Prajapati BP, Kango N. 2021. Prebiotic mannooligosaccharides: Synthesis, characterization and bioactive properties. Food Chemistry. 342(13):128-138.
5. Zheng C, Li F, Hao Z, Liu T. 2018. Effects of adding mannan oligosaccharides on digestibility and metabolism of nutrients, ruminal fermentation parameters, immunity, and antioxidant capacity of sheep. J Anim Sci. 96(1):284-292.
6. Peng Q, Cheng L, Kang K, Tian G. 2020. Effects of yeast and yeast cell wall polysaccharides supplementation on beef cattle growth performance, rumen microbial populations and lipopolysaccharides production. J Integr Agric. 19(3):810-819.
7. Ting L, Xiaowen L, Lei W, Chan L, Qing Q. 2023. Production of xylo-oligosaccharides with degree of polymerization 3–5 from wheat straw xylan by a xylanase derived from rumen metagenome and utilization by probiotics. Food Biosci. 56:103360.
8. Werth BJ, Ashford NK, Penewit K, Waalkes A, Holmes EA, Bryan A, *et al*. 2022. Evolution of cefiderocol resistance in *stenotrophomonas maltophilia* using *in vitro* serial passage techniques. JAC Antimicrob Resist. 4(1):101-132.
9. Liu J, Liu Z, Liu Y, Hou X. 2019. Analysis of cellulolytic bacterial flora in the rumen of linner mongolian sheep. BioResources. 14(4):9544-9556.
10. Shakarami MH, Mohammadabadi T, Motamedi H, Sari M, Teimouri Yansari A. 2019. Isolation and identification of cellulolytic bacteria from gastrointestinal tract of arabian horse and investigation of their effect on the nutritional value of wheat straw. J Appl Microbiol. 127(2):344-353.
11. Li F, Li Z, Li S, Ferguson Jd, Cao Y, Yao J, *et al*. 2014. Effect of dietary physically effective fiber on ruminal fermentation and the fatty acid profile of milk in dairy goats. J Dairy Sci. 97(4):2281-2290.
12. Cao Y, Wang D, Wang L, Wei X, Li X, Cai C, *et al*. 2021. Physically effective neutral detergent fiber improves chewing activity, rumen fermentation, plasma metabolites, and milk production in lactating dairy cows fed a high-concentrate diet. J Dairy Sci. 104(5):5631-5642.
13. Noguchi H, Park J, Takagi T. 2006. Metagene: Prokaryotic gene finding from environmental genome shotgun sequences. Nucleic Acids Res. 34(19):5623-5630.
14. Fu L, Niu B, Zhu Z, Wu S, Li W. 2012. Cd-hit: Accelerated for clustering the next-generation sequencing data. Bioinformatics. 28(23):3150-3152.
15. Li R, Li Y, Kristiansen K, Wang J. 2008. Soap: Short oligonucleotide alignment program. Bioinformatics. 24(5):713-714.
16. Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, *et al*. 2011. Kobas 2.0: A web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res. 39(5):W316-W322.
17. Zhao C, Wang L, Ke S, Chen X, Kenéz Á, Xu W, *et al*. 2022. Yak rumen microbiome elevates fiber degradation ability and alters rumen fermentation pattern to increase feed efficiency. Anim Nutr. 11(6):201-214.
18. Wangkeeree J, Kaewsashan K, Suwanchaisri K, Roddee J, Hanboonsong Y. 2024. Enhancing the efficacy of fungal biocontrol agents for matsumuratettix hiroglyphicus, the vector of sugarcane white leaf disease, through serial passaging. Sugar Tech. 26(3):683-699.
19. Xu N, Liu S, Xin F, Zhou J, Jia H, Xu J, *et al*. 2019. Biomethane production from lignocellulose: Biomass recalcitrance and its impacts on anaerobic digestion. Front Bioeng Biotechnol. 7(5):191-202.
20. Kumar S, Kango N. 2021. Production of mannooligosaccharides from various mannans and evaluation of their prebiotic potential. Food Chem. 334(6):127-138.
21. Zheng C, Zhou J, Zeng Y, Liu T. 2021. Effects of mannan oligosaccharides on growth performance, nutrient digestibility, ruminal fermentation and hematological parameters in sheep. Peer J. 9(8):116-131.
22. Sanjorjo RA, Tseten T, Kang M, Kwon M, Kim S. 2023. In pursuit of understanding the rumen microbiome. Fermentation. 9(2):114-151.
23. Li QS, Wang R, Ma ZY, Zhang XM, Jiao JZ, Zhang ZG, *et al*. 2022. Dietary selection of metabolically distinct microorganisms drives hydrogen metabolism in ruminants. ISME J. 16(11):2535-2546.
24. Gill S, Backhed F, Ley R, Turnbaugh P. 2006. Human gut microbes associated with obesity. Nature. 444(2):41-48.
25. Pinnell LJ, Reyes AA, Wolfe CA, Weinroth MD, Metcalf JL, Delmore RJ, *et al*. 2022. Bacteroidetes and firmicutes drive differing microbial diversity and community composition among micro-environments in the bovine rumen. Front Vet Sci. 9(3):897-906.
26. Xue MY, Sun HZ, Wu XH, Liu JX, Guan LL. 2020. Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. Microbiome. 8(1):64-71.
27. Dai X, Tian Y, Li J, Luo Y, Liu D, Zheng H, *et al*. 2015. Metatranscriptomic analyses of plant cell wall polysaccharide degradation by microorganisms in the cow rumen. Microbiol. 81(4):1375-1386.
28. Halmi MFA, Simarani K. 2024. Response of soil microbial glycoside hydrolase family 6 cellulolytic population to lignocellulosic biochar reveals biochar stability toward microbial degradation. J Environ Qual. 53(4):546-551.

29. Christensen SJ, Krogh K, Spodsberg N, Borch K, Westh P. 2019. A biochemical comparison of fungal gh6 cellobiohydrolases. Biochem J. 476(15):2157-2172.
30. Yamaguchi S, Sunagawa N, Samejima M, Igarashi K. 2024. Thermotolerance mechanism of fungal gh6 cellobiohydrolase. Part ii. Structural analysis of thermotolerant mutant from the *basidiomycete phanerochaete chrysosporium*. J Appl Glycosci. 71(2):63-72.
31. Baramee S, Teeravivattanakit T, Phitsuwan P, Waeonukul R, Pason P, Tachaapaikoon C, *et al*. 2017. A novel gh6 cellobiohydrolase from *paenibacillus curdlanolyticus* b-6 and its synergistic action on cellulose degradation. Appl Microbiol Biotechnol. 101(3):1175-1188.
32. Sharma G, Kaur B, Raheja Y, Kaur A, Singh V, Basotra N, *et al*. 2024. Developing endophytic *penicillium oxalicum* as a source of lignocellulolytic enzymes for enhanced hydrolysis of biorefinery relevant pretreated rice straw. Bioprocess Biosyst Eng. 10(3):12-23.
33. Khusnutdinova AN, Tran H, Devlekar S, Distaso MA, Kublanov IV, Skarina T, *et al*. 2024. Moderately thermostable gh1 beta-glucosidases from hyperacidophilic *archaeon cuniculiplasma divulgatum* s5. FEMS. 100(9):101-120.
34. Kumari S, Magdaleno JS, Grewal RK, Narsing MP, Shaikh A, Cavallo L, *et al*. 2023. High potential for biomass-degrading cazymes revealed by pine forest soil metagenomics. J Biomol Struct Dyn. 9(3):1-12.
35. Polakova M, Horak R, Sestak S, Holkova I. 2016. Synthesis of modified d-mannose core derivatives and their impact on gh38 alpha-mannosidases. Carbohydr Res. 428(7):62-71.
36. 36. Camargo CH, Siviero M, Camargo SE, de Oliveira SH, Carvalho CA, Valera MC. 2007. Topographical, diametral, and quantitative analysis of dentin tubules in the root canals of human and bovine teeth. J Endod, 33(4): 422-26.
37. Wang Q, Xu M, Zhao L, Chen L, Ding Z. 2023. Novel insights into the mechanism underlying high polysaccharide yield in submerged culture of *ganoderma lucidum* revealed by transcriptome and proteome analyses. Microorganisms. 11(3):53-65.
38. Zheng F, Vermaas V, Zheng J, Tu T. 2019. Activity and thermostability of gh5 ndoglucanase chimeras from mesophilic and thermophilic parents. Appl Environ Microb. 85(5):e02079.
39. Boger M, Hekelaar J, van Leeuwen SS, Dijkhuizen L, Lammerts van Bueren A. 2019. Structural and functional characterization of a family gh53 beta-1,4-galactanase from bacteroides thetaiotaomicron that facilitates degradation of prebiotic galactooligosaccharides. J Struct Biol. 205(1):1-10.
40. Gullfot F. 2009. Functional characterization of xyloglucan glycosynthases from gh7, gh12, and gh16 scaffolds. Biomacromolecules. 10(1):1782-1788.
41. Morais S, Cockburn DW, David Y, Koropatkin NM, Martens EC, Duncan SH, *et al*. 2016. Lysozyme activity of the *ruminococcus champanellensis* cellulosome. Environ Microbiol. 18(12):5112-5122.
42. Mu YY, Qi WP, Zhang T, Zhang JY, Mao SY. 2021. Gene function adjustment for carbohydrate metabolism and enrichment of rumen microbiota with antibiotic resistance genes during subacute rumen acidosis induced by a high-grain diet in lactating dairy cows. J Dairy Sci. 104(2):2087-2105.
43. Holman DB, Kommadath A, Tingley JP, Abbott DW. 2022. Novel insights into the pig gut microbiome using metagenome-assembled genomes bioRxiv. 2022:492-507.
44. Chen L, Shen Y, Wang C, Ding L, Zhao F, Wang M, *et al*. 2019. *Megasphaera elsdenii* lactate degradation pattern shifts in rumen acidosis models. Front Microbiol. 10(1):162-171.
45. Papadimitriou K. 2018. Novel insight into the pathogenicity of *streptococcus gallolyticus* subsp. Gallolyticus belonging to the *streptococcus bovis/streptococcus equinus* complex. Virulence. 9(1):662-665.
46. Garcia D, Ferriani B, Jacovaci F, Cabreira J, Bolson DC, Pratti D. 2018. Inclusion of live yeast and mannan-oligosaccharides in high grain-based diets for sheep: Ruminal parameters, inflammatory response and rumen morphology. PLOS ONE. 13(2):193-213.