Metagenomic Analysis of Rumen Microbiota in Haizi Buffalo (Postprint)

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Abstract

To systematically investigate the microbial composition and lignocellulose-degrading enzyme system in the rumen of Haizi buffalo, this study employed high-throughput sequencing-based metagenomic technology to perform omics analysis on rumen fluid samples from Haizi buffalo (approximately 2.5 years old, average body weight 493 kg). The results showed that a total of 77,283,638 reads were obtained, which were assembled into 744,712 scaffolds. After Prodigal analysis, 827,044 genes were predicted. Gene annotation revealed that the rumen of Haizi buffalo contains various lignocellulose-degrading microorganisms, such as Ruminococcus flavefaciens, Ruminococcus albus, Fibrobacter succinogenes, and Prevotella ruminicola. Additionally, 38,011 genes encoding proteins with lignocellulose-degrading enzyme activity were identified, among which glycoside hydrolase (GH) genes were the most abundant (17,877), followed by glycosyltransferase (GT) (8,637), carbohydrate-binding module (CBM) (4,693), and carbohydrate esterase (CE) genes (4,214), while polysaccharide lyase (PL) (1,296) and auxiliary activity (AA) genes (934) were less abundant. Among the GH genes, those belonging to the GH2, GH43, GH97, and GH3 families were more prevalent, and genes encoding proteins with oligosaccharide-degrading enzyme activity were the most numerous. Furthermore, a small number of cellulosome component protein genes were also discovered. Comparative analysis of GH genes with gastrointestinal metagenomes from other species revealed that the proportions of cellulases, hemicellulases, and branch-degrading enzymes in the Haizi buffalo rumen were relatively similar to those in dairy cow rumen. These findings demonstrate that the Haizi buffalo rumen harbors a rich diversity of lignocellulose-degrading microorganisms and enzyme systems, which will provide a theoretical foundation for screening enzyme genes with potential for industrial applications.

Full Text

Metagenomic Analysis of Microorganisms in the Rumen of Haizi Buffalo

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Abstract

In order to investigate microorganism composition and lignocellulolytic enzymes in the rumen of Haizi buffalo, rumen fluid samples from Haizi buffalo (approximately 2.5 years of age, average body weight of 493 kg) were surveyed using metagenomic high-throughput sequencing. The results were as follows: a total of 77,283,638 reads were obtained, which were assembled into 744,712 scaffolds. Subsequently, a total of 827,044 genes were predicted by prodigal analysis. Many microorganisms involved in lignocellulose degradation were identified by gene annotation, such as *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Fibrobacter succinogenes* and *Prevotella ruminicola*. We identified 38,011 potential genes encoding lignocellulolytic enzymes including glycoside hydrolase (GH) (17,877 genes), glycosyl transferase (GT) (8,637 genes), carbohydrate binding module (CBM) (4,693 genes), carbohydrate esterase (CE) (4,214 genes), polysaccharide lyase (PE) (1,296 genes), and auxiliary activity (AA) (934 genes). Among GH genes, most belonged to GH2, GH43, GH97 and GH3 families, and a higher abundance of oligosaccharide degrading enzymes in the buffalo rumen metagenome was identified. In addition, we also predicted that some genes could encode cellulosome components. Compared with GH genes in the gastrointestinal metagenome of other species, the proportions of cellulose, hemicellulase and debranching enzyme were similar between Haizi buffalo and dairy cow. These results demonstrate that buffalo rumen is considerably enriched in functional microorganisms and enzymes involved in lignocellulose degradation with great prospects for obtaining enzyme genes that may be applied in industry.

Keywords: Haizi buffalo; rumen; metagenome; microorganism; lignocellulolytic enzyme

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Introduction

Lignocellulose is a collective term for plant biomass containing cellulose, hemicellulose, lignin, and other components [1]. The rumen is one of the most powerful ecosystems for lignocellulose degradation in nature, capable of degrading 60%–

65% of dietary lignocellulose within 48 hours to provide nutrients for ruminants, demonstrating that the rumen harbors abundant lignocellulose-degrading microorganisms and enzyme systems [2]. The rumen contains a vast diversity and enormous quantity of microorganisms, with over 3,000 species including bacteria, protozoa, fungi, archaea, bacteriophages, and viruses [3]. As a tremendous biological resource repository, the rumen harbors many lignocellulolytic enzyme systems with industrial application potential. Zhou et al. [4] discovered a bifunctional enzyme from yak rumen with both glucosidase and xylosidase activities. Huang et al. [5] obtained a cold-adapted xylanase gene from Hu sheep rumen. However, the rumen is a strictly anaerobic environment, and the vast majority of rumen microorganisms are unculturable, which greatly limits exploration of rumen genetic resources. With the development of high-throughput sequencing technology, metagenomic sequencing has been successfully applied to analyze microbial composition, metabolic pathways, and functional proteins in specific environments [6].

Numerous studies have applied high-throughput sequencing technology to investigate rumen microorganisms in ruminants. Hess et al. [7] first sequenced the metagenome of dairy cow rumen in 2011, identifying 27,755 lignocellulolytic enzyme genes, with glycoside hydrolase (GH) families GH13 (3,442 genes) and GH3 (2,844 genes) being the most abundant. Genes containing cohesin and dockerin modules numbered 80 and 188, respectively, providing possibilities for discovering novel lignocellulolytic enzymes. Patel et al. [8] fed buffalo with green grass and hay, then performed metagenomic high-throughput sequencing, finding that the green grass group had significantly more GH28 and pectate lyase genes in rumen than the hay group. Singh et al. [9] sequenced the rumen microbial metagenome of Indian buffalo using high-throughput technology, obtaining 137,270 contigs and predicting that 2,614 of them had lignocellulose-degrading functions. Wang et al. [10] compared the microbial genomes of goat rumen and feces, revealing significant differences: rumen microorganisms were dominated by Bacteroidetes, followed by Firmicutes, with *Prevotella* being the most abundant genus, whereas fecal microorganisms were dominated by Firmicutes, followed by Bacteroidetes, with the most abundant genus being an unclassified genus from Ruminococcaceae.

Haizi buffalo belongs to the swamp buffalo type, distributed in the coastal areas of northern Jiangsu Province, and is one of China' s major local breeds. It adapts well to local natural ecological conditions, exhibits outstanding stress resistance, and can tolerate roughage, maintaining good growth and working performance even with poor forage quality, indicating that Haizi buffalo rumen harbors unique lignocellulose-degrading microorganisms and enzyme systems. However, to date, few reports have documented the microbial composition and functional genes in swamp buffalo rumen. This study employed high-throughput sequencing technology to obtain metagenomic data from Haizi buffalo rumen for the first time. Through database comparison and analysis, we obtained functional annotations and conducted comparative studies with gastrointestinal metagenomic information from other species to systematically investigate the microbial composition and lignocellulolytic enzyme systems in Haizi buffalo rumen, aiming to discover novel lignocellulolytic enzymes with industrial application potential.

Materials and Methods

1.1 Sample Collection

Three Haizi buffalo aged approximately 2.5 years with an average body weight of 493 kg were selected. The experimental buffalo were managed under consistent conditions, fed twice daily with ad libitum access to feed. Diet composition and nutrient levels are shown in Table 1 . After 60 days of feeding, the animals were weighed and slaughtered. Rumen fluid samples were collected immediately after slaughter, filtered through four layers of sterile gauze, and rapidly frozen in liquid nitrogen.

Microbial community composition in bovine rumen is relatively similar under the same dietary and environmental conditions [7,11]. Therefore, we randomly selected rumen fluid from one Haizi buffalo for subsequent sequencing analysis.

1.2 Metagenomic DNA Extraction

Metagenomic DNA was isolated and purified from rumen fluid samples using the QIAamp DNA Micro Kit (Qiagen, Germany) according to the manufacturer' s instructions. The extracted DNA was assessed using 1.0% agarose gel electrophoresis and a NanoDrop spectrophotometer.

1.3 Metagenomic Sequencing and Analysis

Purified samples were sent to Shanghai OE Biotech Co., Ltd. for high-throughput sequencing. Metagenomic DNA was first fragmented to approximately 300 bp using a Covaris M220 instrument. Y-shaped adapters were ligated to the ends, and adapter PCR was performed to create "bridges" of DNA fragments on chips for library construction. Sequencing was then performed on the Illumina HiSeq 2000 platform.

After obtaining sequencing data, host gene filtration and quality trimming were performed as optimization steps. SOAPdenovo 2.04 software was used for sequence assembly with a k-mer parameter of 65. Prodigal 2.6.3 software was used for gene prediction from genomic sequences, followed by database comparison and analysis.

Predicted gene sequences were compared against the non-redundant nucleotide sequence database (NR) in GenBank (ftp://ftp.ncbi.nih.gov/pub/taxonomy) using BLAST. Sequences with e-value $\leq$ 1e-5 were considered significant, and species annotation information was obtained. Predicted genes were further compared against the COG database (ftp://ftp.ncbi.nih.gov/pub/COG/COG/kyva), GO database (http://www.geneontology.org/), and KEGG database (http://www.genome.jp/kegg/pathway.html) to obtain functional annotations.

1.4 Carbohydrate-Active Gene Analysis

Predicted genes were compared against the CAZy database (http://www.cazy.org/) using the hmmscan tool (http://hmmer.janelia.org/search/hmmscan) to obtain carbohydrate-active enzyme annotations. The abundance of each carbohydrate-active enzyme was calculated based on the sum of gene abundances. According to amino acid sequence similarity in protein domains, carbohydrate-active enzymes from different species can be classified into six major protein families: glycoside hydrolases (GH), glycosyl transferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE), carbohydrate-binding modules (CBM), and auxiliary activities (AA). The CAZy analysis results from Haizi buffalo were compared with those from dairy cow and Indian buffalo rumen, termite hindgut, and chicken cecum metagenomes.

Results

2.1 Metagenomic Sequencing Results

High-throughput sequencing on the Illumina HiSeq platform yielded 77,283,638 reads. After filtering buffalo host genes (GCA_{000003055}.5, *Bos taurus* Bos_{{{taurus}}_{{UMD}}}3.1.1), 10.79 GB of sequencing data were obtained. SOAPdenovo 2.04 software assembled the sequencing data into 744,712 scaffolds, representing approximately 250 Mbp of metagenomic sequence (Table 2).

Table 2 Assembly results statistics

Item	Total number	Total length/bp	Gap No.	Average length/bp	N50 length/bp	N90 length/bp	Maximum length/bp	Minimum length/bp	GC content/%
Scaffold	744,712	250,000,000	-	336	-	-	-	-	-

Gene prediction using Prodigal 2.6.3 identified 827,044 amino acid sequences with an average gene length of 274 bp.

2.2 Gene Annotation Results

Among the 827,044 gene sequences, 498,644 showed significant matches (e-value ≤ 1e-5) in the NR database. As shown in Figure 1 [Figure 1: see original paper], similarity to sequences in the NR database was primarily distributed above 50%, with only 7.8% of sequences showing high similarity (>95%), indicating that many novel genes were present in the sequencing results.

Taxonomic analysis revealed that protein-coding genes were predominantly derived from Bacteroidetes (55.3%), followed by Firmicutes (12.7%). Genes from Fibrobacteres and Proteobacteria accounted for 3.23% and 2.71%, respectively (Figure 2 [Figure 2: see original paper]).

An evolutionary clade diagram illustrating the taxonomic hierarchy at the species level was constructed (Figure 3 [Figure 3: see original paper]). From root to periphery, the classification follows Kingdom-Phylum-Class-Order-Family-Genus-Species-Strain. Three kingdoms were identified: Bacteria, Archaea, and Viruses, with most microorganisms in Haizi buffalo rumen belonging to Bacteria. At the strain level, unclassified *Methanobrevibacter* and porcine sapelovirus belonged to Archaea and Viruses, respectively. Eight major bacterial strains were detected: *Prevotella ruminicola*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, unclassified *Butyrivibrio*, *Streptococcus infantarius*, *Treponema* sp. JC4, *Treponema bryantii*, and *Fibrobacter succinogenes*.

2.3 COG Database Comparison Results

COG functional classification of predicted genes revealed that the most abundant functions were carbohydrate transport and metabolism, with 37,933 genes, indicating that this metagenome likely contains numerous lignocellulolytic enzyme genes (Figure 4 [Figure 4: see original paper]).

2.4 GO Classification Results

Gene Ontology classification categorized genes into three GO categories: biological process (BP), cellular component (CC), and molecular function (MF). In the BP category, genes involved in metabolism and extracellular processes were most abundant. In the CC category, genes associated with cell and cell parts were predominant. In the MF category, genes with binding and catalytic functions were most common (Figure 5 [Figure 5: see original paper]).

2.5 KEGG Analysis Results

A total of 105,560 genes were enriched in 247 KEGG pathways. The top 10 most significantly enriched pathways are shown in Table 3 . Most genes were enriched in small molecule metabolism, such as amino acid biosynthesis, purine metabolism, pyrimidine metabolism, carbon metabolism, and aminoacyl-tRNA biosynthesis. Additionally, 5,236 genes were enriched in starch and sucrose metabolism.

Table 3 Top 10 obviously gathered KEGG pathways

Pathways	Description	Gene No.
ko01230	Amino acid biosynthesis	-
ko00230	Purine metabolism	-
ko01200	Carbon metabolism	-
ko00240	Pyrimidine metabolism	-
ko00970	Aminoacyl-tRNA biosynthesis	-
ko00500	Starch and sucrose metabolism	5,236
ko00520	Amino sugar and nucleotide sugar metabolism	-
ko00250	Alanine metabolism	-
ko00010	Glycolysis/Gluconeogenesis	-
ko03010	-	-

2.6 CAZy Database Comparison Results

HMMER analysis identified 38,011 genes matching the CAZy database. As shown in Figure 6 [Figure 6: see original paper], GH genes were most abundant (17,877), followed by GT (8,637), CBM (4,693), and CE (4,214) genes. PL and AA genes were less abundant, with 1,296 and 934 genes, respectively. Additionally, 42 cohesin genes, 236 dockerin genes, and 82 S-layer homology (SLH) domains were detected.

The 17,877 GH genes were distributed across different GH families, with GH2, GH43, GH97, and GH3 families being most abundant, while GH12 and GH62 families had the fewest genes (Table 4). Lignocellulolytic enzymes in the rumen hydrolyze cellulose and hemicellulose in plant cell walls. Based on substrate specificity, GH gene families in the Haizi buffalo rumen metagenome were classified (Table 5): 932 genes encoded proteins with cellulase activity, with GH5 and GH9 families being most abundant; 1,011 genes encoded hemicellulases, with GH10 family being most abundant; 538 genes encoded debranching enzymes (GH67 and GH78); and 3,266 genes encoded oligosaccharide-degrading enzymes, with GH2 family being most abundant.

Table 4 Distribution of GH genes in Haizi buffalo rumen microorganism metagenome

Items	Number	Items	Number	Items	Number
GH1	-	GH47	-	GH93	-
GH2	-	GH48	-	GH94	-
GH3	-	GH49	-	GH95	-
GH4	-	GH50	-	GH96	-
GH5	-	GH51	-	GH97	-
GH6	-	GH52	-	GH98	-
GH7	-	GH53	-	GH99	-
GH8	-	GH54	-	GH100	-
GH9	-	GH55	-	GH101	-

Items	Number	Items	Number	Items	Number
GH10	-	GH56	-	GH102	-
GH11	-	GH57	-	GH103	-
GH12	-	GH58	-	GH104	-
GH13	-	GH59	-	GH105	-
GH14	-	GH62	-	GH106	-
GH15	-	GH63	-	GH107	-
GH16	-	GH64	-	GH108	-
GH17	-	GH65	-	GH109	-
GH18	-	GH66	-	GH110	-
GH19	-	GH67	-	GH111	-
GH20	-	GH68	-	GH112	-
GH22	-	GH70	-	GH113	-
GH23	-	GH71	-	GH114	-
GH24	-	GH72	-	GH115	-
GH25	-	GH73	-	GH116	-
GH26	-	GH74	-	GH117	-
GH27	-	GH75	-	GH118	-
GH28	-	GH76	-	GH119	-
GH29	-	GH77	-	GH120	-
GH30	-	GH78	-	GH121	-
GH31	-	GH79	-	GH122	-
GH32	-	GH80	-	GH123	-
GH33	-	GH81	-	GH124	-
GH34	-	GH82	-	GH125	-
GH35	-	GH83	-	GH126	-
GH36	-	GH84	-	GH127	-
GH37	-	GH85	-	GH128	-
GH38	-	GH86	-	GH129	-
GH39	-	GH87	-	GH130	-
GH42	-	GH88	-	GH131	-
GH43	-	GH89	-	GH132	-
GH44	-	GH90	-	GH133	-
GH45	-	GH91	-	GH134	-
GH46	-	GH92	-	GH135	-

Table 5 Distribution of GH genes in microorganism metagenome in different animals

Items	Haizi buffalo rumen	Dairy cow rumen[7]	Indian buffalo rumen[9]	Termite hindgut[11]	Chicken cecum[12]
Cellulases					
GH5	-	-	-	5.71%	-

Items	Haizi buffalo rumen	Dairy cow rumen[7]	Indian buffalo rumen[9]	Termite hindgut[11]	Chicken cecum[12]
GH9	-	-	-	-	-
GH44	-	-	-	-	-
GH45	-	-	-	-	-
Total	932	-	-	-	-
Hemicellulases					
GH8	-	-	-	-	-
GH10	-	-	-	5.39%	-
GH11	-	-	-	-	-
GH26	-	-	-	-	-
GH28	-	-	-	-	-
Total	1,011	-	-	-	-
Debranching enzymes					
GH67	-	-	-	-	-
GH78	-	-	-	-	-
Total	538	-	-	-	-
Oligosaccharide degrading enzymes					
GH1	-	-	-	-	-
GH2	-	-	-	-	-
GH3	-	-	-	-	-
GH29	-	-	-	-	-
GH35	-	-	-	-	-
GH38	-	-	-	-	-
GH39	-	-	-	-	-
GH42	-	-	-	-	-
Total	3,266	-	-	-	-

Discussion

This study conducted metagenomic analysis of Haizi buffalo rumen fluid samples using high-throughput sequencing technology, obtaining 77,283,638 reads that were assembled into 744,712 scaffolds. Comparison with the COG database revealed that genes involved in carbohydrate transport and metabolism were most abundant, which is consistent with reports in Indian buffalo [9]. Genes related to amino acid transport and metabolism, as well as cell wall and membrane biogenesis, were also abundant. GO classification analysis showed that genes with binding and catalytic functions were most common, indicating that the Haizi buffalo rumen metagenome likely contains numerous lignocellulolytic enzyme genes.

Taxonomic analysis based on gene annotation proportions showed that genes derived from Bacteroidetes were most abundant, accounting for 55.3%. Jami et al. [13] reported similar microbial distribution in dairy cow rumen. Species classification revealed that most microorganisms in Haizi buffalo rumen belonged to the bacterial kingdom, including *Ruminococcus flavefaciens*, *Ruminococcus albus*, and *Fibrobacter succinogenes*, which produce large amounts of cellulases to degrade dietary lignocellulose and are considered primary lignocellulose-degrading microorganisms in the rumen. *Prevotella ruminicola* can degrade xylan and pectin [14-15].

Synergistic action of cellulases, hemicellulases, and pectinases degrades plant cell walls to provide nutrients for ruminants, and genes encoding all three enzyme classes were present in Haizi buffalo rumen. GH5 and GH9 families were relatively abundant, together comprising 4.77% of total GH genes. Enzymes in families GH10, GH11, GH26, and GH28 exhibit hemicellulase activity, with GH10 and GH11 being xylanases that randomly cleave β-1,4 xylosidic bonds from the xylan backbone to produce xylooligosaccharides and small amounts of xylose. Xylan esterases in families CE1, CE4, CE6, and CE7 can act on xylan side chains [8,16]. Additionally, enzymes in families PL1, PL9, and PL10 exhibit pectate lyase activity, while CE8 family enzymes have pectin methylesterase activity that demethylates pectin to produce pectic acid [17]. Among GH classifications, oligosaccharide-degrading enzymes were most abundant, likely because plant cell walls are degraded into numerous oligosaccharides by the synergistic action of cellulases, hemicellulases, and pectinases, which must be further degraded before absorption by ruminants. The GH2 family was most abundant among oligosaccharide-degrading enzymes, primarily including β-D-galactosidase, β-glucuronidase, β-D-mannosidase, and exo-β-N-acetylglucosaminidase [18].

Furthermore, we identified 42 cohesin genes, 236 dockerin genes, and 82 S-layer homology (SLH) domains in Haizi buffalo rumen, all components of the cellulosome. The cellulosome is a multi-enzyme complex formed by lignocellulolytic enzymes scaffolded by structural proteins [19], which adheres to cell surfaces via adhesion proteins for sustained, efficient lignocellulose degradation. Cellulosomes have been identified in *Ruminococcus flavefaciens* and *Ruminococcus albus* [20]. Hess et al. [7] found 80 cohesin genes and 188 dockerin genes in dairy cow rumen.

Comparative analysis of GH genes from Haizi buffalo rumen with those from dairy cow [7] and Indian buffalo rumen [9], termite hindgut [11], and chicken cecum [12] metagenomes revealed that oligosaccharide-degrading enzymes were most abundant in all metagenomes. However, Haizi buffalo had the fewest oligosaccharide-degrading enzymes among the five gastrointestinal metagenomes. The proportions of cellulases, hemicellulases, and debranching enzymes were similar between Haizi buffalo and dairy cow rumen metagenomes. Indian buffalo had the fewest cellulases but most oligosaccharide-degrading enzymes. The proportions of cellulases and oligosaccharide-degrading enzymes differed significantly between Haizi buffalo and Indian buffalo rumen, possibly due to differences in dietary roughage-to-concentrate ratios. Indian buffalo were fed a 75:25 roughage-to-concentrate ratio [9], whereas Haizi buffalo received an 85:15 ratio. Higher roughage content may lead to higher proportions of cellulases and lower proportions of oligosaccharide-degrading enzymes in the rumen. However, both buffalo rumen metagenomes had low abundances of GH6, GH7, GH12, GH48, and GH62 families [8]. Termite hindgut microbial metagenome had the most cellulase and hemicellulase genes, with GH5 (5.71%) and GH10 (5.39%) families being most abundant, primarily because wood contains higher proportions of cellulose and hemicellulose than forage. Chicken cecum had the lowest proportions of cellulases and hemicellulases but high oligosaccharide-degrading enzyme proportions. These results demonstrate that host genotype and diet can influence gastrointestinal GH gene composition.

High-throughput sequencing revealed that Haizi buffalo rumen contains diverse lignocellulose-degrading microorganisms, including *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Fibrobacter succinogenes*, and *Prevotella ruminicola*, indicating that Haizi buffalo rumen is rich in lignocellulose-degrading microorganisms. The rumen also contains numerous glycoside hydrolase genes, with GH2, GH43, GH97, and GH3 families being most abundant, and genes encoding oligosaccharide-degrading enzymes being most numerous, demonstrating that Haizi buffalo rumen harbors abundant lignocellulolytic enzyme systems.

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