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Metagenomics approach to identify lignocellulose-degrading enzymes in the gut microbiota of the Chinese bamboo rat cecum

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ABSTRACT

Background: Lignocellulose is considered a renewable organic material, but the industrial production of biofuel from lignocellulose is challenging because of the lack of highly active hydrolytic enzymes. The guts of herbivores contain many symbiotic microorganisms that have evolved to hydrolyze plant lignocellulose. Chinese bamboo rats mainly consume high-fiber foods, indicating that some members of the intestinal tract microbiota digest lignocellulose, providing these rats with the energy required for growth.
Results: Here, we used metagenomics to analyze the diversity and functions of the gut microbiota in Chinese bamboo rats. We identified abundant populations of lignocellulose-degrading bacteria, whose main functions involved carbohydrate, amino acid, and nucleic acid metabolism. We also found 587 carbohydrate-active enzyme genes belonging to different families, including 7 carbohydrate esterase families and 21 glycoside hydrolase families. The glycoside hydrolase 3, glycoside hydrolase 1, glycoside hydrolase 43, carbohydrate esterase 4, carbohydrate esterase 1, and carbohydrate esterase 3 families demonstrated outstanding performance.
Conclusions: The microbes and enzymes identified in our study expand the existing arsenal of proficient degraders and enzymes for lignocellulosic biofuel production. This study also describes a powerful approach for targeting gut microbes and enzymes in numerous industries.
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1. Introduction

Lignocellulose is the most abundant and renewable organic material on Earth and is considered a promising substitute for fossil fuels [1,2]. However, its structure is recalcitrant to enzymatic breakdown and currently available hydrolytic enzymes exhibit low activity, preventing industrial-scale biofuel production from lignocellulosic biomass [2,3]. The ability of many herbivores to degrade lignocellulose makes them valuable sources of lignocellulose-degrading enzymes. This ability relies on partnerships with the diverse communities found within the microbiota [4]. In the animal gastrointestinal (GI) tract, the microbiota is closely related to growth and metabolism and improves digestive efficiency, the acquisition of digestive enzymes, the ability to live on suboptimal diets, and vitamin provision [5,6,7]. For many herbivores, the GI microbiome produces enzymes that digest polysaccharides, including cellulose, which is the main component of plant cell walls, which are normally difficult to digest [5].

Chinese bamboo rats (*Rhizomys sinensis*) are well known for their unusual diet and are considered bamboo specialists within the mammalian order and herbivores [8]. They eat high-fiber foods, such as bamboo leaves, poles, roots, shoots, and corn stalks, suggesting that their intestinal microbiota degrades plant polysaccharides, particularly cellulose [9]. Thus, the Chinese bamboo rat is an excellent model for identifying gut biocatalysts that produce digestive enzymes that decompose lignocellulose and convert it to end products such as sugars, hydrogen, and acetate.

Metagenomics is a technology that determines microbiota structure and variation, allowing us to determine microbial composition and function in the GI tract of the host [10]. Metagenomic

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sequencing technology is also extensively used to address the complex process of lignocellulose degradation in herbivores [11,12]. This technology, which is based on shotgun sequencing coupled with biochemical characterization, has already identified several important carbohydrate-active enzyme (CAZyme) families that degrade plant polysaccharides [13,14,15,16]. Here, we used metagenomic sequencing to obtain information on the microbiota of the Chinese bamboo rat cecum by analyzing the microbial community and its functions and to identify the genes and genomes that participate in biomass deconstruction.

2. Materials and methods

2.1. Sample collection and DNA extraction

Three Chinese bamboo rats (The Chinese bamboo rats at the age of 300 d were arrested by a nearby villager on a hill and brought to the meat market, which mainly eat bamboo poles and roots) were purchased from a meat market in Jinsha town of Minqing, Fujian Province, China. Whole fresh cecum content (2 mL) was collected from each rat. The three fresh samples were mixed and immediately frozen at −80°C before analysis. The cecum content DNA was extracted using a modified protocol based on the Meta-G-Nome DNA isolation kit (Epicentre, WI, USA). Briefly, the sample was homogenized in extraction buffer and vortexed, followed by a series of centrifugation steps to remove plant material and other large debris from the cecum. Supernatants were then filtered through a 1.2-μm filter to capture eukaryotic cell debris, followed by a microbe capture step using a 0.2-μm filter. Microbes were collected from the filter, and genomic DNA was extracted. The DNA in the agarose plug was then used for metagenomic sequencing.

2.2. Metagenome library preparation and sequencing

The paired-end sequencing library was prepared using Illumina, Truseq Nano DNA LT Library Preparation Kit (Illumina, California, United States). Subsequently, 200 ng of a genomic DNA was fragmented by Covarivs (CovarissInc, Massachusetts, USA) to generate a mean fragment distribution of 550 bp. Subsequently, the fragments were subjected to end repair and the indexing adapters were ligated to the end of the DNA fragment. The DNA fragment, which ligated adapters, were PCR amplified. The amplified library was analyzed in Bioanalyzer 2100 (Agilent Technologies, USA) using a High Sensitivity DNA chip. Then, the library was cluster generated and paired-end sequenced on the Illumina-MiSeq platform [17].

2.3. Metagenome assembly and analyses of species richness and function

Total genomic DNA was fragmented and ligated with sequencing adaptors. The obtained reads were uploaded to the Metagenome Rapid Annotation Using Subsystem Technology (MG-RAST) server to remove reads with short lengths and poor quality before annotation and analysis [18].

The reads in MG-RAST were classified via the M5NR protein database (http://tools.metagenomics.anl.gov/m5nr/), and predicted genes were aligned in the M5NR database by MG-RAST. Functional annotation and classification relied on the SEED subsystem [19]. The following parameters were applied in the analysis: maximum e-value of 1e-5, minimum percent identity of 60, and minimum alignment length of 30.

BLAST (Basic Local Alignment Search Tool) alignment was used to query predicted protein sequences against an integrated protein database [20]. The following analysis parameters were applied: maximum e-value of le-5 and minimum similarity of 30%. The predicted genes were used for further analyses, such as the identification of Clusters of Orthologous Groups (COG) functional categories, Gene Ontology (GO) classification, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Genes were classified based on GO annotations. The analysis was based on the Blast UniProt results (merging the results obtained with SWISS-PROT and TrEMBL). Pathways were assigned using the KEGG mapping method based on predicted ORF sequences [21]. Corresponding KEGG Orthology (KO) numbers were predicted by KEGG Automatic Annotation Server (KAAS) and mapped to KEGG pathways. Additionally, the relationships between genes and their KEGG annotations were analyzed, and information was mapped to the pathways.

The putative protein-coding regions of the whole metagenome were predicted using FragGeneScan. The presence of carbohydrate-active enzymes of the predicted protein-coding regions was scrutinized in the CAZy database (http://www.cazy.org). To do this, the CAZYmes Analysis Toolkit (CAT) was used to perform the Pfam-based sequence annotation with an e-value of 1e-5 [17]. The results were further analyzed manually to determine CAZyme proportions in the Chinese bamboo rat cecum. The raw data have been deposited into the GeneBank database (GCA_003259765.1) (http://www.ncbi.nlm.nih.gov/nuccore/QLIP00000000).

3. Results and discussion

3.1. Metagenomic library sequencing

We analyzed the obtained metagenomic sequence to identify functional attributes encoded in the gut microbiome of the Chinese bamboo rat cecum. The initial sequence contained 69872827 bases. The average read length was 652.14 bp, and the G + C content was 48.30% (Table 1). Before further processing was performed, the read data were subjected to the MG-RAST online server quality control pipeline [18]. A total of 127,944 protein-coding genes were predicted. The coding ratio was 93.22% (Table 2).

3.2. Microbial diversity analysis of the Chinese bamboo rat cecum

Predicted genes were compared to the M5NR database by MG-RAST, and the best alignment results were obtained by applying specific parameters (e-value < 1e-5, coverage>=60%). Many microorganisms were identified in the Chinese bamboo rat cecum. Bacteria (98.90%) was the predominant domain in Chinese bamboo rat cecum contents; Archaea and Eukarya together composed only 1.10% of microorganisms (Fig. 1). Firmicutes were the most predominant phylum (68.55%), followed by Bacteroidetes (13.46%) and Proteobacteria (9.28%). Bacteroidia was the primary contributor to Bacteroidetes (Fig. 2). In our study, the high proportion of Firmicutes is notably similar to that found in *Ailuropoda melanoleucaa* (60.80%) [22], *Rhinopithecus bieti* (39.36%), [11] and Chinese Mongolian sheep (44.62%) [23] and differs from the findings in Indian buffalo rumen [24]. The content of Firmicutes in our study (68.55%) is significantly higher than *Rhinopithecus bieti* and Chinese Mongolian sheep and has no significant difference from *Ailuropoda melanoleucaa*, perhaps due to the Chinese bamboo rat consume the roots and shoots of bamboo and other highly fibrous plants each day.

Additionally, we found that Clostridia was the predominant class (61.54%), followed by Bacteroidia (11.85%) and Bacilli (4.69%) (Fig. 3). The presence of Clostridia was likely to be important for lignocellulosic biomass degradation [25]. At the genus

Table 1
Gene splicing results.

Contig Number	Max Length(bp)	N50 (bp)	Average Length (bp)	Size (bp)	Gap Number	N Number	GC Content (%)
107144	168,725	884	652.14	69,872,827	0	0	48.3

Table 2
Gene prediction results.

Class	Number
Size (base)	69,872,827
G + C content (%)	48.3
Protein-coding genes	127,944
Min length (base)	63
Max length (base)	18,966
Average length (base)	509.11
Total coding gene (base)	65,138,131
Coding ratio (%)	93.22383793

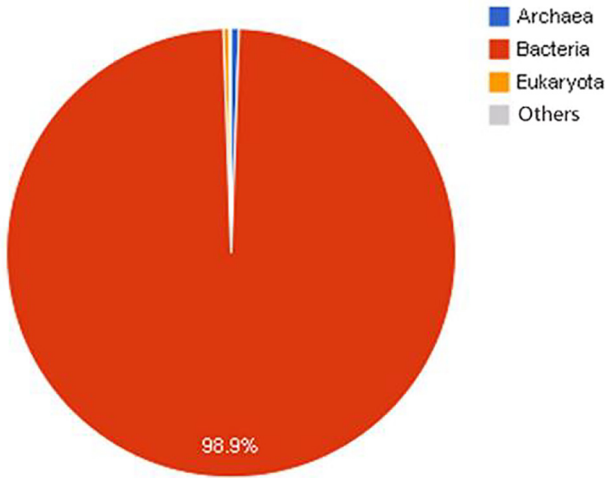

Fig. 1. Microorganisms distribution of Bamboo rat cecum contents in domain.

level, Clostridium was the dominant microbe, accounting for 16.17% of microorganisms, and Bacteroides (7.10%) was the major genus in the Bacteroidetes phylum (Fig. 4). Clostridium is a potential plant biomass degradable bacterium. Researchers found *Clostridium clariflavum*, *Clostridium straminisolvens,* and *Clostridium thermocellum* can efficiently degrade cellulose and hemicellulose [26,27].

Consistently, Bacteroidetes was found predominantly in the rumen [28] and aids in the digestion of complex carbohydrates [29]. But in our study, Bacteroidetes was the second most predominant phylum in the Chinese bamboo rat cecum. The class Bacteroidia was the primary contributor to the Bacteroidetes population (Fig. 3), and Bacteroides was the major genus of Bacteroidetes in the Chinese bamboo rat cecum (Fig. 4). It is reported that Bacteroides encodes key metabolic functions identified in anaerobic food webs, including the ability to process polysaccharides into oligosaccharides and simple sugars and to ferment amino acids in the human gut [30,31]. As the third most predominant phylum in Chinese bamboo rat cecum contents, Proteobacteria (9.28%) was significantly less abundant than Firmicutes and Bacteroidetes. Several microbes belonging to Pseudomonas have very diverse metabolisms and degrade organic solvents such as toluene [32] and phenol [33,34]. This ability may be beneficial for Chinese bamboo rats, given their ability to consume several toxic and pungent insects.

3.3. Functional analysis of the bamboo rat cecum metagenome

The COG functional classification and prediction were based on predicted genes. A total of 37,733 genes were annotated to 22 COG categories. Among these categories, the most abundant function was translation, ribosomal structure, and biogenesis (10.36%); the second most abundant function was general function prediction only (10.08%); the third most abundant function was replication, recombination, and repair (9.95%); and the fourth most abundant functional category was carbohydrate transport and metabolism (9.48%) (Fig. 5, Table S1). We also performed GO functional classification and prediction, and a total of 48,283 genes were annotated to 3929 GO functional classifications. Among biological processes, the genes were mainly categorized under metabolic process (30.17%) and cellular process (28.12%). Among cellular components, the functions of 23,465 genes were categorized under cell (20.38%), and 23,465 genes were categorized under cell part (20.38%). Among molecular functions, 35,392 genes were categorized under catalytic activity (30.74%), and 32,097 genes were categorized under binding (27.88%) (Fig. 6, Table S2).

KEGG pathways were assigned, and a total of 8885 reads were annotated to 338 pathways. Of these, 1127 genes were annotated to organismal systems, 9408 genes were annotated to metabolism, 3096 genes were annotated to genetic information processing, 1594 genes were annotated to environmental information processing, and 1336 genes were annotated to cellular processes (Table S3). More genes were annotated to metabolism than to other functions, and most of these genes were specifically annotated to carbohydrate metabolism, which comprised 1914 genes. The most highly enriched organismal system pathway was the endocrine system with 361 genes. Under genetic information processes, 1207 genes were enriched for translation (Fig. 7). Comparison of the five systems indicated that most gene functions were concentrated under metabolism.

COG categories, GO classifications, and KEGG pathways suggested that the gut microbiome of the Chinese bamboo rat is enriched for carbohydrate, amino acid, nucleotide, and lipid metabolic activity. Comparative COG analysis of our data with panda GI [22] and rumen metagenomes [35,36,37,38] revealed metabolic similarities among pandas, wallabies, cows, termites, and humans, mainly associated with carbohydrate, amino acid, and DNA metabolism.

3.4. Diversity and abundance of CAZymes in the gut microbiota of the Chinese bamboo rat cecum

The plant biomass-degrading capacity of a microbial consortium is closely related to the presence of genes encoding CAZymes [39]. To obtain an overview of the microbial degradation of major plant biomass-associated polymers in the Chinese bamboo rat cecum content, we screened the Chinese bamboo rat cecum gut microbiota by metagenomic sequencing to determine the microbial genes encoding CAZymes. All candidate genes from microorganisms in the Chinese bamboo rat cecum were searched against the CAZy database using dbCAN [40]. The presence of at least one relevant catalytic domain or carbohydrate-binding module indicated a candidate gene. We found 587 CAZyme genes distributed heterogeneously among 7 carbohydrate esterase (CE) fam-

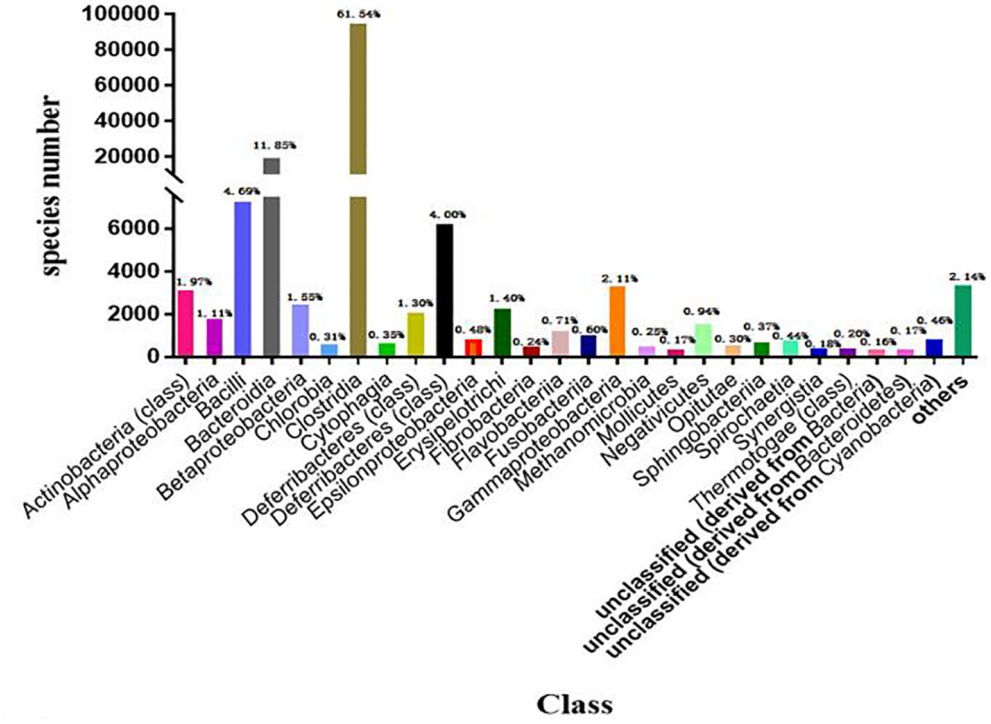

Fig. 2. Microorganism distributions in bamboo rat cecum contents by phylum.

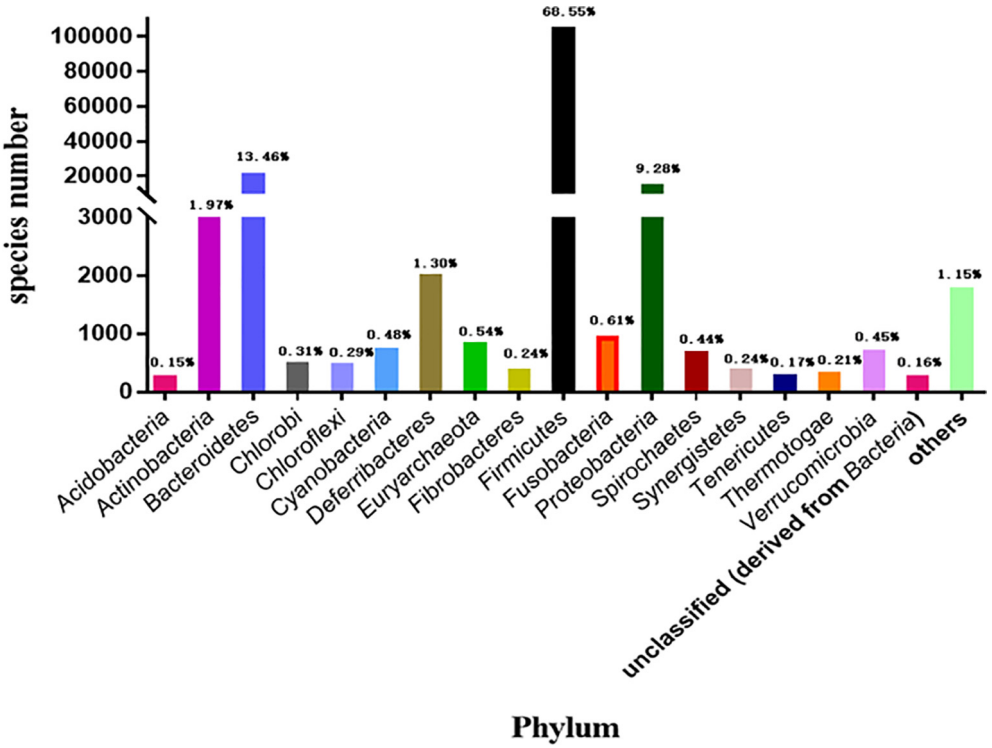

Fig. 3. Microorganism distributions in bamboo rat cecum contents by class.

ilies (42.25%) and 21 glycoside hydrolase (GH) families (57.75%). Interestingly, the GH catalytic modules in the Chinese bamboo rat cecum demonstrated broad diversity, indicated by the presence of 339 genes belonging to 21 GH families. GH3 (93 genes), GH1 (45 genes), and GH43 (42 genes) were the most frequently identified GH families occurring in microorganisms of the Chinese bamboo rat cecum. A total of 248 genes belonged to 7 CE families, and CE4 (91 genes), CE1 (73 genes), and CE3 (48 genes) were the most frequently identified CE families. These enzymes largely demonstrated acetyl xylan esterase functions (Table 3).

GHs are the prominent group of CAZymes that hydrolyze glycosidic bonds in carbohydrate molecules [24]. In cattle rumen, GHs

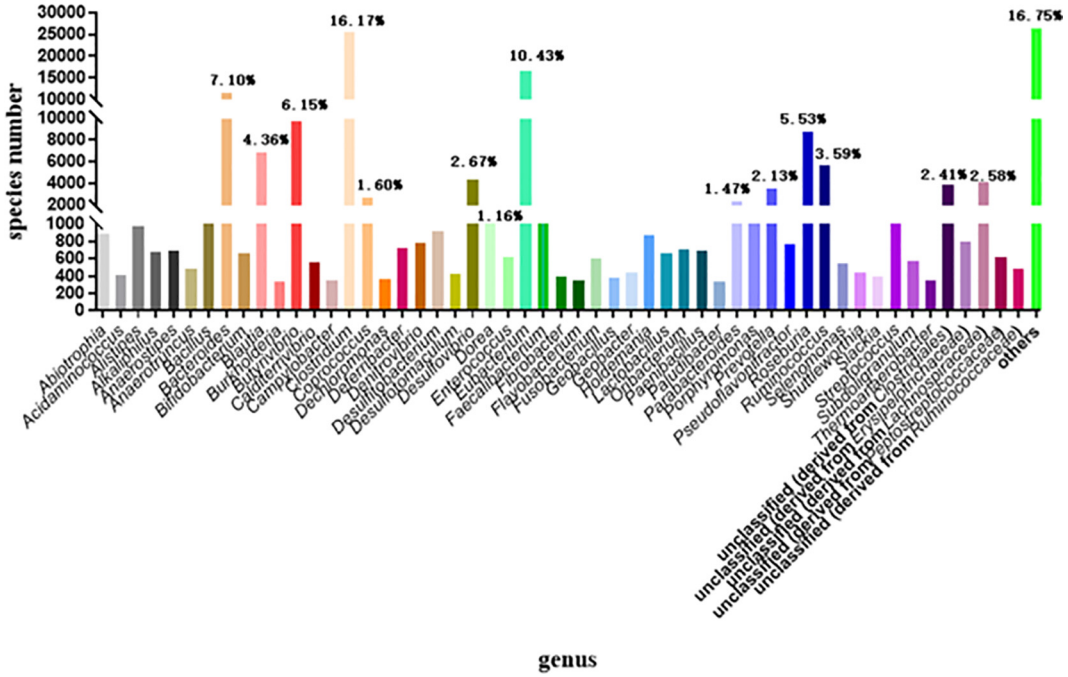

Fig. 4. Microorganism distributions in bamboo rat cecum contents by genus.

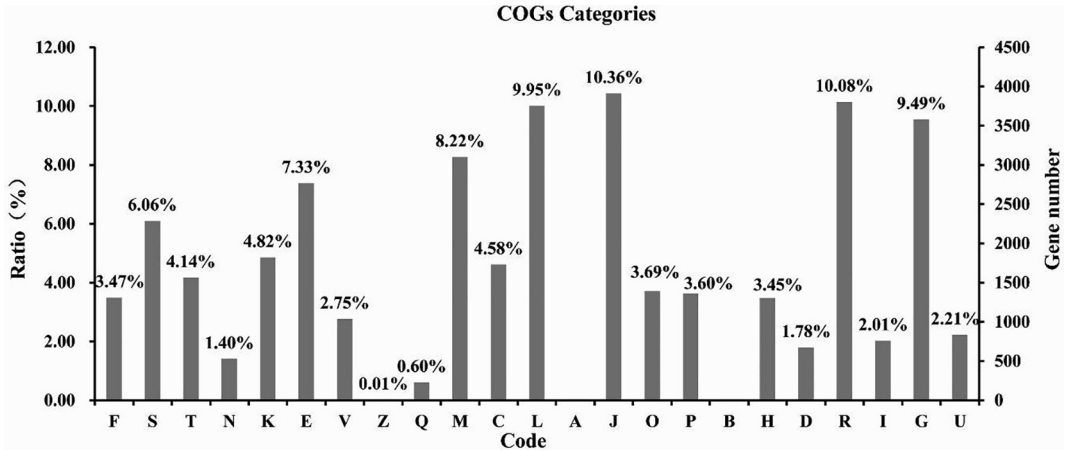

Fig. 5. COG gene categories. Note: "COG" stands for "Clusters of orthologous groups for eukaryotic genomes". The proteins that make up each COG are assumed to be from the same ancestor protein, so they have the same or similar functions. Letter abbreviations: [E] Amino acid transport and metabolism; [C] Energy production and conversion; [J] Translation, ribosomal structure and biogenesis; [R] General function prediction only; [L] Replication, recombination and repair; [H] Coenzyme transport and metabolism; [G] Carbohydrate transport and metabolism; [P] Inorganic ion transport and metabolism; [O] Posttranslational modification, protein turnover, chaperones; [T] Signal transduction mechanisms; [M] Cell wall/membrane/envelope biogenesis; [I] Lipid transport and metabolism; [S] Function unknown; [K] Transcription; [F] Nucleotide transport and metabolism; [N] Cell motility; [V] Defense mechanisms; [U] Intracellular trafficking, secretion, and vesicular transport; and [Q] Secondary metabolites biosynthesis, transport and catabolism.

were observed to be the most predominant and diverse group of catalytic enzymes involved in the hydrolysis of plant polymers [22,41]. In our study, enzyme coding for GH families are highly abundant in most of the genomes, and they account for 57.75% of the enzymes classified in the CAZy database, and GH catalytic modules demonstrated broad diversity. GH variability highlights the great potential of the Chinese bamboo rat cecum for identifying polysaccharide- and cellulose-degrading enzymes. GH3, GH1, and GH43 were the most frequently identified GH family members in the Chinese bamboo rat cecum. The GH3 family is known to have cellobiase activity [42]. It has been reported that GH3 family contains many glycosidases that hydrolyze complex carbohydrates into oligosaccharides [43]. The GH1 family is believed to play important roles in many diverse processes, including chemical defenses during herbivory, lignification, the hydrolysis of cell wall-derived oligosaccharides during germination, and control of active phytohormone levels [44]. The GH43 family includes β-xylosidase, β-1,3-xylosidase, α-L-arabinofuranosidase, arabinanase, xylanase, and galactan 1,3-β-galactosidase activity (www.cazy.org) [24]. In Chinese bamboo rat cecum, the GH family plays an important role to hydrolyze glycosidic bonds.

The complete depolymerization of plant polysaccharides requires the combined activities of different enzyme groups in addition to GHs, including CEs, PLs, and AAs [17]. In our study, 7

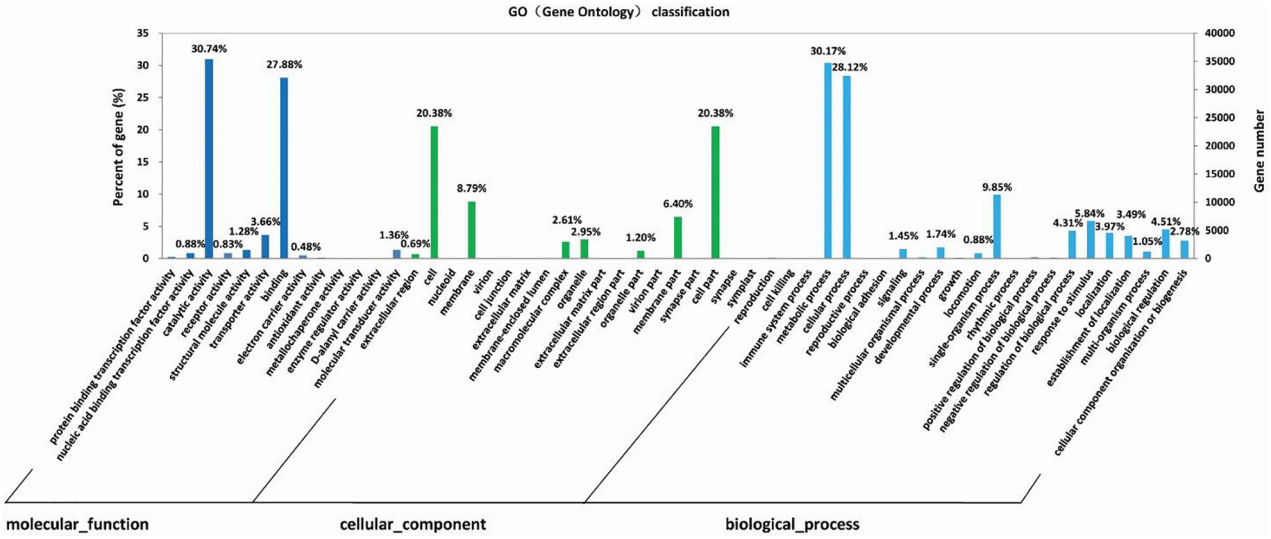

Fig. 6. GO-annotated genes under level 2.

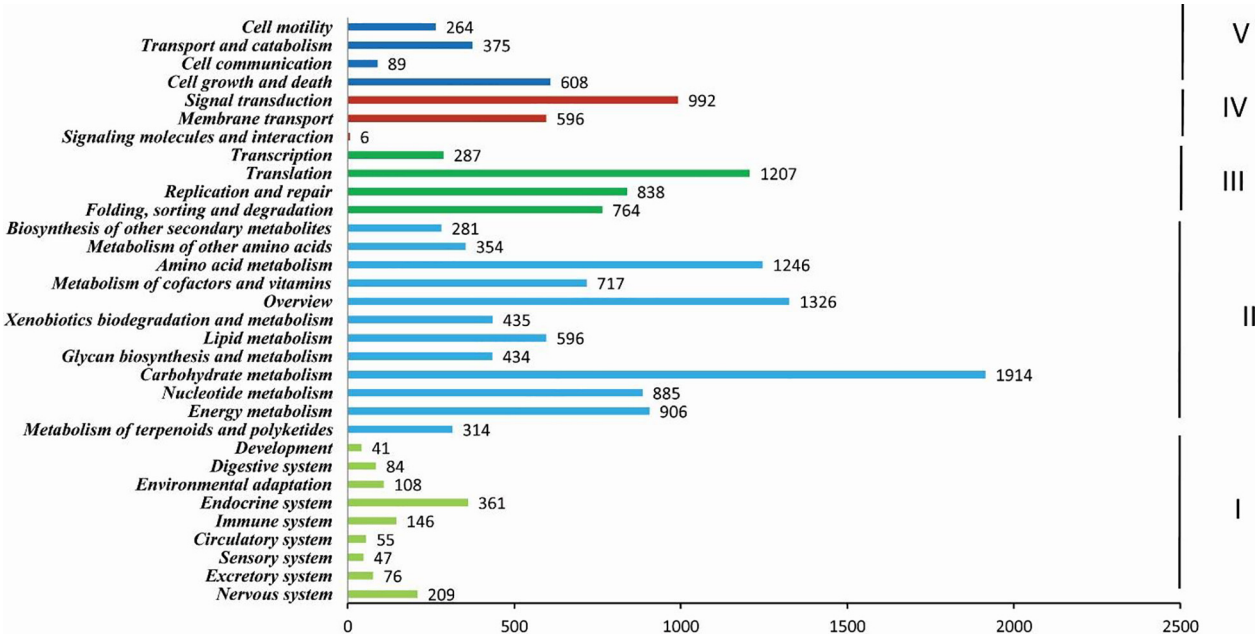

Fig. 7. Pathway annotated classification. Note: (I): Organismal Systems; (II): Metabolism; (III): Genetic Information Processing; (IV): Environmental Information Processing; (V): Cellular Processes.

CE families were also found by annotating the metagenome of Chinese bamboo rat cecum contents. CEs such as CAZymes can be harnessed to overcome challenges associated with the acetylated backbone structures of many hemicelluloses, which present a challenge when the objective is to convert corresponding polysaccharides to fermentable sugars or recover hemicellulose for biomaterial applications [45]. Zhang et al., [46] reported that enzymes from CE1, CE2, CE3, CE4, and CE7 were mostly annotated as acetyl xylan esterases, which have been demonstrated to facilitate xylan solubilization by removing acetyl substituents, based on sequence similarity. In Chinese bamboo rat cecum, CE4, CE1, and CE3 were the CE families with the highest numbers of genes identified. In Chinese bamboo rat cecum, GH family and CE family digest hemicelluloses and hydrolyzing glycosidic bonds.

4. Conclusions

The metagenomic analysis and gene annotation results obtained for putative cellulose-metabolizing symbionts in the microbial environment in the present study clarified that bamboo rats partially digest bamboo fibers. It is increasingly clear that bamboo rats have undergone suitable evolutionary adaptation for highly specialized herbivory, and the main metabolic pathways are carbohydrate, amino acid, and nucleotide metabolism. CEs and GHs were the main CAZyme families in the Chinese bamboo rat cecum, and Firmicutes, Bacteroidetes, and Proteobacteria were identified as the main contributors of CAZymes inhabiting the Chinese bamboo rat cecum.

Table 3
CAZyme target gene annotations.

Predicted enzyme function	CAZy family	CAZy class	Gene number
Acetyl xylan esterase	CE4	CE	91
Acetyl xylan esterase	CE3	CE	48
Acetyl xylan esterase	CE12	CE	21
Acetyl xylan esterase	CE2	CE	6
Acetyl xylan esterase	CE6	CE	6
Acetyl xylan esterase	CE7	CE	3
Acetyl xylan esterase, feruloyl esterase	CE1	CE	73
Cellobiohydrolase, β-glucosidase	GH9	GH	8
Endo-β-1,4-galactanase	GH53	GH	10
Endo-β-1,4-glucanase, Endo-β-1,4-xylanase, β-mannosidase	GH5	GH	9
Exo-α-L-1,5-arabinanase	GH93	GH	2
Xyloglucanase	GH16	GH	16
Xyloglucanase	GH74	GH	2
α-galactosidase	GH36	GH	10
α-galactosidase	GH57	GH	10
α-galactosidase	GH97	GH	7
α-galactosidase	GH110	GH	4
α-galactosidase	GH27	GH	4
α-galactosidase, α-glucuronidase	GH4	GH	7
α-glucuronidase	GH67	GH	1
α-L-fucosidase	GH29	GH	23
α-L-fucosidase	GH95	GH	5
β-1,4-mannanase	GH26	GH	2
β-glucosidase	GH3	GH	90
β-glucosidase, β-mannosidase	GH1	GH	45
β-mannosidase	GH2	GH	32
β-xylosidase/α-L-arabinofuranosidase	GH43	GH	42
β-xylosidase/α-L-arabinofuranosidase	GH51	GH	10

The results of this study enrich the metagenome studies of Chinese bamboo rats and provide a genetic resource of plant cell wall degrading microbial enzymes for biofuel production. The discovery of these enzymes can be used for industrial degradation of lignocellulose. In addition, these enzymes also facilitate the conversion of lignocellulose into usable small molecules that can be used by organisms (i.e., glucose, alcohol), which can broaden the application of lignocellulose in food, animal husbandry, and other agricultural fields. So the identified enzymes in this study not only provide novel insights for the use of new biological energy sources, but also provide a reference for the development and utilization of lignocellulose new energy. These metabolites also play important roles in maintaining the normal growth and development of the Chinese bamboo rat.

Ethical approval

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the College of Animal Science, Fujian Agriculture and Forestry University. Experiments involving live animals were conducted according to the "Regulations for the Administration of Affairs Concerning Experimental Animals." Animals involved in this study were humanely sacrificed as necessary to ameliorate their suffering.

Conflict of interest

The authors declare no competing interests.

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Supplementary material

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