Metagenomics Reveals Rumen Microbial Diversity and Function Postprint

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Abstract

Ruminant rumen harbors a vast and complex microbial community intimately linked to host digestion, nutrient metabolism, and immune function, together constituting a "superorganism" . Since the vast majority of rumen microorganisms are non-culturable, traditional anaerobic culture-based methods exhibit significant limitations. Metagenomics, through high-throughput sequencing approaches, enables comprehensive characterization of microbial diversity and accurate identification of novel functional genes. Moreover, metagenomics reveals the interplay between host genes and the microbiome. As omics technologies continue to advance, metagenomics holds promising prospects for rumen microbiome research.

Full Text

Metagenomics Reveals Rumen Microbial Diversity and Functions

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Abstract

The rumen of ruminant animals harbors a vast and complex microbial community that is intimately associated with host digestion, nutrient metabolism, and immune function. The host and its symbiotic microorganisms together constitute a "superorganism." Since the vast majority of rumen microorganisms cannot be cultivated, traditional culture-dependent research methods suffer from significant limitations. Metagenomics overcomes these constraints through high-throughput sequencing, enabling comprehensive characterization of microbial diversity and accurate discovery of novel functional genes. Furthermore, metagenomics reveals the intricate interactions between host genetics and the microbiome. With continuous advances in omics technologies, metagenomics holds broad application prospects for rumen microbiome research.

Keywords: metagenomics; rumen; microbiome

The global ruminant population has exceeded 4.4 billion, representing an important source of food and economic value [1]. The rumen provides an ideal environment for microbial growth with its large capacity and optimal temperature. This ecosystem contains over 3,000 microbial species, including bacteria, protozoa, fungi, and archaea [2], as well as bacteriophages and viruses [3]. Research indicates that each milliliter of rumen fluid contains approximately 10^{11} bacteria, 10^{10} bacteriophages, 10^9 archaea, 10^6 protozoa, and 10^6 fungal spores [4]. These rumen microorganisms are closely linked to host nutrition, metabolism, and immunity [5]. They utilize dietary cellulose, hemicellulose, and proteins to produce volatile fatty acids (VFAs) and synthesize microbial protein, thereby providing essential nutrients for the host. Simultaneously, methanogens generate methane from carbon dioxide and hydrogen, contributing to global climate change [6]. Additionally, rumen microorganisms maintain host health by degrading toxic substances in feed [7] and producing immunoprotective compounds such as mannan oligosaccharides [8]. Improper regulation of rumen microorganisms can compromise animal health; for example, excessive concentrate intake causes rapid proliferation of lactic acid bacteria, leading to lactic acid accumulation, death of Gram-negative bacteria, release of lipopolysaccharides from cell walls, and ultimately rumen acidosis [9].

Metagenomics, first proposed by Handelsman et al. [10] in 1998, refers to the study of all microbial genomes in an environment, thereby overcoming the limitations of traditional cultivation methods. Lederberg [11] coined the term "superorganism" to define the intimate relationship between host and symbiotic microbes, recognizing that ruminants possess two sets of genetic material: their own and that of their symbiotic microbiome. The implementation and completion of the human gut metagenome project (MetaHIT) [12] has deepened our understanding of host-microbe relationships. Research groups such as the Australian Rumen Pangenome and European RuminOmics have applied metagenomic approaches to investigate rumen microbial community composition, phylogeny, and metabolic functions. As shown in [Figure 1: see original paper], although metagenomics emerged relatively recently, it has developed rapidly and become a global research hotspot.

1. Metagenomics Methods and Workflow

Early metagenomic technologies focused on discovering genes encoding specialized biomolecules from various environments. The general workflow involves: (1) extracting total DNA from environmental samples, (2) constructing metagenomic libraries using expression vectors such as Fosmid, Cosmid, and bacterial artificial chromosomes, and (3) introducing these vectors into host bacteria like *E. coli* to express active products. Currently, three screening methods exist for metagenomic libraries: sequence-based, function-based, and substrate-induced gene expression. Ufarté et al. [13] provided detailed descriptions of Fosmid library construction and screening protocols for carbohydrate-active enzyme genes, while Daniel [14] reviewed major library construction and functional screening methods. To date, metagenomics has been successfully applied to discover lipases [15] and cellulases [16] from soil and rumen environments.

The advent of next-generation sequencing has substantially enriched metagenomics, with amplicon sequencing and whole-genome sequencing emerging as the two primary directions. Amplicon sequencing reveals microbial community structure and abundance information, while whole-genome sequencing enables further functional analysis. Using platforms such as Illumina and Roche 454 FLX, researchers sequence phylogenetic marker genes like the 16S/18S small subunit (SSU) rRNA gene. After quality filtering and chimera removal, reads are processed using Mothur [17] or QIIME [18] to generate operational taxonomic units (OTUs). Representative sequences from each OTU are then compared against microbial databases including Greengenes [19], SILVA [20], and RDP [21] to obtain taxonomic and abundance information. Di Bella et al. [22] reviewed several common high-throughput sequencing platforms and their applications. Additionally, some studies have reported functional prediction directly from 16S rRNA gene data [23].

Alternatively, metagenomic DNA fragments can be sequenced directly, with raw reads assembled into contigs containing longer base sequences. Software such as MEGAN [24] can classify reads or contigs onto corresponding microbial genomes. Contigs are then ordered into scaffolds, which can be used to draft microbial genomes with gaps between scaffolds. Both reads and contigs can be analyzed for gene prediction using MetaGeneMark [25] or FragGeneScan [26], followed by annotation against protein, nucleic acid, and metabolism databases such as COG [27], PFAM [28], and KEGG [29] to reveal microbial community composition, function, and metabolic characteristics. Due to the variety of databases and complex operations, integrated systems like MG-RAST [30] have been developed for metagenomic data management and analysis. Thomas et al. [31] provided comprehensive protocols and recommendations covering sampling to data analysis for metagenomic studies.

2. Metagenomics Reveals Rumen Microbial Diversity

Rumen microbial diversity refers to the composition and population structure of microorganisms in the rumen habitat, primarily measured at two spatial scales: α-diversity and β-diversity. Diversity indices (Chao1, ACE, Shannon, etc.) reflect combined metrics of richness and evenness, where richness indicates the number of microbial species and evenness represents their relative proportions. Early understanding of rumen microorganisms relied mainly on microscopic observation, limiting knowledge to morphological characterization of relatively large microbes. As research progressed, it became clear that numerous other microorganisms existed in the rumen, but cultivation limitations prevented their isolation and identification [4].

Molecular technologies, particularly fingerprinting techniques, have enabled researchers to analyze microbial DNA or RNA sequences directly without cultivation, reducing analytical errors and greatly enhancing our understanding of uncultured rumen microorganisms. For example, the large rumen bacterium *Quinella ovalis* has never been cultured successfully but was taxonomically classified through analysis of its small subunit ribosomal RNA gene sequence [32]. Current molecular biology techniques for studying microbial diversity include single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), ribosomal intergenic sequence analysis (RISA), and terminal-restriction fragment length polymorphism (t-RFLP) [16]. While these methods can reflect microbial community composition to some extent, their resolution remains insufficient, revealing significantly less diversity than actually exists [33]. Bokulich et al. [34] systematically compared the advantages and disadvantages of these methods and illustrated their applications.

With decreasing costs of high-throughput sequencing, metagenomics has been increasingly applied, leading to large-scale discovery of uncultured microorganisms. Compared with traditional molecular methods, high-throughput sequencing directly obtains base sequence information. Different microorganisms possess relatively conserved base sequences, with greater differences in conserved regions among distantly related microbes. These conserved regions can serve as marker genes. In metagenomic studies, sequence similarities of 97%, 95%, and 90% are used as thresholds for OTU delineation at species, genus, and family levels, respectively [2]. Unless otherwise specified, 97% sequence similarity is used as the default threshold for species-level OTU clustering.

2.1 Rumen Microbial Community Structure

Kim et al. [2] analyzed all 13,478 bacterial and 3,516 archaeal 16S rRNA gene sequences from rumen sources in the RDP database. At the species level, this yielded 5,271 bacterial OTUs and 943 archaeal OTUs. The vast majority of bacterial OTUs belonged to Firmicutes (2,958 OTUs), Bacteroidetes (1,610 OTUs), and Proteobacteria (226 OTUs), suggesting these three phyla dominate the rumen. Additionally, nearly all rumen-derived archaea belong to Euryarchaeota, with *Methanobrevibacter* being the predominant methanogen. Using a similar approach, Fouts et al. [35] screened all rumen-derived bacterial and archaeal 16S rRNA gene V1–V3 region sequences from NCBI, SILVA, and RDP databases, obtaining 14,332 bacterial and 2,484 archaeal sequences that clustered into 4,670 bacterial OTUs and 486 archaeal OTUs–slightly lower than Kim et al.' s results. Since the RDP database lacks eukaryotic 18S sequences, Fouts et al. [35] selected 1,027 and 1,803 18S sequences from SILVA and NCBI, respectively, for fungal diversity analysis, yielding 168 fungal OTUs. Notably, due to incomplete fungal information in databases, the taxonomic and abundance results for fungi have limited reliability [36]. Some researchers argue that the high conservation of fungal 18S rRNA sequences precludes their use for identification or comparison [37], indicating that rumen fungi require further investigation.

Fouts et al. [35] sequenced the 16S/18S small subunit rRNA gene V1-V3 region from rumen contents of 12 dairy cows fed alfalfa and hay, identifying 4,370 bacterial OTUs, 10 archaeal OTUs, and 52 fungal OTUs. Compared with all OTUs in the databases, each microbial domain showed reduced numbers, with archaeal OTUs decreasing most dramatically. At the genus level, the top five bacterial genera were *Prevotella*, *Oscillibacter*, *Coprococcus*, unclassified Ruminococcaceae, and *Butyrivibrio*, accounting for approximately 40% of total bacteria. While 46 fungal genera exist in the rumen, individual rumens contained a maximum of only 40 OTUs, indicating low fungal abundance. At the genus level, *Nectria*, *Penicilliopsis*, *Cystofilobasidium*, and *Delphinellan* showed high abundance, representing over 25% of total fungi. Additionally, individual cows averaged 2,122 bacterial OTUs, while Chao1 index estimates suggested each cow harbored 3,116-5,439 bacterial OTUs, indicating that nearly half of OTU sequences remained undetected. This demonstrates that the current sequencing depth (5,000 reads) cannot fully capture rumen bacterial community structure. For fungi, however, 1,000 reads were sufficient, achieving over 98.4% Good’ s coverage.

Jami et al. [36] investigated inter-individual variation in rumen microbial composition among 16 Holstein dairy cows fed identical diets through 16S rRNA gene V2-V3 region amplicon sequencing, identifying 4,986 OTUs with an average of 1,800 OTUs per cow. Only 157 OTUs (4%) were shared among all cows, while approximately half of OTUs were present in four or more cows. Bray-Curtis similarity between individuals was 51%, indicating substantial variation, yet all animals harbored a core microbiome comprising 32 genera. Considering phylogenetic relationships, weighted UniFrac analysis revealed 82% similarity between samples, suggesting that while rumen microbiota may differ taxonomically between individuals under identical feeding conditions, their functional capacities likely converge.

2.2 Microbial Distribution Characteristics in Rumen Habitats

Larue et al. [37] first used DGGE and automated ribosomal intergenic spacer analysis (ARISA) to investigate compositional differences between solid and liquid phases of sheep rumen contents. Recent studies using high-throughput sequencing have further confirmed these differences. Mullins et al. [38] found that in dairy cows fed total mixed rations (TMR), the liquid phase contained higher proportions of *Streptococcus bovis*, *Prevotella ruminicola*, and *Butyrivibrio fibrisolvens*, while the solid phase was dominated by *Eubacterium ruminantium*, *Fibrobacter succinogenes*, and *Ruminococcus albus*.

McCann et al. [39] used pyrosequencing to study rumen microorganisms in beef cattle fed low-quality forage, finding higher proportions of *Paludibacter* and *Succiniclasticum* in the liquid phase compared with the solid phase, whereas *Prevotella* and *Treponema* were more abundant in the solid phase. Pitta et al. [40] examined how dietary shifts from low-protein, high-fiber bermudagrass to high-protein, high-soluble-nutrient winter wheat affected microbial community structure in rumen contents and phases. The bermudagrass diet yielded the highest microbial diversity in the liquid phase, with 149 genera identified, compared with only 118 genera in the winter wheat group. *Prevotella* was the most abundant genus in both diets and was more prevalent in the liquid phase, contradicting McCann et al.' s findings [39]. This discrepancy may arise from the metabolic versatility of *Prevotella* strains that can utilize various substrates. Additionally, microorganisms attached to solid particles likely play important roles in fiber degradation.

Rumen epithelium-adherent microorganisms, due to their close structural association with the host, may participate in important physiological processes such as VFA absorption. Petri et al. [41] combined DGGE, real-time quantitative PCR (qPCR), and pyrosequencing to investigate how diet and induced ruminal acidosis affect epithelial microbiota. DGGE profiles showed greater similarity between roughage and mixed-roughage groups than between high-concentrate groups. qPCR results indicated that *Fibrobacter succinogenes* populations were significantly affected by roughage diets. Pyrosequencing identified 149 OTUs, with Firmicutes being the dominant phylum on rumen epithelium. qPCR quantification of *Megasphaera elsdenii*, *Streptococcus bovis*, and *Prevotella* showed good agreement with pyrosequencing-derived abundance data. Notably, rumen epithelial microbiota remained more stable than solid and liquid phase communities in response to dietary changes [41].

3. Metagenomics and Rumen Microbial Functions

The rumen microbial system is extremely large and complex, with microorganisms engaging in symbiotic, parasitic, competitive, and predatory relationships. Host health and nutrient digestion depend entirely on normal microbial function. The rumen microbiome represents a vast biological resource reservoir containing many genes awaiting discovery. Using metagenomic approaches, researchers have actively explored rumen microbial functions, particularly those related to important nutritional and physiological processes.

3.1 Fiber Degradation

Rumen microorganisms possess strong cellulolytic capabilities, producing acetate, propionate, and butyrate that provide energy for the host. The fiber degradation system is highly complex, involving a series of enzymes including hemicellulases, xylanases, glucosidases, endoglucanases, amylases, phenolic acid esterases, and xylan acetyl esterases [42]. Rumen microorganisms have evolved several specialized fiber degradation mechanisms. Cellulosomes are multi-enzyme complexes found in anaerobic organisms, comprising various cellulases and hemicellulases assembled through anchoring and adhesion mechanisms that attach to bacterial cell walls via cell adhesion proteins, enabling efficient and complete degradation of native cellulose materials [43].

Screening for highly active fiber-degrading genes has been a major research focus. Ferrer et al. [44] first discovered nine novel endoglucanase genes from a dairy cow rumen metagenomic library. Dai et al. [45] investigated the yak rumen microbiome to explore fiber-degrading functional genes, screening 223 bacterial artificial chromosome (BAC) clones with fiber-degrading capacity and predicting 10,070 open reading frames (ORFs). Among these, 150 ORFs belonged to glycoside hydrolase (GH) genes, predominantly from families GH5, GH9, and GH10, but lacking GH48, an important component in most microbial cellulase systems. Analysis of cellulolytic contigs longer than 10 kb revealed that 25 originated from Bacteroidetes and 4 from Firmicutes, often linked with SusC/SusD-type outer membrane protein genes. Hess et al. [46] discovered 27,755 carbohydrate-active genes in the rumen, with 57% of 90 randomly selected genes expressing enzymes with cellulolytic activity when tested. Brulc et al. [47] identified 35 glycoside hydrolase family genes in three beef cattle fed identical diets, but only 3 carbohydrate-binding module families and 3 anchoring modules, suggesting that during fiber degradation, microorganisms first attack side-chain groups to complete colonization rather than hydrolyzing main-chain cellulose and hemicellulose.

Butyrivibrio proteoclasticus is a butyrate-producing Gram-positive bacterium that can ferment inulin, pectin, and xylan, and is widely distributed in the rumen [33]. Kelly et al. [48] sequenced the complete genome of *B. proteoclasticus* B316, revealing three types of carbohydrate-binding modules (CBM2a, CBM6, and CBM13) and multiple glycoside hydrolases and glycosyltransferases including GH2, GH3, and GH13.

3.2 Methanogenesis

Denman et al. [49] investigated the effects of bromochloromethane (BCM-CD), a methane inhibitor, on rumen microbial fermentation. BCM-CD significantly increased rumen hydrogen concentration while enhancing the abundance of hydrogen-consuming bacteria such as *Prevotella* and *Selenomonas*, thereby producing more propionate. Ross et al. [50] demonstrated that dietary supplementation with grape marc and tannin-lipid mixtures reduced methane emissions in dairy cows. Although the additives differed, they induced similar changes in the rumen microbiome, significantly affecting methanogenesis-related microorganisms and steering the community toward lower methane emission profiles.

Methane production correlates not only with methanogen abundance but also with other rumen microorganisms. Hydrogen concentration and interactions between methanogens, hydrogen-producing bacteria, and hydrogen-consuming bacteria are important factors influencing methane output [51]. Kittelmann et al. [52] found strong positive correlations between *Methanobrevibacter* and Fibrobacteraceae populations, suggesting functional linkages. Ng et al. [53] confirmed functional associations between *Methanobrevibacter ruminantium* and *Butyrivibrio proteoclasticus*, where the latter provides substrates (formate, hydrogen, carbon dioxide) for methanogenesis while receiving nutrients such as glutamate from methanogens. Acetogens can produce acetate from carbon dioxide and hydrogen. Hydrogen serves as both a substrate and important energy source for methanogens [54], and acetogens can compete with methanogens for hydrogen, thereby reducing methane production. However, selecting appropriate acetogens is prerequisite for effective hydrogen competition. Kelly et al. [55] analyzed the complete genome of *Eubacterium limosum* SA11, finding it could grow autotrophically using carbon dioxide/hydrogen and heterotrophically using glucose and methanol. However, its metabolic versatility suggests it may not be an ideal candidate for competing with methanogens for hydrogen.

To improve methanogen gene databases and elucidate methane production mechanisms at deeper levels, several methanogen genomes have been sequenced, including *Methanobrevibacter ruminantium* M1, as shown in . Methanogen genome sizes vary, as do methanogenesis pathways. Besides the carbon dioxide/hydrogen pathway, some can produce methane from formate and acetate. Methanogens may occupy specific microenvironments within the rumen, with methane output depending on their efficiency in utilizing environmental nutrients. Understanding the symbiotic and interactive relationships between methanogens and surrounding microorganisms will facilitate development of more effective methane mitigation strategies.

4. Summary and Outlook

Metagenomic technologies have broadened our understanding of rumen microecology, revealing that ruminants and their rumen microorganisms form an integrated whole. A stable, efficient, and balanced microbial community is essential for normal rumen function and a key determinant of ruminant production efficiency. Over the past decade, metagenomic studies of rumen microecology have provided general insights into ruminant nutrition mechanisms, particularly regarding roughage digestion. However, research on oral and intestinal microecology in ruminants remains relatively limited, though these microbial communities may be connected to rumen microorganisms.

The core of metagenomics involves comparison with existing databases to understand phylogeny, community structure, and functional information. Therefore, improving metagenomic applications requires database refinement, increased read lengths, and enhanced data analysis platform construction. However, traditional isolation and cultivation methods should not be completely abandoned. Improvements in cultivation techniques will substantially increase the diversity of culturable rumen microorganisms, and studies on pure culture physiology and genomics will facilitate interpretation of omics data and understanding of microbial phenomena. The Hungate 1000 project (www.hungate1000.org.nz) and FibeRumBa (http://www.jcvi.org/rumenomics) will help characterize more uncultured microorganisms and their genetic mechanisms, thereby improving database information.

Metagenomic analysis has certain limitations, as DNA sequences only reflect metabolic potential rather than real-time gene transcription and expression. Therefore, future metagenomics will integrate with metatranscriptomics, metaproteomics, and other multi-omics technologies to reveal microbial community structure, phylogeny, metabolic functions, and regulatory patterns at DNA, RNA, and protein levels. These technologies will rapidly develop and apply across diverse ecological fields, enabling comprehensive system-level understanding of microbial communities and their functions, and facilitating discovery of novel microbial strains and enzyme resources. As sequencing costs decrease and accuracy improves, metagenomics will become a mainstream method in microbial research, much like PCR technology today.

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Note: Figure translations are in progress. See original paper for figures.