Postprint: Analysis of Rumen Bacterial Diversity Changes in Dairy Cow In Vitro Fermentation Based on 16S rRNA Sequencing

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Abstract

This study aimed to analyze the temporal dynamics of rumen microbiota in dairy cows during in vitro fermentation using 16S rRNA sequencing technology, thereby providing support for investigating proliferation patterns of rumen microorganisms and refining in vitro fermentation analytical methods. The experiment was conducted using the in vitro gas production technique. Fresh rumen fluid was collected from three healthy dairy cows, mixed with artificial saliva, and injected into fermentation bottles containing fermentation substrates, followed by incubation in a shaking water bath at 39°C. Fermentation fluid was sampled at 0, 6, 12, 24, and 48 h, and changes in rumen bacteria were analyzed using 16S rRNA sequencing. The results demonstrated: 1) All sequences were classified into 14 phyla and 59 genera. Over time, at the phylum level, the relative abundances of Fibrobacteres, Bacteroidetes, and Lentisphaerae decreased, with Fibrobacteres and Bacteroidetes being significantly lower at 48 h compared to other time points ($P<0.05$), and Lentisphaerae being significantly lower at 24 h compared to other time points ($P<0.05$). Conversely, the relative abundances of Spirochaetae and Proteobacteria increased, with Spirochaetae being significantly higher at 48 h compared to other time points ($P<0.05$), and Proteobacteria being significantly higher at 12, 24, and 48 h compared to other time points ($P<0.05$). At the genus level, the relative abundances of Butyrivibrio and Ruminococcus increased over time, both being significantly higher at 48 h compared to other time points ($P<0.05$); whereas the relative abundances of Succiniclasticum and Prevotella decreased, with Succiniclasticum being significantly lower at 24 and 48 h compared to other time points ($P<0.05$), and Prevotella being significantly lower at 48 h compared to other time points ($P<0.05$). 2) The experiment obtained 15,792 operational taxonomic units (OTUs) for diversity analysis, with the rarefaction curve reaching a plateau. Both Simpson

and Shannon indices indicated that microbial diversity exhibited a trend of initially decreasing and subsequently increasing. In conclusion, during the in vitro fermentation process of dairy cow rumen, the relative abundances of major bacterial phyla and genera, as well as microbial diversity, changed over time, with these changes being more pronounced due to functional differences among rumen bacteria throughout the fermentation process. Therefore, when employing the in vitro fermentation method for research, the relationship between functional differences of rumen bacteria and alterations in the fermentation environment caused by nutrient depletion from substrates and artificial saliva should be fully considered.

Full Text

Diversity Changes of Racterial Community in the Rumen of Dairy Cows in Vitro Fermentation Analyzed by 16S rRNA Sequencing Technology

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Abstract

This study aimed to analyze the temporal dynamics of rumen microbial community in dairy cows during in vitro fermentation using 16S rRNA sequencing technology, providing support for exploring rumen microbial propagation patterns and improving in vitro fermentation methodologies. The fermentation was conducted using an in vitro gas production method. Fresh rumen fluid was collected from three healthy dairy cows, mixed with artificial saliva, injected into fermentation flasks containing substrate, and incubated at 39 °C with shaking. Fermentation fluid was sampled at 0, 6, 12, 24, and 48 h for analysis of rumen bacterial dynamics via 16S rRNA sequencing. The results showed that: (1) All sequences were classified into 14 phyla and 59 genera. At the phylum level, the relative abundances of Fibrobacteres, Bacteroidetes, and Lentisphaerae decreased over time, with Fibrobacteres and Bacteroidetes being significantly lower at 48 h compared to other time points ($P<0.05$), and Lentisphaerae being significantly lower at 24 h ($P<0.05$). Conversely, the relative abundances of Spirochaetae and Proteobacteria increased, with Spirochaetae being significantly higher at 48 h ($P<0.05$) and Proteobacteria being significantly higher at 12, 24, and 48 h ($P<0.05$). At the genus level, the relative abundances of Butyrivibrio and Ruminococcus increased, both being significantly higher at 48 h ($P<0.05$), while the relative abundances of Succiniclasticum and Prevotella decreased, with Succiniclasticum being significantly lower at 24 and 48 h ($P<0.05$) and Prevotella being significantly lower at 48 h ($P<0.05$). (2) A total of 15,792 operational

taxonomic units (OTUs) were obtained. Diversity analysis revealed that the rarefaction curves reached a plateau stage, and both Simpson and Shannon indices indicated that bacterial abundance initially decreased then increased. In conclusion, the relative abundances and diversity of major rumen bacterial phyla and genera changed significantly over time during in vitro fermentation, with functional differences among rumen bacteria leading to more pronounced temporal changes. Therefore, when using in vitro fermentation methods, the relationship between bacterial functional differences and fermentation environment changes caused by nutrient depletion in both substrate and artificial saliva should be fully considered.

Keywords: dairy cows; in vitro fermentation; microbial community; 16S rRNA

Introduction

The rumen is a complex ecosystem containing diverse archaea, fungi, and protozoa that play crucial roles in maintaining rumen homeostasis and influencing dairy cow health. These highly specialized rumen microbes produce energy through digestion and metabolism that can be utilized by the host, are critical for nutrient digestion, and may enhance resistance to invading pathogens [1]. Bacteria are the most abundant microorganisms in the rumen, with cellulolytic bacteria such as *Fibrobacter*, *Ruminococcus*, and *Butyrivibrio* being primarily responsible for degrading dietary fiber. *Fibrobacter succinogenes* exhibits strong cellulolytic capacity, producing acetate and succinate as fermentation products, and demonstrates high antibiotic tolerance. Additionally, *Prevotella* within the phylum Bacteroidetes represents a dominant rumen genus capable of degrading protein, starch, and polysaccharides but not cellulose. In summary, multiple microorganisms interact and constrain each other to maintain rumen homeostasis, which is essential for promoting digestion and metabolism, maintaining cow health, and improving production performance. Therefore, studying rumen microbial community dynamics is critically important.

The in vitro gas production method is a widely used, relatively simple, and highly reproducible technique for evaluating feed digestibility and assessing feed additive effects [2]. Most in vitro fermentation studies have investigated the effects of substrate concentrate-to-forage ratios and additives on rumen microbial communities [3-7]; however, few reports have examined temporal changes in rumen microbial communities during in vitro fermentation using normal diets as substrate. Understanding these community changes in gas production systems is therefore practically significant for improving current research methodologies. The 16S rRNA gene, encoding rRNA in bacteria, is present in all bacterial genomes and exhibits high conservation and specificity. With advances in PCR technology, 16S rRNA sequencing has become an important method for analyzing microbial community composition and studying ecosystem diversity, offering superior accuracy, comprehensiveness, and precision compared to traditional

techniques [8]. This study employed 16S rRNA sequencing to analyze temporal dynamics of rumen microbial communities in dairy cows during in vitro fermentation, providing support for exploring microbial propagation patterns and improving in vitro fermentation analysis methods.

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1 Materials and Methods

1.1 Establishment of In Vitro Fermentation System

The fermentation substrate consisted of total mixed ration provided by Xigang Dairy Farm in Nanjing, Jiangsu Province, with a concentrate-to-forage ratio of 50:50. The substrate was dried, ground, and passed through a 20-mesh sieve before use. Diet composition and nutrient levels are presented in .

Fermentation fluid was prepared by mixing fresh rumen fluid with artificial saliva. Fresh rumen fluid was collected from three healthy mid-lactation Holstein dairy cows. Specifically, rumen fluid was collected via rumen fistula 2 h after morning feeding, filtered through four layers of gauze, and transferred into pre-warmed (39 °C) thermos flasks filled with CO . After filling, flasks were immediately sealed and transported to the laboratory, where the fluid was filtered again through four layers of gauze and mixed uniformly using a magnetic stirrer. The entire process was maintained under anaerobic conditions with continuous CO infusion. Artificial saliva was prepared according to Theodorou et al. [9], with composition detailed in . The preparation process per liter involved adding 0.1 mL Solution A, 200 mL Solution B, 200 mL Solution C, and 1 mL Solution D to 558.9 mL distilled water. After CO saturation, the mixture was placed in a 39 °C water bath for 5-6 h, then 40 mL Solution E was added. The solution was mixed, saturated with CO , heated to 39 °C, and continuously infused with CO for 0.5-1.0 h until colorless. Prepared rumen fluid and artificial saliva were mixed under anaerobic conditions at a 1:9 volume ratio, pH was adjusted to 6.7, and the mixture was maintained at 39 °C as fermentation fluid.

This experiment used 160 mL sterile glass bottles as fermentation flasks. Each flask contained 1 g of fermentation substrate and was infused with CO for 30 s before rapid dispensing of 100 mL fermentation fluid using an automatic dispenser. Flasks were immediately sealed with butyl rubber stoppers and aluminum caps, shaken, and incubated at 39 °C in a shaking incubator for 48 h.

1.2 Experimental Design

A single-factor experimental design was employed. Fermentation flasks were removed at 0, 6, 12, 24, and 48 h of incubation and placed in an ice bath to terminate fermentation. Two milliliters of fermentation fluid were collected from each flask into cryovials, sealed, and stored in liquid nitrogen. Each time point

had three replicates, totaling 15 samples. Additionally, three flasks containing only artificial saliva were incubated for equipment parameter calibration.

1.3 Determination of Cumulative Gas Production

Gas production was measured and recorded at 6, 12, 24, and 48 h during incubation. Following the method of Zhu et al. [10], a pressure transducer (IGER, UK) was used to periodically measure gas production from anaerobic rumen microbial fermentation. Cumulative gas production was calculated by correcting for gas production in blank fermentation flasks based on measured gas volumes and pressures.

1.4 16S rRNA Sequencing and Data Analysis

DNA was extracted from samples, followed by PCR amplification, purification, and MiSeq library construction for sequencing. Sample data were distinguished based on index and barcode sequences, processed to filter low-quality regions at both ends, adapter sequences, and sequences containing ambiguous bases (N). After assembly, 744,200 clean-reads sequences were obtained (average 49,613 per sample) with a high-quality sequence ratio of 99.47%. Following removal of primer-mismatched sequences (4 bp), primer sequences, and chimeras, 341,727 optimized sequences were obtained from 15 samples (average 22,782 per sample; range 14,195-31,305), with an average length of 456 bp.

Using QIIME software and the RDP classifier [11], sequences were taxonomically classified against Silva, RDP, and Greengenes bacterial databases [12] based on Bergey' s taxonomy at six levels: kingdom, phylum, class, order, family, and genus, with a default threshold of 80%. Community composition at phylum and genus levels was statistically analyzed, taxonomic histograms were generated, and rumen microbial community heatmaps were constructed using gplots software [13]. Finally, species diversity analysis was performed based on operational taxonomic units (OTUs) [14] using USEARCH software [13].

1.5 Statistical Analysis

Data were analyzed using one-way ANOVA in SPSS 17.0. Results are expressed as mean ± standard deviation, with $P<0.05$ considered statistically significant.

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2 Results

2.1 Sequencing Quality and Rationality

The 16S rRNA V3-V4 region was successfully amplified from different samples, yielding correctly sized bands with appropriate concentration and single bands, indicating no PCR-inhibiting impurities in purified genomic DNA. High-throughput sequencing on the MiSeq platform generated 748,172 raw reads (av-

erage 49,878 per sample). After removing low-quality regions, adapter sequences, and ambiguous bases, 744,200 clean-reads were obtained (average 49,613 per sample) with a quality rate of 99.47%. Following removal of primer-mismatched sequences (4 bp), primers, and chimeras, 341,727 optimized sequences were obtained from 15 samples (average 22,782 per sample; maximum 31,305, minimum 14,195), with an average length of 456 bp.

Rarefaction curves were constructed by random sampling to evaluate the relationship between sequence number and OTU richness. When curves plateau, sequencing depth is considered adequate, as additional sequencing provides minimal new OTU detection. In this study, rarefaction curves for all 15 samples ([Figure 1: see original paper]-A) indicated that not all samples reached complete plateau, but coverage curves ([Figure 1: see original paper]-B) reached saturation, demonstrating that sequencing depth was reasonable and deeper sequencing would contribute minimally to new OTU detection in rumen fluid.

2.2 Taxonomic Analysis

At the phylum level ([Figure 2: see original paper]-A), the relative abundance of Firmicutes initially increased then decreased over time. In contrast, Bacteroidetes and Fibrobacteres showed overall decreasing trends. The relative abundances of Spirochaetae, Lentisphaerae, and Planctomycetes initially decreased then increased, while Tenericutes showed an increasing trend.

As shown in , after 48 h fermentation, Fibrobacteres abundance was significantly lower than at other time points ($P<0.05$) at 0.22%. Bacteroidetes abundance was also significantly lower at 48 h ($P<0.05$) at 30.69%. Lentisphaerae abundance was significantly lower at 24 h ($P<0.05$) at 3.41%. Planctomycetes abundance was significantly lower at 6, 12, and 24 h ($P<0.05$), reaching its lowest point at 24 h (0.59%). Firmicutes abundance was significantly higher at 6 h ($P<0.05$) at 36.69%. Proteobacteria abundance was significantly higher at 12, 24, and 48 h ($P<0.05$), peaking at 24 h (18.23%). Spirochaetae abundance was significantly higher at 48 h ($P<0.05$) at 7.05%. Tenericutes abundance was significantly higher at 48 h ($P<0.05$) at 5.58%.

At the genus level, 72 genera had relative abundances above 0.01%, with unclassified bacteria accounting for up to 42.26% of total abundance. As shown in [Figure 2: see original paper]-B, *Prevotella*, *Streptococcus*, and *Succinivibrio* proportions initially increased then decreased over time. *Butyrivibrio* and *Treponema* showed opposite patterns, while *Fibrobacter* and *Succiniclasticum* exhibited decreasing trends.

As presented in , *Prevotella* abundance was significantly higher at 12 h ($P<0.05$) at 31.19%. *Streptococcus* abundance was significantly higher at 6 h ($P<0.05$) at 23.03%. *Succinivibrio* abundance was significantly higher at 12 h ($P<0.05$) at 5.94%. RC9_gut_group abundance was significantly lower at 24 h ($P<0.05$) at 1.73%. *Butyrivibrio* abundance was significantly lower at 12 h ($P<0.05$) at 0.26%. *Treponema* abundance was significantly lower at 12 h ($P<0.05$) at

0.24%. *Fibrobacter* abundance was significantly lower at 48 h ($P<0.05$) at 0.22%. *Succiniclasticum* abundance was significantly lower at 24 and 48 h ($P<0.05$) at 2.22% and 2.42%, respectively.

Heatmaps intuitively display data density using gradient color bands and can aggregate large datasets effectively. Two-way cluster analysis of 18 phylum-level bacterial groups ([Figure 3: see original paper]-A) showed that 15 samples could be horizontally clustered into two major groups: Bacteroidetes and Firmicutes formed one cluster, while the remaining 16 phyla formed another (which could be further divided into two subclusters). Vertically, samples were divided into two major clusters: the first containing 5 samples (0 and 6 h) and the second containing 10 samples (12, 24, and 48 h). At the genus level, distinct temporal differences in bacterial composition were observed ([Figure 3: see original paper]-B). Two-way cluster analysis of 59 genera revealed three horizontal clusters: *Prevotella*, *Streptococcus*, and other genera. Vertically, samples formed two clusters: the first containing 3 samples (6 h) and the second containing 12 samples (further divisible into two subclusters).

2.3 Sequencing Results and -Diversity Analysis

As shown in , Ace and Chao indices did not differ significantly over time ($P>0.05$), while Shannon and Simpson indices showed significant differences ($P<0.05$). All sample rarefaction curves reached plateau, indicating adequate sequencing depth. Coverage rates exceeded 97% across all samples, demonstrating that sequencing data were sufficient to capture the majority of microbial information.

2.4 pH and Cumulative Gas Production

As presented in , pH was significantly lower at 24 h compared to other time points ($P<0.05$). Cumulative gas production increased progressively over time, being significantly higher at 48 h ($P<0.05$).

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3 Discussion

Firmicutes, Bacteroidetes, and Proteobacteria represent the three dominant bacterial phyla in the rumen [15]. In this study, Bacteroidetes showed the highest relative abundance, consistent with findings from studies on Alaskan and Norwegian moose, sika deer, water buffalo, and Svalbard reindeer fed winter diets, as well as gayal fed mixed leaf and straw diets [16-17]. Qin et al. [18] and Tun et al. [19] also confirmed that Bacteroidetes and Firmicutes dominate gastrointestinal microbiomes in both humans and felines. Tajima et al. [20], Singh et al. [21], and Peng et al. [22] reported that Bacteroidetes predominate in rumen bacterial communities of Holstein cattle and water buffalo, with minor phyla including Acidobacteria and Cyanobacteria. Research indicates that Acidobac-

teria and Cyanobacteria are better adapted to aquatic environments [23] and cannot grow in the strictly anaerobic rumen environment. Bacteroidetes are primary degraders of non-fibrous carbohydrates in the rumen, possessing genes related to non-fibrous polysaccharide degradation [24], while cellulolytic bacteria dominate in later fermentation stages. Therefore, Bacteroidetes abundance decreased after 12 h.

Dairy cows lack endogenous fiber-degrading enzymes and rely on rumen microorganisms for fiber digestion [25]. Fibrobacteres play crucial roles in fiber digestion and decomposition. Liu et al. [26] reported that *Fibrobacter* abundance increased significantly from 0 to 16 and 48 h, playing a key role in plant fiber degradation. However, this study showed significant Fibrobacteres reduction from 12 to 48 h, likely due to methodological differences. In this in vitro fermentation system, fiber-degrading bacteria competed as cellulose and hemicellulose decreased over time, leading to reduced Fibrobacteres abundance. In contrast, Liu et al. [26] used the nylon bag method with continuous substrate supply at each time point, potentially alleviating competition among fiber-degrading bacteria. Additionally, the increasing abundance of major fiber-digesting genera *Ruminococcus* and *Butyrivibrio* after 12 h in this study may have competed with Fibrobacteres. Research also suggests that *Streptococcus bovis* can inhibit acid-intolerant bacteria such as fiber-degrading bacteria [27]. In this study, *Streptococcus* abundance increased significantly while fiber-degrading bacteria decreased, potentially contributing to reduced Fibrobacteres abundance.

Most Proteobacteria genera can compete with other bacteria and grow moderately under low carbon concentrations [28]. During prolonged fermentation, readily degradable nutrients like starch and protein were largely depleted, and increased Proteobacteria abundance may reflect moderate growth through competition with fiber-degrading bacteria for limited resources. Firmicutes contains a large proportion of Clostridia, with *Streptococcus* and *Succiniclasticum* as dominant genera [29], consistent with our findings. Firmicutes includes numerous fiber-degrading genera such as *Ruminococcus*, *Butyrivibrio*, and *Pseudobutyrivibrio* [30], whose abundances increased after 12 h, contributing to increased Firmicutes abundance.

At the genus level, numerous studies have identified *Prevotella* as the most abundant rumen genus. *Prevotella* are highly active protein-degrading bacteria that can also utilize starch and pectin, with members occupying different ecological niches due to genetic relatedness or high genetic variation [31-33], consistent with our results. *Prevotella* are hydrogen-consuming bacteria that ferment carbohydrates and lactic acid to propionate via succinate and acrylate pathways [34-35], potentially reducing methane production by competing with methanogens for hydrogen. In this study, *Prevotella* abundance decreased over time while *Methanobrevibacter* increased, warranting further investigation into their relationship. As starch and protein were continuously metabolized and utilized by *Prevotella*, these nutrients decreased over time. After 12 h, highly active fiber-degrading genera became dominant. Although Li et al. [16] sug-

gested *Prevotella* may play a potential role in fiber degradation, they are not primary cellulolytic bacteria and their growth may be inhibited by other fiber-degrading bacteria, causing their abundance to peak then decline.

Succiniclasticum, isolated from bovine rumen by Van Gylswyk in 1995, converts succinate to propionate and may be a normal rumen inhabitant capable of degrading both starch and cellulose [36]. Its decreasing abundance in this study may reflect competition with other starch- and cellulose-degrading bacteria for limited nutrients. *Succinivibrio*, first discovered in bovine rumen, primarily ferments dextrin to acetate and succinate with amylolytic activity. Its abundance increased before 12 h during starch fermentation, then decreased as fiber-degrading bacteria dominated after 12 h. *Streptococcus bovis* degrades starch but not cellulose, similar to *Succinivibrio*, but its abundance increased before 6 h, possibly due to higher amylase activity. *Treponema*, *Butyrivibrio*, and *Ruminococcus* are major fiber-degrading bacteria whose abundances increased after 12 h, likely reflecting their primary role in fiber digestion during this stage.

-diversity reflects intra-sample species diversity, including Chao, Shannon, Simpson, and Ace indices. Chao and Ace indices reflect community richness, while Shannon and Simpson indices reflect species diversity. Higher Chao, Ace, and Shannon values and lower Simpson values indicate greater species richness. The observed variation ranges in these indices indicate temporal differences in bacterial composition and diversity. Non-significant differences in Chao and Ace indices suggest stable total bacterial numbers over time, while significant differences in Shannon and Simpson indices indicate significant temporal changes in bacterial diversity. Compared to 0 h, Shannon index decreased significantly while Simpson index increased significantly at 48 h, indicating reduced bacterial diversity, possibly related to substrate consumption and nutrient depletion in artificial saliva.

Gas production comprehensively reflects feed fermentability; higher fermentable organic matter content correlates with greater microbial activity and gas production [37]. In this study, cumulative gas production increased significantly over time, indicating sustained high microbial activity. pH is a critical factor affecting rumen fermentation; prolonged low pH reduces cellulolytic activity [38]. Normal rumen fermentation requires pH 6-7. In this study, pH ranged from 6.26 to 6.70, all within the appropriate range.

4 Conclusion

During in vitro rumen fermentation in dairy cows, the relative abundances and diversity of major bacterial phyla and genera changed significantly over time, with functional differences among rumen bacteria leading to more pronounced temporal changes during fermentation. Therefore, when using in vitro fermentation methods, the relationship between bacterial functional differences and

fermentation environment changes caused by nutrient depletion in both substrate and artificial saliva should be fully considered.

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