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Short communication: Variability in fermentation end-products and methanogen communities in different rumen sites of dairy cows

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ABSTRACT

The objective of this study was to investigate differences in fermentation and methanogen communities in samples collected from 3 sites in the rumen of dairy cows. The study involved 3 ruminally cannulated nonlactating Chinese Holstein dairy cows fed a diet of 40% forage and 60% concentrate feeds. Four handfuls of whole ruminal contents were collected from the cranial sac, middle of the ventral sac, and caudodorsal blind sac of the rumen of the cows at 0, 2.5, and 6 h after the morning feeding. Concentrations of VFA, ammonia, and dissolved methane and hydrogen were analyzed. Methanogen populations and communities were analyzed targeting 16s rRNA genes. Dissolved methane concentration and pH were highest in samples from the cranial rumen. Ruminal fluid from the cranial rumen also had greater copy numbers of the *Methanobrevibacter* and higher Simpson indexes compared with samples from middle of the ventral rumen. In summary, cranial rumen had higher dissolved CH_4 concentration than middle and hind rumen, which might be caused by the greater population of *Methanobrevibacter* with higher ruminal pH.
Key words: rumen fermentation, methanogen, methane, hydrogen

Short Communication

Methane in the rumen is produced by a specialized group of microbes called methanogenic archaea, a process that prevents accumulation of hydrogen in the rumen. It was reported that dissolved (**d**)CH_4 was higher in ruminal fluid from the front, cranial sac of the rumen than from the middle rumen (Wang et al., 2016a), suggesting that CH_4 production may vary within rumen sites. The cranial sac of rumen usually has a higher pH and lower VFA concentration compared with other rumen sites (Bryant, 1964; Duffield et al., 2004; Wang et al., 2016a), which may be caused by rumination and consequent entry of saliva (Bryant, 1964; Duffield et al., 2004; Wang et al., 2016a). Ruminal pH is an important factor influencing rumen methanogens (Lana et al., 1998; Wenner et al., 2017), and thus may affect methanogenesis and dCH_4 distribution within the rumen. Studies are lacking concerning investigating the differences in methanogenesis and methanogen population among rumen sites.

This experiment was approved by the Animal Care Committee, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China. Three nonlactating Chinese Holstein rumen-fistulated (100-mm internal diameter; Anscitech Co. Ltd., Wuhan, China) dairy cows (BW = 460 ± 36.5 kg, DMI = 6.25 ± 0.690 kg/d, methane emission = 210 ± 24.8 L/d; mean ± SD) were used in this experiment. The cows were housed in a tiestall barn, fed a TMR twice daily at 0600 and 1600 h to allow approximately 5% refusals, and had free access to fresh water. The TMR contained (DM basis) 40.0% corn silage, 24.0% corn grain, 6.0% soybean meal, 10.2% wheat middling, 13.2% dried distillers corn grain with solubles, 2.0% soybean oil, 0.5% sodium chloride, 1.7% calcium carbonate, 0.6% calcium hydrophosphate, 0.8% magnesium sulfate, and 1.0% vitamin/trace-mineral premix (Jiuding Feed Co. Ltd, Changsha, China).

Whole ruminal contents samples were collected by same person from 3 sites in the rumen, as described by McCracken et al. (1999). The sites were cranial sac, middle of the ventral sac, and caudodorsal blind sac of the rumen and were defined as front, middle, and hind rumen, respectively. Four handfuls of whole ruminal contents were collected from the 3 sites at 0, 2.5, and 6 h after the morning feeding, and sampling was repeated in 2 consecutive days. Ruminal fluid was separated by squeezing through 4 layers of cheesecloth.

The apparatus used to extract dCH_4 and dH_2 in the liquid fraction was as described in Wang et al. (2014).

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Extracted CH_4 and H_2 were analyzed using GLC (Agilent 7890A, Agilent Inc., Palo Alto, CA). Concentrations of dH_2 and dCH_4 in the original liquid fraction were calculated as described in Wang et al. (2016a).

The pH of the ruminal fluid was measured immediately after samples were collected using a portable pH meter (Starter 300; Ohaus Instruments Co. Ltd., Shanghai, China), which was calibrated before each sampling using standard buffers (pH 4.0 and 7.0). Concentrations of VFA were analyzed using GLC (Agilent 7890A, Agilent Inc., Palo Alto, CA) according to Wang et al. (2014). Concentration of ammonia was analyzed using the procedure of Weatherburn (1967).

Aliquots of ruminal fluid collected at 0, 2.5, and 6 h after the morning feeding were frozen immediately in liquid nitrogen and stored at −80°C. Two hundred fifty microliters of the ruminal fluid were pipetted into 2-mL enzyme-free centrifuge tubes for DNA extraction. Microbial DNA extraction of samples followed the protocol described by Yu and Morrison (2004). Three hundred microliters of TE buffer (Tris 10 m*M*, EDTA 1 m*M*, pH = 8.0) was used to elute total DNA. The concentration and purity of total DNA were measured using a ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quantitative real-time PCR (**qPCR**) was performed using the procedures detailed by Jiao et al. (2014). The qPCR assays were performed on a LightCycler 480 (Roche Molecular Systems Inc., Pleasanton, CA), with a total volume of 10 μL, using SYBR Premix Ex Taq (Takara Inc., Dalian, China). The PCR reaction contained 1 (template DNA) + 5 (SYBER Green Mix, Takara Inc.) + 0.25 (forward primer) + 0.25 (reverse primer) + 3.5 (double-distilled H_2O) = 10 μL. All samples were assayed in triplicate and all standard curves met the following requirements (R^2 >0.99, 90% < E < 120%). The PCR efficiency, for sort E, is calculated by the slope of the standard curve ($E = 10^{(-\text{slope})\text{-}1}$). Details are described by Rutledge and Côté (2003). Forward (**F**) and reverse primer (**R**) selected from the literature for qPCR of methanogen groups were 5′-GGATTAGATACCCSGGTAGT-3′(F) and 5′-GTTGARTCCAATTAAACCGCA-3′(R) for total methanogens (Hook et al., 2010); 5′-CGWAGGGAAGCTGTTAAGT-3′ (F) and 5′-TACCGTCGTCCACTCCTT-3′ (R) for *Methanobacteriales* (Yu et al., 2005); and 5′-CCTCCGCAATGTGAGAAATCGC-3′ (F) and 5′-TCWCCAGCAATTCCCACAGTT-3′ (R) for *Methanobrevibacter* (Huang et al., 2016).

The template DNA representing 3 rumen sites for each cows used in PCR for sequencing of amplicons were pooled at equal volume ratio of total DNA extracted from ruminal fluid sampled from 3 time points on 2 consecutive days. The 16S rDNA V4-V5 hypervariable regions of the methanogen genomic DNA were used for PCR amplification with the primers 5′-TGYCAGCCGCCGCGGTAA-3′(524F) and 5′-YCCGGCGTTGAVTCCAATT-3′ (Arch958R). The PCR reactions were performed in a triplicate 20-μL mixture containing 0.8 μL of each primer, 10 ng of template DNA, 2 μL 2.5 m*M* deoxyribonucleoside triphosphate, 0.4 μL of FastPfu polymerase (Transgen, Beijing, China), and 4 μL 5 × FastPfu Buffer (Transgen). The thermal cycling programing was performed as 3 min for an initial denaturation step at 95°C, 27 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s, and elongation at 72°C for 45 s, with a final extension at 72°C for 10 min. The PCR products were excised from 2% agarose gels and purified using a QIAquick Gel extraction kit (Qiagen, Hilden, Germany). Amplicons from each reaction mixture were quantified fluorometrically, normalized, and pooled at equimolar ratios based on the concentration of each amplicon. Amplicons were sequenced with the Illumina MiSeq platform (Illumina, San Diego, CA) at Majorbio Bio-Pham Technology (Shanghai, China). Raw reads were submitted to the NCBI Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra) under accession number SRP121400. Quality control of the sequence reads were performed using MOTHUR v.1.39.5 (Schloss et al., 2009) and followed the protocol described by Kozich et al., (2013). The high-quality reads were clustered into operational taxonomic units (**OTU**) at 97% similarity using Usearch v.7.0 (Edgar, 2013). Representative sequences defined by abundance from each OTU were using PyNAST (Caporaso et al., 2010) against SILVA archaea database v.128 (Quast et al., 2013). Taxonomy analysis were using the RDP classifier v.11.1 (Wang et al., 2007) with a minimum support threshold of 80%. A principal coordinate analysis (**PCoA**) was performed based on Bray-Curtis similarity distances (Bray and Curtis, 1957).

Fermentation and qPCR data were averaged to get the mean value of 2 consecutive days, which were further analyzed statistically using the linear mixed model of SPSS 17.0 software (SPSS Inc., Chicago, IL), with rumen site (n = 3), sampling time (n = 3), and cow (n = 3) as fixed effects, sampling time (n = 3) as repeated measures, and interaction between rumen site and cow. No interactions between rumen site and sampling time were found; thus, it was removed from the model. Diversity indexes estimated from 16s rRNA gene library sequences were performed using a linear mixed model with rumen site (n = 3) and cow (n = 3) as fixed effect. Significance was declared at $P \leq 0.05$.

Three cows were employed, and samples collected at 3 sampling times had great variabilities in dCH_4, dH_2, and molar percentage of acetate and propionate (P

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