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Impact of subacute ruminal acidosis (SARA) adaptation on rumen microbiota in dairy cattle using pyrosequencing

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ABSTRACT

The objective of this study was to evaluate the changes in bacterial populations in the rumen of dairy cattle following adaptation to subacute ruminal acidosis (SARA) using 16S rRNA gene pyrosequencing. Rumen contents were collected from four cattle adapted to either a 40% (control diet, COD) or 70% (SARA induction diet, SAID) concentrate feeds. DNA was extracted from each of the samples. Bacterial 16S rRNA genes of ruminal DNA extracts were PCR amplified with 2 bar coded primer sets and sequenced by 454 pyrosequencing. At a high taxonomic level, the percentage of Proteobacteria and Bacteroidetes were reduced by SAID feeding, whereas Firmicutes and Actinobacteria were more abundant in the SAID than in the COD group. At the genus level, as compared with the COD group, the abundances of *Prevotella*, *Treponema*, *Anaeroplasma*, *Papillibacter*, *Acinetobacter* and unclassified populations including unclassified Lentisphaerae, and unclassified bacteria were lower ($P < 0.05$), while the percentages of *Ruminococcus*, *Atopobium*, unclassified Clostridiales and *Bifidobacterium* were increased ($P < 0.05$) in the SAID group. Feeding of SAID reduced ($P < 0.001$) the diversity of the rumen microbial community. Taken together, our findings provide a comprehensive picture of current knowledge of the community structure of the rumen bacterial ecosystem during SARA, and enhance our understanding about the ruminal microbial ecology that may be useful in the prevention of ruminal acidosis.

1. Introduction

In the dairy industry, it is common practice to feed dairy cattle with a high concentrate diet in order to improve milk production. However, high concentrate diets can reduce rumen pH and the acetate/propionate (A/P) ratio, increase the volatile fatty acids (VFA) concentration and cause major changes in the microbiota of the rumen [1]. These changes are usually coupled with an increased incidence of metabolic diseases [2], for example subacute ruminal acidosis (SARA), or non-lactic acid acidosis, which is characterized by daily episodes of low ruminal pH, between 5.8 and 5.5 [3]. Field studies have revealed the presence of SARA in 11–29% of cattle in early lactation and in 18–26% of mid-lactation cattle [4,5]. Even on well managed dairy farms, SARA still represents a common and economically important problem, which leads to the notion that SARA is the most important nutritional disease that affects dairy cattle [6].

Several studies have been conducted on the rumen microbial changes in association with SARA. The most common bacteria detected in the rumen of cattle and steer linked to SARA are *Lactobacillus* spp. and *Streptococcus bovis* [7]. The dominant bacterial phyla include Firmicutes, Bacteroidetes, and all subgroups of Proteobacteria [8]. However, to our knowledge, most of the studies about the microbial communities related to SARA were based on culture techniques, conventional gene cloning and sequencing, which suffered from the neglect of the uncultured bacteria, or both cloning bias and limited throughput, leading to masking and underestimation of the actual diversity of the microbial community [9]. Hence, more comprehensive information about the microbial communities is needed to elucidate its association with SARA.

The advent of high-throughput pyrosequencing technology addresses these methodological shortcomings by recovering uncommon, exceedingly rare species [10]. 454 pyrosequencing is a widely accepted sequencing method, which was developed by Margulies et al. [11]. Pyrosequencing has been used in microbial ecology in a variety of ecosystem, including cecum of laying hens [12], the ileum of pig [13] and the hindgut and rumen of cattle [14–20]. These studies supported that pyrosequencing facilitated greater understanding of microbial diversity. Therefore, in this paper, we used 454 pyrosequencing to analyze the bacterial communities of rumen during SARA and compared the composition of rumen microbial community in dairy cattle fed high-forage (control diet) or high-grain diets (SARA induction diet).

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Although ruminal acids are considered to be the main contributors to the pathophysiology of acidosis, other toxic factors of microbial origin, including ethanol, amines, and especially bacterial lipopolysaccharides (LPS), have been implicated [21,22]. LPS is a component of the cell wall of Gram-negative bacteria, and regarded as a strong inducer of the acute phase response, which is a nonspecific immune mechanism aimed at restoring disturbed homeostasis. Some researchers have suggested that, under the conditions of low rumen pH, Gram-negative bacteria release LPS and cause defects in the barrier function of the epithelium, leading to inflammation [23]. Although data on bacteriological changes and changes in ruminal LPS have been obtained in experiments in which acute ruminal and subacute ruminal acidosis were induced [24,25], fewer studies have been conducted directly to explore the correlation between bacteriological changes, monitored by molecular techniques, and the accumulation of ruminal LPS. Therefore, the second objective of this study was to investigate the relationship between fluctuations in LPS and the shifts in structure of the microbial community in the rumen.

2. Materials and methods

2.1. Animals and experimental design

The experimental design and procedures were approved by the Animal Care and Use Committee of Nanjing Agricultural University following the requirements of the Regulations for the administration of Affairs Concerning Experimental Animals.

Four multiparous, early-lactation Holstein cattle fitted with 10-cm ruminal cannulas (Bar Diamond, Parma, ID) were used in the experiment. The cattle (average body weight, 460 ± 16.4 kg; 84 ± 25 days in milk at the beginning of the trial) were grouped and subjected to the experimental treatments in a 2 × 2 crossover design (2 × 2 with 2 treatments and 2 periods, replicated with 4 cattle). The treatments were a control diet (COD; 40% concentrate feed, dry matter basis) and a SARA induction diet (SAID; 70% concentrate feed, DM basis) (Table S1). The diets were formulated to meet or exceed the energy requirements (at 18 kg/d DMI) of a Holstein cattle yielding 20 kg of milk/d with 3.50% milk fat and 3.10% true protein. The cattle were fed at 0700 and 1800 h (half of the daily feed allowance was provided at each feeding). Each experimental period consisted of 21 d. During the first 2 d, the dietary concentrate level was gradually increased in the SAID group (by approximately 15 percentage units/d compared with COD). This was followed by 9 d of adaptation to the diet, and then by 9 d of sampling. The COD group had 11 d of adaptation and 9 d sampling periods. During the adaptation periods, the cattle were housed in tie stalls. Feeding was ad libitum to about 5% orts. The cattle had free access to fresh water during the trial.

2.2. Sampling and measurements

Samples of ruminal contents were collected at 0, 4 and 8 h following the morning feed on days 12, 17 and 21 of each experimental period. In addition, on day 21 of the experimental period, samples of ruminal fluid were taken every 2 h, from 0800 until 2000, to evaluate the postprandial response of ruminal pH. Ruminal samples were collected from four locations in the rumen (approximately 250 g each). Aliquots of the rumen samples were filtered through four layers of sterile cheesecloth. The pH of rumen fluid was immediately determined by pH meter, and subsamples of filtrate were centrifuged at 6000× *g* for 15 min at 4 °C; and the supernatant was stored at −20 °C until analyses of volatile fatty acid, lactic acid and LPS. VFA concentrations in ruminal fluid were analyzed using the gas chromatographic method followed exactly as previously described by Mao et al. [26]. Concentration of lactic acid was analyzed following the method described by Baker and Summerson [27]. Absorbance was measured using a Shimadzu UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan) at 560 nm. LPS were determined using the Limulus amebocyte lysate test (XiaMen HouSiJi Company, China) according to the directions of the manufacturer. To evaluate the effect of treatment on the risk of SARA, the duration for which the rumen pH was <5.8 from 0 to 12 h post-feeding was also estimated, assuming an exponential function of the pH curve between two adjacent time points.

2.3. DNA isolation

Samples of rumen contents collected on days 12, 17 and 21 before the morning feed (0 h) in each period, were directly used for DNA extraction according to a bead-beating method using a mini-bead beater (Biospec Products, USA) followed by phenol–chloroform extraction [26]. The DNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). DNA was quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France) following staining using the Quant-it Pico Green dsDNA kit (Invitrogen Ltd. Paisley, UK).

2.4. DNA pyrosequencing

PCR amplification of the V1-V3 region of bacterial 16S rDNA was performed using universal primers (27F 5′-AGAGTTTGATCCTGGCTCAG-3′, 533R 5′-TTACCGCGGCTGCTGGCAC-3′) incorporating the FLX Titanium adapters and a sample barcode sequence [28]. The cycling parameters were as follows: 5 min initial denaturation at 95 °C; 25 cycles of denaturation at 95 °C (30 s), annealing at 55 °C (30 s), elongation at 72 °C (30 s); and final extension at 72 °C for 5 min. Three separate PCR reactions of each sample were pooled for pyrosequencing. The PCR products were separated by 1% agarose gel electrophoresis and purified by using the QIAquick Gel extraction kit (Qiagen). Amplicons were quantified using the Quant-iT Pico Green dsDNA Assay Kit (Invitrogen). Equal concentrations of amplicons were pooled from each sample. Amplicons were sequenced as recommended by the manufacturer's instructions. The end fragments were blunted and tagged on both ends with one of twenty four ligation adapters that contained a unique 10-bp sequence and a short four-nucleotide sequence (TCAG) called the sequencing key; these were recognized by the system software and the priming sequences.

2.5. Analysis of pyrosequencing data

A total of 110,520 raw reads were obtained from the 454 pyrosequencing runs. All pyrosequencing reads were filtered according to barcode and primer sequences. The resulting sequences were further screened and filtered for quality and length. Sequences that were less than 200 nucleotides, contained ambiguous characters, contained over two mismatches to the primers, or contained mononucleotide repeats of over six nucleotides were removed [17]. The high-quality sequences were assigned to samples according to barcodes. Sequence collections were then depleted of chimeras using B2C2 (http://www.researchandtesting.com/B2C2.html). High quality pyrotags were then assigned to taxa using the SILVA database (SILVA 108) [29]. The candidate sequences were screened, and preclustered to eliminate outliers; a distance matrix was generated from the resulting sequences. Sequences were clustered into OTUs using the furthest neighbor algorithm. Representative sequences from OTUs at a 0.03 distance were obtained and classified using the RDP's Bayesian classifier. Any sequences not identified as of bacterial origin, because they were either nonbacterial microbes,