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RESEARCH ARTICLE

Diversity shifts of rumen bacteria induced by dietary forages in dairy cows and quantification of the changed bacteria using a new primer design strategy

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Abstract

The partial 16S rRNA gene sequences (100 to 500 bp) were widely used to reveal rumen bacterial composition influenced by diets, while quantification of the changed uncultured bacteria was inconvenient due to difficult designing of specific primers based on short sequences. This study evaluated the effect of forage resources on rumen bacterial diversity and developed new strategy for primer design based on short sequences to quantify the changed uncultured bacteria. Denaturing gradient gel electrophoresis (DGGE) analysis and subsequent band sequencing were used to reveal the distinct rumen bacteria composition in cows fed with two forage sources (single corn stover *vs*. mixed forages including alfalfa hay and corn silage). The bacterial diversity in the rumen of dairy cows fed with corn stover was lower than that with mixed forages ($P<0.05$). The bacterium named R-UB affiliating to uncultured Succinivibrionaceae was identified, and it was abundant in the rumen of cows fed with mixed forages compared to corn stover. The full length 16S rRNA gene sequences with identity of >97% to the R-UB 16S rRNA gene sequence were obtained from GenBank and used to design specific primers to quantify uncultured bacterium R-UB. All sequences of amplicon from the new primers were of 100% identity to R-UB sequences indicating the high specificity of new primers. Quantitative PCR confirmed that abundance of R-UB in the rumen of cows fed with corn stover was lower than those fed with mixed forages ($P<0.01$). New strategy for designing primers based on partial 16S rRNA genes to quantify targeted uncultured bacteria was successfully developed. The rumen bacteria descending significantly in the cows fed corn stover compared to those fed mixed forages was identified as uncultured R-UB from Succinivibrionaceae.

Keywords: rumen, bacterial diversity, forage source, primers, qPCR

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1. Introduction

Forage in the diet is an important nutrients source, and it supplies energy to dairy cows. In the diet of ruminants, alfalfa and corn silage are forages with high quality. However, there is limited production of alfalfa and corn silage, but high production of corn stover in China. Ruminal degradation

of corn stover is rather difficult, which easily results in low efficiency of carbohydrate utilization in the rumen (Wanapat and Khampa 2007; Pang *et al.* 2008; Wang 2011). Rumen bacteria are the largest and important population during forage degradation (Kamra 2005; Welkie *et al.* 2010). Previous studies have found that corn stover significantly influenced the ruminal microbial metabolome especially with regard to short chain fatty acids, amines and amino acids compared with alfalfa hay in lactating dairy cows (Zhao *et al.* 2014). Meanwhile corn stover resulted in decreased rumen microbial protein production and milk production (Zhu *et al.* 2013). Characterization of microbial composition or function in the rumen of dairy cows fed with different diets could facilitate nutrient utilization by regulating microbial communities.

In recent years, many molecularly based culture-independent techniques such as denaturing gradient gel electrophoresis (DGGE), clone library, and pyrosequencing have been used to study the rumen bacteria diversity or community structure (Sadet *et al.* 2007; Wu *et al.* 2012; Thoetkiattikul *et al.* 2013). However, these techniques could not accurately quantify the abundance of bacteria because of the PCR biases of universal primers and insufficient sequencing depth (Pinto and Raskin 2012; Pawluczyk *et al.* 2015), and these methods may overestimate relative abundance of the bacteria represented (Stiverson *et al.* 2011). Quantitative PCR (qPCR) was more accurate and popular in quantifying targeted bacteria. Lots of qPCR primers targeting the known rumen bacteria 16S rRNA genes have been designed (Yu *et al.* 2005; Wanapat and Cherdthong 2009; Liu *et al.* 2014). The targeted uncultured bacteria revealed from DGGE or pyrosequencing analysis based on partial 16S rRNA gene sequences (100 to 500 bp) was difficult to be quantified because short sequences had less choice to design specific primers as the template. In other words, acquiring longer or full length 16S rRNA genes based on partial genes could facilitate the design of qPCR primers and quantification of the changed uncultured bacteria revealed by diversity analysis.

We hypothesize that qPCR primers could be designed based on short 16S rRNA gene sequences, and used to quantify the changed rumen bacteria induced by dietary forages in dairy cows. Therefore, the objective of this study was to evaluate the rumen bacterial profile changes in cows fed different forage sources and develop new strategy for primer design based on partial 16S rRNA gene sequences to quantify the changed rumen bacteria.

2. Materials and methods

2.1. Animals, diets and rumen sampling

Thirty-two Chinese Holstein dairy cows similar in body weight ((550±50) kg) were randomly assigned to two diets with different forage. Cows in the group MF (mixed forages diet) were fed with diets containing alfalfa hay and corn silage as the forage source, while cows in group CSA (corn stover diet) were only fed with corn stover as dietary forage (Appendix A) for 91 d. All cows were kept in individual pens and fed total mixed ration (TMR) three times daily and were milked three times. The animal use protocols were approved by the Animal Care Committee of the Institute of Animal Science (Beijing, China). Dry matter intake (DMI) and milk production were recorded daily. Milk samples were collected weekly and used for milk composition determination using a Foss-Milkoscan TM Minor (MilkoScan FT120, Foss Electric A/S, Hillerod, Denmark). For each animal, rumen fluid samples were obtained using an oral lavage tube before morning feeding (0 h) and 2 h after morning feeding (2 h) on day 91. The samples were immediately frozen in liquid nitrogen for DNA extraction.

2.2. DGGE and sequencing

Total DNA of the ruminal microbes was extracted using the CTAB isolation buffer (100 mmol L^{-1} Tris-HCl, pH 8.0; 1.4 mol L^{-1} NaCl; 20 mmol L^{-1} EDTA (sodium salt); 2% hexadecyltrimethylammonium bromide) plus bead beating method (Minas *et al.* 2011). Every four microbial DNA samples of the cows from the same diet group were mixed together for DGGE analysis. The V3 region of the 16S rRNA gene was amplified by PCR using primers 338F (5′-ACTCCTACGGGAGGCAG CAG-3′) and 533R (5′-TTACCGCGGCTGCTGGCAC-3′), the 338F primer has a 40-base GC clamp attached to its 5′ end (Kawai *et al.* 2002; Huse *et al.* 2008). All PCR amplifications were performed with a MyCycler™ thermal cycler (Bio-Rad, USA) according to Li *et al.* (2012). The DGGE gel was run at 60°C and 85 V for 16 h using a DCode™ Universal Mutation Detection System (Bio-Rad, USA). After the gels were silver stained, the gel images were scanned using GS-800 calibrated densitometer (Bio-Rad, USA). The diversity index was calculated based on numbers and density of bands according to publications (Sadet *et al.* 2007). The intense DGGE bands were excised, and the DNA was recovered by boiling in TE buffer. The recovered DNA was used as the template in PCR re-amplification using primers 338F and 533R. PCR products were ligated into the pMD18-T Easy vector (TaKaRa, Dalian, China) and the recombinant plasmids were transformed into *Escherichia coli* JM109 cells (TaKaRa, Dalian, China). Positive clones were selected for sequencing at BGI (Beijing, China). The taxonomy of the sequences was analyzed using the SINA aligner in Silva (Pruesse *et al.* 2012).

2.3. Designing qPCR primers of bacterium R-UB

The bacterium (referred to as R-UB) exhibited significant