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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Rumen bacterial diversity in Philippine native cattle (*Bos primigenius* Bojanus) fed cellulase treated rice straw

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Abstract

The effect of cellulase treated rice straw on the molecular diversity of rumen bacteria in Philippine native cattle was investigated. A 3×3 Latin Square Design (LSD) was used to evaluate the effect of cellulase supplementation on the rumen bacterial diversity of Philippine native cattle fed rice straw. Three different treatments consisted of Treatment 1: urea-molasses-treated rice straw (UMTRS); Treatment 2: cellulase mixed with urea-molasses pre-treated rice straw (UMCTRS) and Treatment 3: Cellulase treated rice straw (CTRS). Changes in rumen bacterial diversity due to the dietary treatments were determined by Polymerase Chain Reaction – Denaturing Gradient Gell Electrophoresis (PCR- DGGE). The DGGE was used to separate PCR product of the amplified 16S rDNA or its regions. The sequence analysis of purified PCR product amplified from excised DGGE bands was used to determine the phylogenetic placement of each individual band in the community. Results of DNA sequencing showed the presence of more amplicons in UMCTRS and CTRS indicating that these treatments led to increase in the population of fibrolytic microorganisms in the rumen. Results of an *in situ* study also showed that these two treatments have ability to improve feeding value. We concluded that cellulase has a positive effect on feeding value of rice straw especially with the addition of cellulase to urea-molasses pre-treated rice straw.

Keywords: Rice straw; cattle; cellulose; rumen

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Introduction

Livestock production in many developing countries is constrained because of poor nutrition. Cereal crops (maize, rice, wheat, sorghum, etc.) straw and stover are the main sources of feed for ruminants especially during the dry season and/or winter despite the fact that these straws and stover have poor nutritional value because of their low energy and nitrogen and high fibre content. Slow and incomplete digestion of fibrous substrates often limit the overall digestive process in the rumen and can significantly influence animal performance in livestock production systems that use forages as a major component of the diet. There are many strategies that have been developed to stimulate the digestion of fibrous components in ruminant feeds. These include the use of fibrolytic enzymes such as cellulase, which hydrolyses the fibre in feeds. The rate of cellulase is to increase the rate and extent of fibre digestion meant for better feed conversion. Cellulase preparations can be used to drive specific metabolic and digestive processes in the gastrointestinal tract of animal and may augment natural digestive processes to increase nutrient availability and feed intake (McAllister et al., 2001).

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The use of fungi and/or their enzymes that metabolize lignocelluloses is a potential biological treatment to improve the nutritional value of straw by selective delignification (Jalc, 2002). However, it is difficult to control the optimal conditions for fungal growth such as pH, temperature, pressure, O_2 and CO_2 concentration when treating the fodder directly with hydrolytic microorganisms like fungi. With the recent developments in fermentation technology and enzyme production system, the costs of feed enzymes are expected to decline in the future. Commercial products like enzymes could play important roles in the future of ruminant production systems (Beauchemin et al., 2004).

Most commercially available exogenous fibre-degrading enzyme products consist of cellulases and xylanases. Commercial enzymes used in the livestock feed industry are generally of fungal (mostly Trichoderma and Aspergillus) (Sarnklong et al., 2010). Enzyme treatment alone or in combination with other treatments were proven in many studies that it can increase the degradability of cereal straw by rumen microorganisms (Liu and Orskov, 2000; Wang et al., 2004; Zhu et al., 2005; Eun et al., 2006; Fazaeli et al., 2006; Rodrigues et al., 2008).

Fibrolytic enzyme preparations have become valuable tools in the last decade for improving digestive processes in the ruminant (Murad et al., 2009; Azzaz, 2009). To date, little is known about the way exogenous fibrolytic enzymes improve feed and how it affects rumen microorganisms. Several potential modes of action have been proposed. These include: (a) increase in microbial colonization of feed particles (Yang et al., 1999), (b) enhancing attachment and/or improve access to the cell wall matrix by ruminal microorganisms and by doing so, accelerate the rate of digestion (Nsereko et al., 2000), and (c) enhancing the hydrolytic capacity of the rumen due to added enzyme activities and/or synergy with rumen microbial enzymes (Morgavi et al., 2000).

Exogenous fibrolytic enzymes have been used to improve fibre digestibility. The use of exogenous enzymes in ruminant diet is a technology still in the developmental stage and positive effects have been recently reported. However, enzyme supplementation appears to depend on such factors as enzyme activities, type and dose of enzyme, enzyme application method, diet and animal physiological status (Giraldo et al., 2008; Colombatto et al., 2003) even the level of animal productivity (Beauchemin et al., 2003; Giraldo et al., 2008) as well as the expensive expenditure of exogenous enzyme.

With the development and application of a variety of cultivation-independent molecular techniques, it has become clear that cultivation-based methods have only identified approximately 10 to 20% or less of the total microbial population harboured in the rumen (Edwards et al., 2008).

The key is to link analysis of the community structure to ruminal function by measuring the gene expression, which is translated into protein and metabolic function (fibre degradation, amino acid deamination, methanogenesis etc.). The ultimate challenge is to understand and describe the biology at the molecular level so that the information can be used to manipulate feeding systems to maximize efficiency of digestion in the rumen.

The basic prerequisites for ecological studies involve the identification and enumeration of community members. Historically, most knowledge on natural microbial community composition has been derived using microbiological techniques, such as selective enrichment, pure culture isolation and most-probable-number estimates. The use of sequences of the small subunit (16S) of ribosomal ribonucleic acid (rRNA) for the identification and taxonomy of microorganisms has revolutionized studies in these areas. The results obtained using molecular techniques such as 16S rRNA has the advantage of being independent from the conditions of growth and culture techniques. Muyzer and Smalla (1998) developed a new genetic fingerprinting technique, called denaturing gradient gel electrophoresis (DGGE), which is used to evaluate microbial ecology. DGGE allows for the analysis of multiple samples simultaneously, and several studies have been performed to assess the structure of the bacterial communities and determine their dynamics based on environmental perturbations or seasonal, spatial and geographical variability (Crump et al., 2004). This study was conducted to evaluate the effect of cellulase treated rice straw on the molecular diversity of rumen bacteria in Philippine native cattle.

Materials and Methods

Experimental animals, design and treatments

Three rumen fistulated cattle with an average body weight of 360 ± 46.54 kg were allocated with three dietary treatments for 3 repeated trials of 11-day period using a 3×3 Latin square design. The experimental animals were in good body condition prior to and throughout the duration of the study. A 3X3 Latin Square Design (LSD) was used to evaluate the effect of using enzyme in feeding value of cattle fed with treated rice straw on its rumen microbial diversity.

The diets were provided to the animals at 3% of their body weight on a dry matter basis. Each of the dietary treatments was fed to animals in sequential order for three successive periods with 7 days neutralization period in between periods. Treatments consisted of Treatment 1 (UMTRS): Urea and molasses treated rice straw (5% urea + 5% molasses); Treatment

2 (UMCTRS): Urea, molasses and cellulase treated rice straw (5% urea + 5% molasses + 2.5% BIOTECH-Cellulase from *Trichoderma reesei* RUT-C40) and Treatment 3 (CTRS): BIOTECH-Cellulase treated rice straw.

They were fed with 70% rice straw and 30% concentrate for 11 d (7 days for adaptation, 4 days as experimental period). On the 11th d, sample of rumen was obtained for microbial identification. On the 11th d, after *in sacco* incubation and rumen fluid collection, animals were rested for 7 d, allowing them to graze freely in the pasture area. After the rest period, the animals were brought to the metabolism stalls and were given the next dietary treatment.

Rumen bacterial diversity

Changes in rumen bacterial diversity due to the dietary treatments were determined by Polymerase Chain Reaction–Denaturing Gradient Gell Electrophoresis (PCR-DGGE) following the procedure by Ercolini (2004) and Muyzer and Smalla (1998). The DGGE had been used to separate PCR product of the amplified 16S rDNA or its regions.

Extraction of genomic DNA from rumen fluid

DNA was extracted from rumen fluid sample using DNAeasy plant DNA Minikit (Quiagen). Extracted DNA quality and quantity was verified spectrophotometrically (NANO drop).

Agarose gel electrophoresis

Extracted DNA samples were mixed to 10× loading dye solution (bromophenol blue) prior to electrophoresis at 100V. About 7 µL of DNA was loaded in each well of 1% molecular screening agarose gel (W/V) (Promega Medison, Ca) submerged horizontally in 0.50X Tris-acetate EDTA (TAE) buffer. During electrophoresis, DNA molecules were allowed to migrate to 2/3 of the length of the gel for about 30 min or until the blue loading dye is 1 to 2 cm from the bottom. After which gels were stained by immersing in ethidium bromide solution (0.5 µg/mL) (Promega Medison, Ca) for a few seconds and then destained in distilled water for 20 min. DNA bands on agarose gels were visualized under transilluninator UV at 254 nm and recorded using Bio-Rad Doc 2000 documentation system (Berkeley, Ca. USA).

Polymerase chain reaction (PCR) amplification of 16S rDNA

The total DNA extracted from each rumen fluid sample and diluted to15 ng/µl was used as a template in PCRs. The DNA was amplified by PCR using Universal primer set consisting of 0008-a-S-19 (5′-GAGTTTGATCCTGGCTCAG-3′) and 1528-a-A-17 (5′-AAGGAGGTGATCCAGCC- -3′) to obtain the whole 16SrDNA (Srivastava et al., 2008; Petrov and Petrova, 2009). The PCR was carried out using a total of 20µl containing 0.5 µM of each primer, 1X buffer 0.16 m*M* dNTP, 1.5 m*M* $MgCl_2$, 0.4 U Tag DNA polymerase (Vivantis, USA) and 50ng of DNA template. It was ran at initial denaturation step of 94°C for 3 min., followed by 30 cycles of denaturation for 1 min., annealing at 45°C for 30 sec. and extension at 72°C for 90 sec. and a final extension at 72°C for 10 min. Amplification of PCR products was verified through electrophoresis 1% agarose gel stained with ethidium bromide and visualized on a trans-illuminator UV at 254 nm. Images of gels were captured using Bio-Rad Gel Doc 2000 documentation system (Berkely, Ca. USA).

The product was then subjected to second PCR amplification using 926r and 341f-GC primers to amplify the V_3 region of 16S rDNA. PCR mixture (30µl) contains same as what is used in the first amplification except for primers; 0.75 µl of 926r and 341 f-GC of each were used. It was ran with initial denaturation step of 94°C for 5 min followed by 28 cycles: denaturation 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1min, and for elongation at 72°C for 30 min.

Denaturing gradient gel electrophoresis (DGGE)

The PCR products were subjected to DGGE using a DCode^{TM} Universal Mutation Detection System (Bio-Rad, Berkely, Ca. USA). The PCR amplicons were resolved in 8% (W/V) polyacrylamide gel containing 30-60% denaturing gradient (100% denaturant consisted of 40% (v/v) formamide and 7 M urea). Thirty-five (35) µL of PCR product and dye mixture was loaded in each well (20-well comb) of polyacrylamide gel set in 0.5X TAE (20mM Tris; pH 7.4, 10 mM Acetic acid, glacial, 0.5 M EDTA) running buffer. The gels were run at 60°C and 100 V for 10 min then 60 V for 15-17 h. After electrophoresis, gels were stained with ethidium bromide (0.5µg/ml) (Promega Medison, Ca) and destained in distilled water for 20 min before finally photographed under UV using Bio-Rad Gel Doc 2000 Documentation System. Representatives of bands that were clear or distinct and had high intensity were randomly excised from DGGE gels for sequencing.

Sequencing analysis of PCR-DGGE fragments

Distinct bands were excised from DGGE gel and transferred into 1.5 ml Eppendorf tubes containing 50 µl sterilized nano pure (SNP) water. Thereafter, the gels were crushed using 1 ml sterile pipette tips and centrifuged at 10k rpm for 30 sec followed by 30 min heating at 37°C to extract the desired DNA. From this, 1 µl was used as a template for PCR amplification using 00341 forward primer without the GC clamp and

0926 R primer set. The PCR was conducted as that in 00341 F-GC and Eub 926 R conditions and the resulting product was verified through agarose gel electrophoresis under transillumination UV. Images of gels were captured using Bio-Rad Gel Doc 2000 documentation system. The resulting unpurified PCR products were sent directly to 1st BASE Inc., Malaysia for sequencing. Quality sequences were compared to 16S rDNA sequences submitted to the National Centre for Biotechnology Information (NCBI) nucleotide database using nucleotide basic local alignment search tools (BLASTn, Altschul et al., 1997). Also DNA sequence alignments of presented amplicons with local vector database (Vector NTI) were used to show similarity of amplicons in the BLAST- NCBI database. Sequences with >97% homology to the required sequences were identified.

Results and Discussion

Bacterial diversity analysis

The sequence analysis of purified PCR product amplified from excised DGGE bands was used to determine the phylogenetic placement of each individual band in the community (Liu et al., 2008).

There were 12 distinct 16S rRNA V3 (variable region 3) amplicons in the DGGE electro photogram (Fig. 1) representing predominant bacterial groups in the rumen of cattle in response to 3 feeding treatments. Only those amplicons that passes quality chromographic profile were considered for sequence comparison in the NCBI-BASTn.

In cattle, the amplicons A, B, C, E, K and L were identified as uncultured rumen bacterium clone CTL2D06 (Table 1). Amplicons G and H were recognized as uncultured rumen bacterium clone UG-B0_128. Amplicons C in line 8, D, F, I and J were identified as *Butyrivibrio crossotus* strain DSM 2876T, uncultured rumen bacterium clone D-B-37, uncultured rumen bacterium clone D-C-CA84, uncultured rumen bacterium clone MXMP-B6 and uncultured rumen bacterium clone MXMP-I2 respectively. The bacterial species were organisms that had highest identity with the query sequence although other bacterial species belonging to phyla Firmicutes, Bacteroides and Clostridia is an order in Phyum Firmicutes, were also identified. Related sequences were 53-86% belonging to these phyla.

Accession numbers and corresponding percentage similarity to the queried sequences are indicated in Table 1. In this study uncultured rumen bacterium clone D-B-37 is well established in cattle fed with urea-molasses treated rice straw (UMTRS) and cellulase treated rice straw (CTRS). Uncultured bacterium clone D-C- CA84 is well established in animals fed with urea-molasses and cellulase treated rice straw (UMCTRS). Uncultured rumen bacterium clone CTL2D06, Uncultured rumen bacterium clone UC-BO-128, Uncultured rumen bacterium clone MXMP-B6, Uncultured rumen bacterium clone MXMP-12 and Uncultured rumen bacterium clone CTL2D06 were found in all treatments.

All of the sequenced bacteria in this study are observed in cattle fed with UMCTRS except uncultured rumen bacterium clone D-B-37 that only presented in cattle fed with CTRS. Some studies using fibrolytic enzymes alone showed that it did not significantly increase the degradability of rice straw because the ability of these enzymes to break down esterified bonds within lignin-carbohydrate complexes may have been limited (Sarnklong et al., 2010). However, when used in combination with other pre-treatments, they could increase degradability and *in vitro* fermentation characteristics as shown by work of Eun et al. (2006) on xylanase or cellulase treated rice straw in combination with ammonia treatment. The works of Liu and Orskov (2000) also on rice straw involving treated with cellulase from *Penicillum funiculosum* in combination with steam pre-treatment and the study of Wang et al. (2004) when rice straw was treated with

Table 1: Identification of PCR-DGGE bands submitted to Nucleotide BLAST- NCBI database in Philippines native cattle

PCR-DGGE Band	Most closely related taxon	Genbank Accession NO.	% Similarity
A	Uncultured rumen bacterium clone CTL2D06	GQ327514.1	99
	Uncultured bacterium clone 1103206092250	EU843793.1	99
B	Uncultured rumen bacterium clone CTL2D06	GQ327514.1	99
C	Uncultured rumen bacterium clone CTL2D06	GQ327514.1	99
	Butyrivibrio crossotus strain DSM 2876T	FR733670.1	89
D	Uncultured rumen bacterium clone D-B-37	AB612556.1	95
E	Uncultured rumen bacterium clone CTL2D06	GQ327514.1	99
F	uncultured rumen bacterium clone D-C-CA84	AB612868.1	88
G	Uncultured rumen bacterium clone UG-B0_128	JF797427.1	97
H	Uncultured rumen bacterium clone UG-B0_128	JF797427.1	98
I	Uncultured rumen bacterium clone MXMP-B6	JX218739.1	100
J	Uncultured rumen bacterium clone MXMP-I2	JX218852.1	98
K	Uncultured rumen bacterium clone CTL2D06	GQ327514.1	99
L	Uncultured rumen bacterium clone CTL2D06	GQ327514.1	99

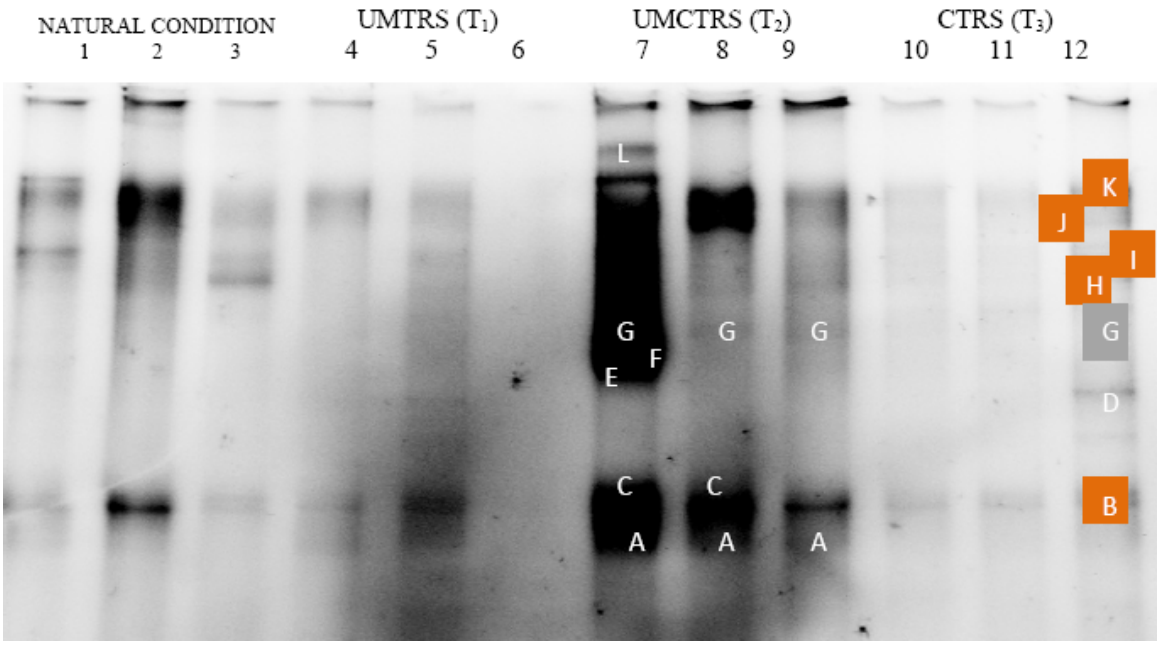

Fig. 1: 16srDNA DGGE profile of bacterial groups from cattle fed with different treated rice straw as resolved by denaturing gradient gel electrophoresis (DGGE): lines 1,2 and 3 are before condition in rumen of animals (T_0), lines 4, 5 and 6 are UMTRS (T_1) fed to animals; lines 7, 8 and 9 are UMCTRS (T_2) fed to animals and lines 10, 11and 12 are CTRS (T_3) fed to animals.

multi-enzymes (xylanase, beta-glucanase, carbo-xymethyl cellulase and amylase) in combination with NaOH showed synergistic effect on the nutritive improvement of rice straw.

DNA sequence alignment of presented amplicons in cattle with local vector database (Vector NTI) showed that amplicons A and B have 78% similarity based in the BLAST- NCBI database (Table 1) and both amplicons have a sequence similarity 68% compared to amplicon C. with band C while they are shown 68% similarity with band C.

Amplicons C was identified to be *Butyrivibrio crossotus* strain DSM 2876T while amplicon F to be an uncultured rumen bacterium clone D-C-CA84 present only in animals fed with treatment 2 (UMCTRS).

Amplicons K and L had 100% identity with each other. Amplicon J with amplicons K and amplicons L had 62% similarity. Amplicons H with amplicons I and amplicons G showed 52% similarity. Kong et al. (2010) reported the uncultured rumen bacterium clone CTL2D06 from rumen of cow fed different forages. Uncultured bacterium clone 1103206092250 was observed as a rumen fluid fibre adherent microbiome from steer, 1h after feeding (Brulc et al., 2008; Brulc et al., 2009).

Uncultured rumen bacterium clone D-B-37 was detected by Kobayashi (2011) from rumen microbiota of cattle. Uncultured rumen bacterium cloneUG-BO-128 was reported by Hook et al. (2011) from rumen of dairy cattle (Holstein). Uncultured rumen bacterium clone MXMP-B6 and MXMP-12 were reported by Forster et al. (2012) from rumen of *Ovibos moschatus (*Mammals: Bovidae family), USA, Alaska.

Conclusion

There were 12 distinct 16S rRNA V3 (variable region 3) amplicons observed in the DGGE electro photogram representing predominant bacterial groups in the rumen of cattle in response to feeding treatments. Uncultured rumen bacterium clone D-B-37 was well established in cattle fed with CTRS and uncultured bacterium clone D-C- CA84 was well established in animals fed with UMCTRS. All of the sequenced bacteria in this study were observed in cattle fed with UMCTRS, however, only the D-B-37 was present in animals fed with CTRS. DNA sequence in cattle showed presence of more amplicons in UMCTRS indicating that this treatment led to increase in the population of fibrolytic microorganisms in the rumen.

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