Amplified Ribosomal DNA Restriction Analysis Method to Assess Rumen Microbial Diversity of Ruminant

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Abstract—Rumen degradation characteristic of feedstuff is one of the prominent factors affecting microbial population in rumen of animal. High rumen degradation rate of faba bean protein may lead to inconstant rumen conditions that could have a prominent impact on rumen microbial diversity. Amplified Ribosomal DNA Restriction Analysis (ARDRA) is utilized to monitor diversity of rumen microbes on sheep fed low quality forage supplemented by faba beans. Four mature merino sheep with existing rumen cannula were used in this study according to 4 x 4 Latin square design. The results of study indicated that there were 37 different ARDRA types identified out of 136 clones examined. Among those clones, five main clone types existed across the treatments with different percentages. In conclusion, the ARDRA method is potential to be used as a routine tool to assess the temporary changes in the rumen community as a result of different feeding strategies.

Keywords—ARDRA method, clones, microbial diversity, ribotypes, ruminants.

I. INTRODUCTION

FOR a long time, methods to identify and study diversity of bacteria often relied on the traditional methods of plating bacteria on agar [1]. With this technique, rumen bacteria have been indicated to belong to some small number of predominant species [2]. Recent observation, however, showed that the prior observation represents only a small portion of the total diverse population that may occupy the rumen [3].

Currently, molecular techniques based 16S ribosomal RNA (rRNA) and their encoding genes have been used extensively to study rumen microbes both qualitatively and quantitatively [4]-[7]. ARDRA is one of several methods that can be applied to monitor the diversity of rumen microbes. ARDRA is a technique where rRNA gene fragments (rDNA) are enzymatically amplified using conserved primers.

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Furthermore, the amplified fragment is subjected to restriction analysis using various restriction enzymes. The resulting patterns generated then can be used for identification of bacterial species and for strain differentiation within species [8].

Several studies have reported the use of ARDRA in monitoring genotypic community changes over a period of time or over different environmental conditions [9], [10]. Blaszczyk et al. [11] also reported to detect molecular changes between normal and bulking sludge samples informing about disadvantageous changes in the microbial population.

ARDRA might be used to assess rumen microbial diversity of rumen microbes due to the change of rumen condition as a consequence of provision of feed having different characteristics of its chemical components, especially in its protein content. Faba beans (*Vicia faba*) are legume seeds, which are particularly high in crude protein concentrations (25-42%) [12]. Nevertheless, high degradation rate (85-90%) of faba beans in the rumen [13]-[15] should be taken into account in order to optimize its use as a protein supplement [16], [17].

Natsir [18] has noted the importance of synchronizing the availability of N obtained from the supplement of faba beans and energy derived from both supplement and forages as the basal diet. Provision of faba beans in small portion more than once a day can reduce the high amplitude of fluctuations of rumen conditions such as pH after feeding which may maintain fiber degradation rate of the basal diet [19], which in turn may have profound effects on the diversity of rumen microbes [20]. The purpose of this study was to evaluate whether providing the same amount of faba beans supplement delivered at the different frequencies to the sheep could have an impact on the diversity of rumen microbes of sheep using ARDRA method as a tool of assessment.

II. MATERIALS AND METHODS

A. Animal and Feeding

The experiment was carried out according to 4x4 latin square design (4 treatments x 4 periods) [21]. The treatments were T0 = Forages without faba beans supplement, T1 = T0 + 450 faba beans, T2 = T0 + 225 g faba beans given in the morning and 225 g faba beans given in the afternoon, and T3 = T0 + faba beans supplement given every three hours the rate of 58.2 g. Faba beans supplement was given in the morning starting at 09.00 and the amount given was depending upon

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the treatment. Each experimental period lasted 20 days, in which the last three days of each period was allocated for collecting rumen fluid samples, blood samples, and samples for rumen microbial for metagenomic study.

B. Sampling

Samples for genomics and rumen fermentation studies were taken in each period of the experiment. The ruminal contents (solid + liquid) were sampled at 6 h after feeding, the time when rumen fermentation rate for each treatment was predicted to be close to highest and microbial patterns reflecting the balance of substrate being fermented under the 4 dietary conditions. For both treatment T2 and T3, faba beans were given after samples were taken. For DNA extraction, sample of approximately 50 g was collected from each animal in each period of sampling and transferred directly to laboratory and stored at -20 °C for later analysis. Rumen liquid was also sampled during the sampling for genomic study. Rumen fluid was measured for rumen pH onsite and the rest of the rumen fluid was stored for later analysis of rumen-NH_3.

C. DNA Extraction

The rumen samples collected beforehand were thawed and pooled according to the treatment and mixed thoroughly before taking samples for DNA extraction. Approximately 50 mg of mixed sample was hardened by using liquid nitrogen before grounded. The grounded sample was extracted for DNA using DNeasy Tissue Kit (QIAGEN). The basic procedure of the kit is to lyse the microorganisms in the ruminal sample by the combination of heat, detergent and proteinase K. The released DNA is then purified via a spin filter.

D. PCR Amplification of the 16S rRNA Genes

PCR amplification of 16S rDNA was conducted in a PTC-100 thermocycler (MJ Research. Inc., USA). The reaction mixture contained 10x AmpliTaq Gold reaction buffer, 200 μM of deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP) (Pharmacia LKB Biotechnology), 1.25 units of AmpliTaq Gold (Applied Biosystem), 0.44 unit of each oligonucleotide primer and 250 ng of template DNA in a total volume of 50 μl. The primers used for amplification for bacterial 16s rDNA were 519f and 1492r (Life Technologies, USA).

The PCR reaction was performed following the procedures of [22]. The PCR product was cut from 1% (W/V) agarose gel stained with ethidium bromide and purified through a Rapid Gel Extraction spin column according to the recommendation of the manufacturer (Life Technologies, USA). Only one PCR reaction was required to provide sufficient DNA concentration for further construction of 16S rDNA libraries.

E. Construction of 16S rDNA Libraries and Amplified rDNA Restriction Analysis

Construction of 16S rDNA and Amplified rDNA restriction analysis were carried out following the procedures of [22]. The method of dice similarity was applied to estimate the genetic distances between a pairs of clones [23]. The unweighted pair group method with arithmetic means was used to create Dendrograms using Mega software [24]

F. Sequencing of Cloned 16S rDNA PCR Fragments

Clones of ten ribotypes were selected according to the dendrograms derived from the results of ARDRA. Plasmid DNA for sequencing was isolated with the Qiagen plasmid kit (Qiagen GmbH, Hilden, Germany). Sequencing of cloned 16S rDNA PCR fragments was performed with an automated laser fluorescent DNA sequencer (Pharmacia Biotech) as described by the manufacturer. Sequencing reactions were carried out with the ABI Prism BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystem) with primers M13 forward and M13 reverse corresponding to regions in the plasmid pGEM-T. The sequences were compared with similar rDNA sequences retrieved from the DNA databases, by using the BLAST search program in the National Centre for Biotechnology Information (NCBI).

III. RESULTS

Provision of faba beans at different frequency is intended to minimized changes on the conditions of the rumen due to the provision of high degradable feedstuff. Rumen pH dropped slightly (pH less than 6.0) on the animal fed on supplements either once or twice a day. The rumen pH rebound when it was provided 8 times a day (Table I). pH = 6.0 is regarded as the threshold pH for optimum fiber degradation in the rumen [25]. Provision of faba beans as supplement either once, twice, or eight times a day, significantly improved the concentration of rumen NH_3. The average increment of rumen NH_3 for animal fed faba bean supplement was 85% higher than those fed basal diet without supplement, indicating that provision of faba beans has profound effects on rumen fermentation condition of the animal (Table I). Provision of faba beans for sheep consuming oaten chaff as basal diet improved rumen fermentation pattern of the animal and also improved the estimation of microbial protein supply for the animal [18].

As the rumen condition is affected by provision of faba beans delivered at different frequencies, the structure and diversity of rumen microbes was also influenced by animal feeding behavior. The assessment of diversity of rumen microbes for each treatment is presented in Table I. Initially, 136 reamplified 16S rDNA fragments were digested with the endonuclease *HhaI* and resulted in the identification of 37 ribosomal types.

TABLE I
SUMMARY OF AMPLIFIED rDNA RESTRICTION ANALYSIS (ARDRA) FOR EACH TREATMENT

Treatment	Rumen pH	Rumen NH_3 (mg/L)	Number of clones	Number of ADRA types	Genotype diversity (%)
T0	6.17	185	34	20	58.8
T1	5.82	379	34	16	47.1
T2	5.91	305	34	16	47.1
T3	6.05	343	34	18	52.9

T0 = oaten chaff and lucerne *ad libitum* + nil supplement, T1 = oaten chaff and lucerne *ad libitum* + faba beans fed once daily, T2 = oaten chaff and

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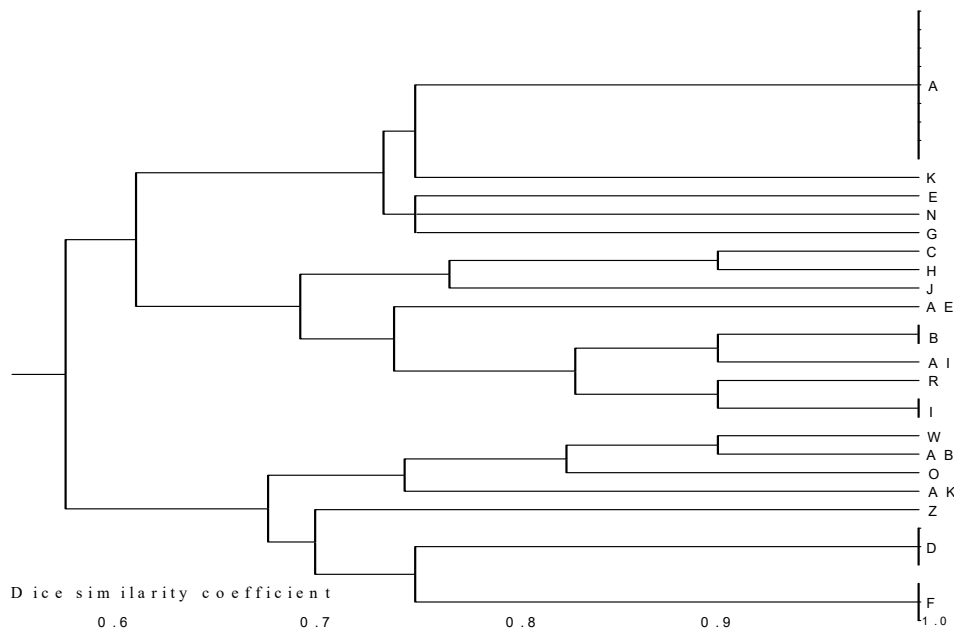Vol:10, No:12, 2016

lucerne *ad libitum* + faba beans fed twice daily, T3 = oaten chaff and lucerne *ad libitum* + faba beans fed 8 times daily.

Among the 37 different ARDRA types identified out of 136 clones examined, five clone types, designated as A, B, C, D, and E, were found to occur in all samples, contributing 17.2, 11.0, 6.6, 6.6 and 4. Among the 37 different ARDRA types identified out of 136 clones examined, five clone types, designated as A, B, C, D, and E, were found to occur in all samples, contributing 17.2, 11.0, 6.6, 6.6 and 4.4% of clones, respectively. Collectively across dietary treatments, these profiles accounted for 45.6% (62/136) of the clone numbers. In addition, clone type F occurred with relatively high frequency among the clones for treatments T0, T1, and T3 but was absent in T2. The percentage of this clone across treatments was 9.6%. Together the 6 types (A, B, C, D, E, and F) accounted for 55.1% (75/136) of all clones.
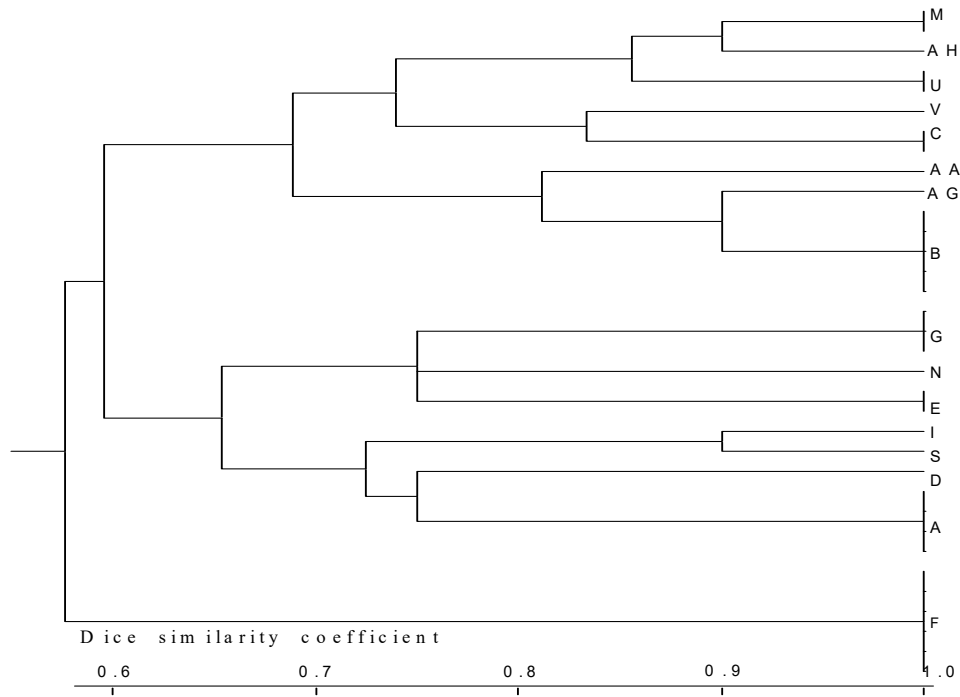
ARDRA profile A was the most abundant in all samples, being, 26.5, 11.8, 8.8, and 20.6% for T0, T1, T2 and T3, respectively. The distribution of type B was 5.9, 14.7, 5.9 and 17.6%, respectively. The percentage of type C was 2.9, 5.9, 5.9 and 11.8%, respectively. For type D, the percentage was 8.8, 2.9, 8.8 and 5.9%, respectively, and the distribution of type E was 2.9, 5.9, 5.9 and 2.9% for T0, T1, T2 and T3, respectively. The percentage of type F, which was abundant in all treatments except in T2, was 8.8, 17.6 and 11.8% for T0, T1 and T3, respectively. The ARDRA analysis also revealed rybotypes regarded as a single type clones for each dietary treatment. The number of clones of this type was 15, 8, 9 and 8 for T0, T1, T2 and T3, respectively, representing 44.1%, 23.5, 26.5, and 23.5% of the all clones for T0, T1, T2 and T3, respectively. In addition, the analysis also was able to identify some clone types that could be categorized as "unique" type, a clone that occurred in one treatment only. For example, clone type P and U only occurred in T3 and T1, both of which were 5.9%.
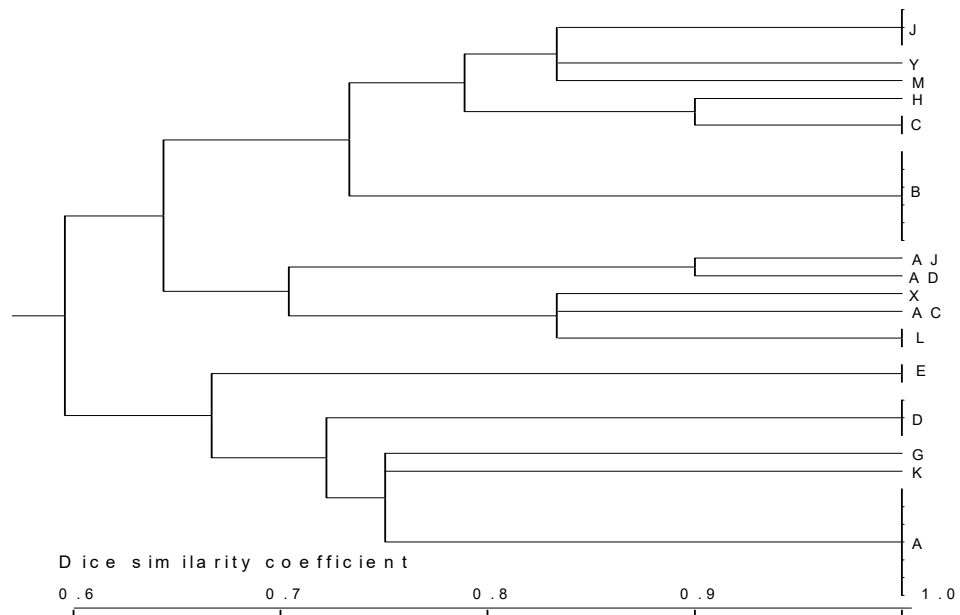
The relationships among the 34 clones from each rumen sample are shown in Fig. 1. Using a Dice similarity coefficient of 0.60, two main clusters can be distinguished in all samples. The main rybotypes (A to F) were distributed into those clusters differently for each treatment.
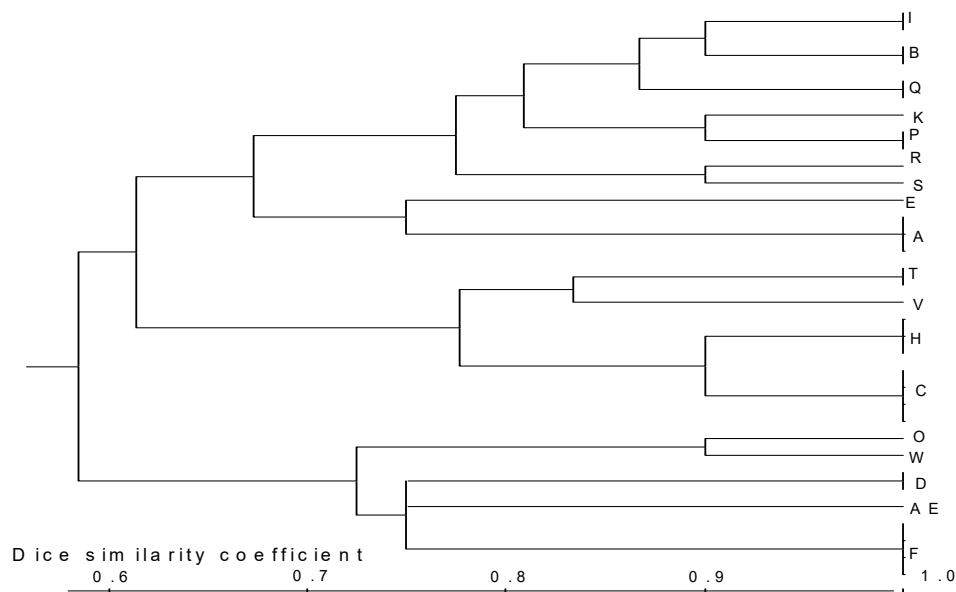

(a) Dendogram for treatment T0

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(b) Dendogram treatment T1

(c) Dendogram for treatment T2

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(d) Dendogram for treatment T3

Fig. 1 Dendrogram constructed from ARDRA for treatment T0 (a), T1 (b), T2 (c), and T3 (d) and each ribotype is designated by a capital letter

Numbers and position of bands obtained from 16S rDNA restriction analysis of each ruminal microbial of differences protein source sample were the basis for creating dendrograms. Intracluster similarities and distances between isolates depict their genetic relationships. Based on the dendrograms derived from ARDRA (Fig. 1), clones of 10 ribotypes (A, AG, AJ, B, C, D, E, F, G, and P) were selected and sequenced, so as to cover most of the microbial diversity in the clone libraries. The ten-nucleotide sequences, each one representing one 16S ribotype, are presented in Table II.

TABLE II
NEAREST NEIGHBOURS OF THE 16S rDNA CLONES

Rybo-Type	Nearest neighbours	Similarity	% of clone			
A	*Ruminococcus albus*	95	26.5	11.8	20.6	8.8
AG	*Unidentified rumen bacterium*	91	0	2.9	0	0
AJ	*Unidentified rumen bacterium*	90	0	0	2.9	0
B	*Prevotella ruminocola*	96	5.9	14.7	17.6	5.9
C	*Butyrivibrio fibriosolvens*	92	2.9	5.9	5.9	11.8
D	*Clostridium sp*	91	8.8	2.9	8.8	5.9
E	*Ruminococcus flavafaciens*	93	2.9	5.9	2.9	5.9
F	*Succiniclasticum ruminis*	96	8.8	17.6	0	11.8
G	*Eubacterium cellulosolvens*	91	2.9	8.8	2.9	0
P	*Uncultured–fiber attaching rumen bacterium*	92	0	0	0	5.9

IV. DISCUSSION

Intention to maintain optimal conditions of the rumen for fibre digestion can be achieved by providing faba beans supplement more than twice a day. This feeding strategy can reduce fluctuation of rumen pH. If rumen pH falls below 6.0, it could significantly reduce the fibre digestion of the ration. The rumen pH of 6.0 is regarded as a threshold point for fibre degradation [25].

Amplified rRNA restriction and confirmation analysis is a useful method for confronting isolates undergoing condition changes or samples from different environments [26], [27]. The ARDRA clearly identified both similarities and differences among the different rumen samples. It appears that microbes comprising ARDRA profiles A to F were less affected by rumen conditions such as rumen pH and rumen NH_3 concentration, generated by the different treatments. These types were found in all 4 libraries. Therefore, for sequencing purposes, the clone types of profile A-F were chosen plus 4 other rybotypes, providing much of the microbial diversity in the clone libraries.

The sequence of ribotype A was identified as belonging to *Ruminococcus albus* with a similarity of 95% of database sequence. Clones from ribotype B were identified belonging to *Prevotella ruminococcus* with a similarity of 96%. The other dominant ribotypes, C, D, E, and F were also not affected by the rumen conditions. The high percentage of ruminococci detected in this experiment was not surprising as they are commonly found in a high percentage on the animal fed on either roughage- or concentrate-based diet, which differentiated from such other important cellulolytic as *F. succinogenes, R. albus* and *R. flavefaciens* or from other secondary cellulolytic bacteria as *Butyrivibrio fibrisolvens*, *Eubacterium cellulosolvens*, *Clostridium longisporium*, and *Clostridium locheadii* [1], [3], [4]-[7], [28]-[30].

Even though *R. albus*, *R. flavefaciens* and *F. succinogenes*, are usually the predominant cellulolytic bacteria [31], in this experiment, the sequence analysis only detected *R. albus* at a high percentage compared to *R. flavefaciens* and did not detect the existence of *F. succinogenes* at all among the 136 clones isolated. This may have been related to time of sampling and the rumen conditions, especially rumen pH at the time when rumen samples were withdrawn when rumen pH was quite

low, less than 6.0. The high number of *R. albus* detected in this study might be related to its capacity in metabolizing carbohydrate under acidic conditions [28], [31]. This phenomenon has been observed by [32] which reported a relatively high number of *R. albus* in the rumen of animals receiving concentrate diets. In addition, although most ruminococci ferment cellobiose, some species can also metabolize soluble sugars (e.g. glucose) arising from starch degradation. This may explain why the number of cellulolytic or hemicellulolytic bacteria are not necessarily diminished when grain supplements are added to the diet, even though fibre digestion in the rumen is usually reduced quite significantly [32]. Acute episodes of high grain feeding, with recovery of fibre degradation rates when grain was subsequently withheld, would suggest the survival of large numbers of fibrolytic bacteria even with greatly depressed pH [19], [20].

A high number of *Prevotella/Bacteroides* in rumen samples has been previously reported. Russell and Wilson [33] indicated that *Prevotella* was a common ruminal bacterium that was found in high numbers in the rumen of animals fed virtually all diets. *Prevotella*, which is not a cellulolytic bacterium, cannot digest native cellulose. However, it does produce an extracellular *ß*-1,4, endogluconase that hydrolyses carboxymethyl cellulose (CMC), a soluble cellulose derivative and an *in vitro* study indicated that *P. ruminocola* was able to grow at pH as low as 5.1 [34].

It was interesting to note that the ARDRA revealed the presence of one ribotype, with close genomic similarity to *Succiniclasticum ruminis*, in treatments T0, T1 and T3. These bacteria have an important role in converting succinate to propionate [35], however why this ribotype did not appear in the treatment T2 was unclear.

Martin-Laurent et al. [36] reported that even though ARDRA method can be used to monitor differences among sludge samples, it is not capable of detecting changes in the community regardless of its capacity in detecting the dominant genera within the sample. Despite the ability of ARDRA analysis to identify similarities and differences which exist in the rumen due to different treatments or different sampling times, as with other analytical procedures, this method carries potential biases. Sampling collection, cell lysis, DNA extraction, PCR amplification, and the cloning process are potential` sources of bias [1], [3]. As discussed by [37] that even though the ARDRA patterns of different species can be observed in the diversity of a composed dendrogram, preferential amplification can occur affecting the relative frequency of ARDRA types recovered. This potential problem can be caused by differences in DNA extraction efficiency, differences in copy number of 16S rDNA, differences in genome sizes and by preferential annealing during PCR. In addition, the accuracy with which different 16S rDNA fragments are distinguished by ARDRA very much depends on the number of restriction enzymes used [37], [38].

V. Conclusion

ARDRA analysis was successful in identifying similarities

and differences of a microbial population derived from different rumen samples. Use of ARDRA method as a routine tool of analysis to identify the temporary changes due to different diets or different sampling times in the rumen community is a one step further in defining how the rumen population deals with the non-steady state created by supplementary feeding strategies.

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