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MICROBIAL DIVERSITY IN RUMEN ECOSYSTEM OF LACTATING COWS GRAZING DIFFERENT LEVELS OF HIGHLY DIGESTIBLE PERSIAN CLOVER.

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SUMMARY

16S rDNA clone libraries were analysed to investigate the microbial diversity in rumen contents taken at 6.30 a.m. and 7.00 p.m. from two lactating cows grazing either medium (MA) or high (HA) levels of highly digestible Persian clover. Ruminal pH was 6.4 and 5.7 for the MA cow and 6.4 and 5.2 for the HA cow at 6.30 a.m. and 7.00 p.m. respectively. Total genomic DNA was extracted from samples of ruminal content, and used for Amplified Ribosomal DNA Restriction Analysis (ARDRA) to examine the microbial diversity present in each sample. No less than 36 different ARDRA profiles were obtained from 120 clones from 4 libraries. However, only 2 of these ARDRA profiles were found to occur in all samples (designated A and B, with 49 clones). Clones obtained from HA cow had the diversity of 0.40 and 0.43 at 6.30 a.m. and 7.00 p.m. respectively and appeared to be lower than that of the clones obtained from MA cow which were 0.56 and 0.60 at 6.30 a.m. and 7.00 p.m. respectively. Distinct differences in microbial community structure were found in all samples of two cows. Using a Dice similarity coefficient of 0.60, the ARDRA profiles can be separated into 2 major clusters. The first cluster includes the ARDRA profiles A and B, representing about 63.3% and 36.6% of the total clones analysed at 6.30 a.m. and 7.00 p.m. respectively, in HA cow; and 33.3% and 29.9% at 6.30 a.m. and 7.00 p.m. respectively, in the MA cow. The second cluster is comprised mainly of single clones representing a unique ARDRA profile, and the profiles obtained are distinctly different between low and high pH samples from both cows. Further work needs to be done on clones selected from the dendrogram distribution to sequence the rDNA and determine their phylogenetic affiliation.

Keywords: Amplified ribosomal DNA restriction analysis, bacteria diversity, rumen pH

INTRODUCTION

The dependence of the rumen bacterial community structure on an animal's diet is a well-documented fact (Dehority and Orpin 1997). Most profound changes in the community structure occur during the weaning period and, in adult animals, during major dietary changes such as the switch from roughage to high-grain diet or transfer from hay/concentrate to pasture feeding. Supplementation to elevate rapidly fermentable carbohydrate levels in the diet used to improve the productivity of cattle, often induces acidosis, with pH declines attributable to the increase in the concentration of short chain fatty acids and lactic acid. Although variations in the microbial population structure can be detected at different levels of acidosis (Goad *et al*. 1998), these characterizations are all cultivation-based, and it is uncertain whether the full range of microbial diversity has been recovered. Recently, PCR- and hybridization-based analyses of either small subunit ribosomal RNA, or the gene(s) encoding this molecule, have supported new ways to evaluate microbial diversity without the need for bacterial cultivation (Amann *et al*. 1995), all useful for the identification and typing of microorganisms. We report here the use of one of these methods, named ARDRA (for amplified rDNA restriction analysis) to assess shifts in rumen microbial communities associated with differences in ruminal pH in lactating dairy cows grazing different levels of highly digestible Persian clover. The method is relatively fast, straightforward to use, and requires little or no sequence information about the amplified 16S rDNA fragments prior to comparative or functional analysis.

MATERIALS AND METHODS

Animals and sample collection.

The experiment was conducted using 2 rumen fistulated, lactating Holstein cows. The animals were allocated to either medium allowance (MA: pasture offered at approximately 15 kg DM/cow/day) or high allowance (HA: pasture offered at 50 kg DM/cow/day). The two allowances generated DM intakes of about 11 and 20 kg DM/cow/day. These allowances were aimed to result in differences in average rumen pH, the minimum pH reached, and the proportion of the day that pH is below 6.0. The cows were allocated to treatments and grazed Persian clover (Trifolium resupinatum L.) in individual plots for 14 days prior to the commencement of rumen sampling. Ruminal fluid was collected using a copper probe with a syringe attached and inserted through the cannula into the mid ventral region of

the rumen. Approximately 30 ml of this fluid was collected, dispensed into a tube, and its pH was measured immediately. The whole ruminal contents were also sampled twice daily, one sample was taken at 6.30 a.m. after milking and another at 7.00 p.m. Approximately 50 ml of digesta was collected, immediately frozen in liquid nitrogen and stored at –20^{o}C prior to DNA extraction.

Total DNA extraction and PCR amplification of the 16S rRNA genes.
Ruminal contents were thawed, then approximately 50 mg of sample was ground in liquid nitrogen twice with mortar and pestle and the powder was used for DNA extraction using the DNeasy Tissue Kit (QIAGEN). PCR amplification of 16S rRNA genes was conducted with a PTC-200 thermocycler (MJ Research, Inc., USA). The reaction mixtures (50µl) contained 5µl of 10x AmpliTaq Gold reaction buffer and 1.25 Units of AmpliTaq Gold DNA polymerase (both from Applied Biosystems), 200µM of each deoxyribonucleotide triphosphate (Pharmacia LKB Biotechnology), 0.44 µM of each oligonucleotide primer (519f 5′-CAGCMGCCGCGGTAATWC and 1492r 5′-GGTTACCTTGTTACGACTT), and 250ng of template DNA. The PCR reaction was performed with an initial denaturation step at 94^{o}C for 2 min., followed by 25 cycles of denaturation at 94^{o}C for 2 min., annealing at 45^{o}C for 1.50 min. and extension at 72^{o}C for 2 min. A final extension at 72^{o}C for 10 min was also included. The PCR products were subjected to gel electrophoresis in 1.5% (w/v) agarose gels and stained with ethidium bromide. The gel regions containing the desired PCR products were recovered from the gels using a sterile razor blade, and the DNA was purified using a Rapid Gel Extraction spin column (Life Technologies, USA) according to the manufacturer's recommendations.

Construction of 16S rDNA libraries.
The PCR products were ligated into the pGEM-T cloning vector (Promega, USA) and the ligation mixtures were used to transform *Escherichia coli* JM109 competent cells, according to the manufacturer's specifications. White colonies (presumed to contain recombinant clones of the 16S rRNA genes from the sample) were selected and propagated on LB agar plates with ampicillin (50µg ml^{-1}). Clones were confirmed to contain inserts of the desired length by PCR by using the same procedures outlined above, but with T7 and SP6 (pGEM-T-specific) primers from Life Technologies (US).

Amplified rDNA restriction analysis (ARDRA).
Aliquots (8µl) of each PCR product amplified by the T7 and SP6 primer set were taken and digested with the restriction endonuclease *Hha*I (New England Biolabs) for at least 2h. The resulting DNA fragments were then separated by electrophoresis in 2% (w/v) agarose gels. The 50 bp DNA step Ladder (Promega) was used as a DNA marker. Gels were stained with ethdium bromide and DNA fragments visualized under UV illumination. Restriction fragments shorter than 200bp were not considered in the analysis because of the possible interference of residual primers and primer dimers. Genetic distances between pairs of clones were calculated from the Dice similarity coefficient method (Nei and Lei, 1979) using RAPDistance Program version 1.04 (Armstrong *et al*. 1994). Dendrograms were constructed by the unweighted pair group method with arithmetic means using the Mega software program (Kumar *et al*. 2001). Since diversity indices commonly used by ecologists cannot be easily applied to ARDRA patterns (Borneman *et al*. 1996), we simply calculated diversity by dividing the number of different patterns by the total number of clones that were analysed.

RESULTS

The results of the ARDRA analyses to date are shown in Table 1. We have identified 36 different ARDRA patterns among the 120 clones examined to date. Only 2 dominant clone types, designated as A and B, were found to occur in all samples, and these two ARDRA profiles account for ~41% of the clones examined so far (49/120). Clones obtained from the HA cow had the diversity of 0.40 and 0.43 at 6.30 a.m. and 7.00 p.m. respectively and appeared to be lower than that of the clones obtained from MA cow which were 0.56 and 0.60 at 6.30 a.m. and 7.00 p.m. respectively.

Table 1. Summary of rumen pH at a time of sampling and amplified rDNA restriction analysis

Cows	Sampling time	Rumen pH	Number of clones	Number of ARDRA type	Genotypic Diversity
Medium allowance (MA)	6.30 a.m.	6.4	30	17	0.56
	7.00 p.m.	5.7	30	18	0.60
High allowance (HA)	6.30 a.m.	6.4	30	12	0.40
	7.00 p.m.	5.2	30	13	0.43

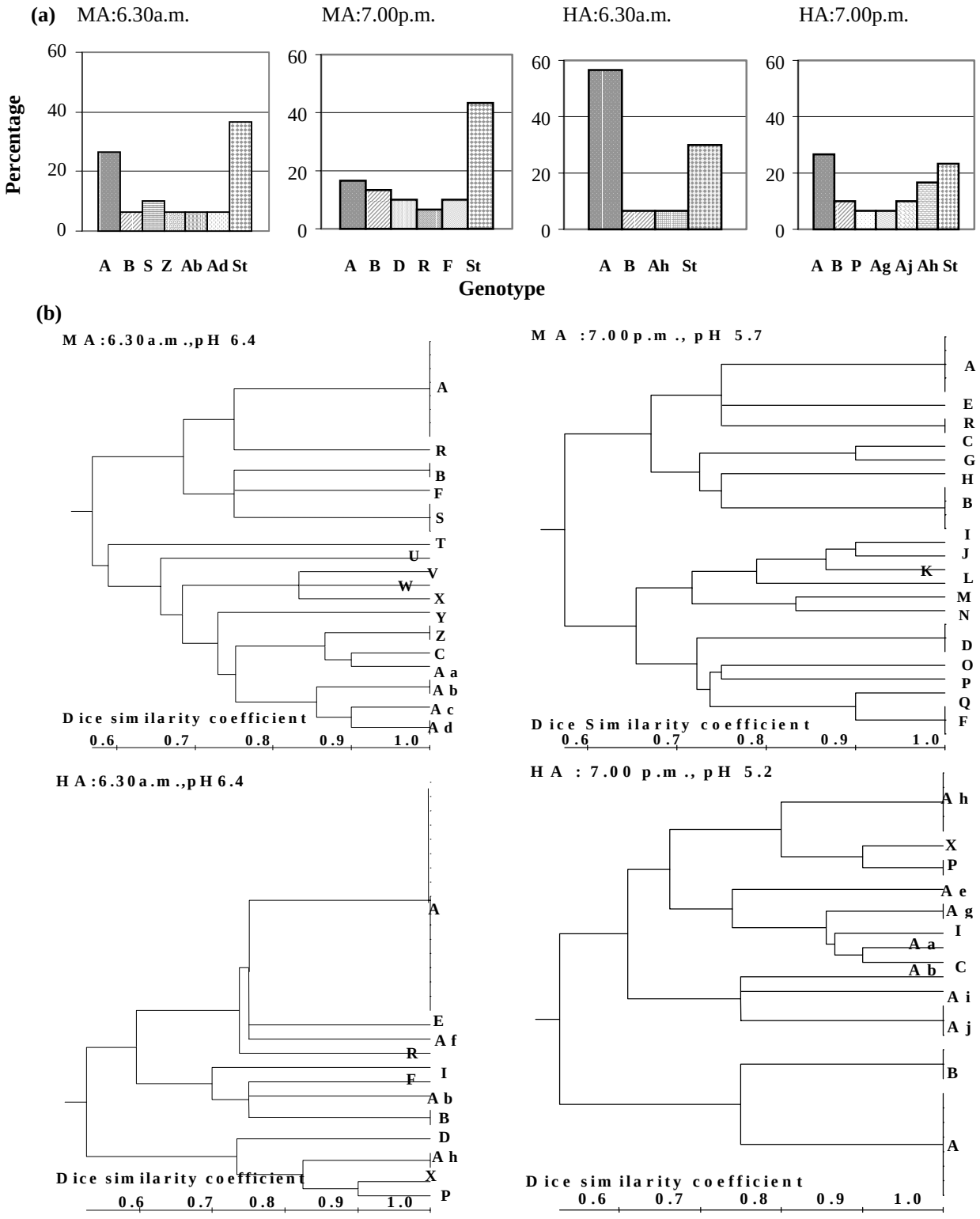

Figure 1. Relationship between 16S rDNA ARDRA types and percentage of clones. (a) Genotype compositions. St, single-type clones, which found only once in 30 clones of the clone library. (b) Dendrograms constructed from ARDRA. Each ribotype is designated by a capital letter

As shown in Figure 1a, ARDRA profile A was the most numerous in the MA cow, being 26.7 and 16.6% of the total clones analyzed at 6.30 am and 7.00 p.m., respectively. Profile A was also numerically dominant in HA cows, as high as 56.6% in the 6:30 am sample and declining to 26.6% in the 7:00 pm sample. Among the other widely distributed ARDRA profiles the F, B, P and Ah patterns were found less in rumen samples of relatively high pH. "Unique" ARDRA profiles were more abundant in the libraries prepared from the MA cow (36.7% and 43.3% of the total clones analyzed so far from 6.30 a.m. and 7.00 p.m. samples, respectively) as compared to the HA cow (30% and 23.3% of total clones from the 6:30 am and 7:00 pm samples, respectively). The relationships among the 30 clones from each rumen sample are shown in Fig. 1b. Using a Dice similarity coefficient of 0.60, 2

main clusters can be roughly distinguished in all samples. The first cluster which includes two main genotypes, A and B, representing about 63.3% and 36.6% of the total clones analysed at 6.30 a.m. and 7.00 p.m. respectively in the HA cow and 33.3% and 29.9% at 6.30 a.m. and 7.00 p.m. respectively in the MA cow. The second cluster appears to be variable and constituted mainly of "unique" clones, which were distinctly different between the low and high pH samples in both cows. The dominating pattern A type was abundant at high pH rumen samples but found less in low pH rumen samples and was consistent in both cows.

DISCUSSION

Although the number of clones in our libraries is relatively small, the ARDRA analysis clearly identified both similarities and differences among the different rumen samples. First, it appears that microbes comprising ARDRA profiles A and B are little affected by either time of sampling, or ruminal pH. These ARDRA profiles were the most numerous in all 4 libraries, and likely represent bacterial species that can adapt readily to fluctuations in ruminal pH and/or type of carbohydrate(s) available for growth. A good candidate would be the ruminal *Prevotellas* which are both acid-tolerant, and capable of growth with both starches and fiber. Despite these similarities, the dendogram analyses indicates that these four rumen samples harbour a different array of microbial species. The low diversity in HA cow, as compared to MA cow, might be explained by the fact that ruminal pH remains low (~5.2) in the HA cow for a much longer period, because of the high intake of highly digestible Persian clover throughout the day. Secondly at high intakes achieved under HA condition, the continuity of intake and the high turnover rate of ruminal digesta result in altered rumen dynamics for fluid and solid fractions (Williams, unpublished data). This in turn may select for a microbial population dominated by relatively few genera, or species, of acid tolerant microbes. Elias *et al.* (1996) found decreased ruminal pH significantly decreased the number of cellulolytic bacteria in dairy cows supplemented with high levels of concentrate, and Suda *et al*. (1997) also reported that a significant decline in ruminal pH in goats fed 90% concentrate plus 10% lucerne hay were also accompanied by a significant decrease in the number of gram-negative cocci.

In conclusion, ARDRA can provide very useful data with regard to microbial diversity and change in community structure in rumen. Additional work to increase the number of clones from each samples and apply nucleotide sequence analysis to clones representing each of the predominant ARDRA profiles is proceeding , to allow the correct assignation of the clones to specific bacterial groupings, and to shed further insight into the species composition for each sample.

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