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Isolation of *Pseudobutyrivibrio ruminis* and *Pseudobutyrivibrio xylanivorans* from rumen of Creole goats fed native forage diet

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Abstract We isolated and identified functional groups of bacteria in the rumen of Creole goats involved in ruminal fermentation of native forage shrubs. The functional bacterial groups were evaluated by comparing the total viable, total anaerobic, cellulolytic, hemicellulolytic, and amylolytic bacterial counts in the samples taken from fistulated goats fed native forage diet (*Atriplex lampa* and *Prosopis flexuosa*). Alfalfa hay and corn were used as control diet. The *roll tubes* method increased the possibility of isolating and 16S rDNA gene sequencing allowed definitive identification of bacterial species involved in the ruminal fermentation. The starch and fiber contents of the diets influenced the number of total anaerobic bacteria and fibrolytic and amylolytic functional groups. *Pseudobutyrivibrio ruminis* and *Pseudobutyrivibrio xylanivorans* were the main species isolated and identified. The identification of bacterial strains involved in the rumen fermentation helps to explain the ability of these animals to digest fiber plant cell wall contained in native forage species.

This paper is drawn from a thesis submitted by Diego Grilli in partial fulfillment of the requirements for a PhD at PROBIOL, Universidad Nacional de Cuyo, Mendoza, in 2012.

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Introduction

The ability to digest fiber observed in some goat breeds exceeds to that observed in other domestic ruminants (Silanikove and Brosh 1989), so the study of ruminal fibrolytic bacteria is important for goat production. The genetic diversity of rumen microbial communities based on 16S rDNA sequences has identified a high diversity of rumen microbes with the majority of them not yet cultured (Edwards et al. 2004). These uncultivated microorganisms are totally unknown in terms of function. To increase our knowledge about functional role of bacterial species on ruminal fermentation, the isolation and identification of microorganisms are required. The methods for identifying functional groups of ruminal bacteria have been widely used for several decades (Stewart and Bryant 1988). These methods involve viable bacteria counts, which consider live and functional bacteria contained in the rumen (Dehority 2003). These functional groups of rumen bacteria are estimated by culturing ruminal samples in habitat-simulating medium containing several different energy sources (Hungate 1966). Most probable number (MPN) procedures have been used rather extensively

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to estimate numbers of cellulose, xylan, pectin, starch-fermenting bacteria in ruminal contents, intestinal contents or feces from cows (Dehority 1966; Dehority 1969), sheep (Grubb and Dehority 1975), buffaloes (Sinha and Ranganathan 1983), steers (Kern et al. 1974), lambs (Fonty et al. 1983), and reindeer (Orpin et al. 1985). The MPN procedures provide a suitable means to study the effects of diet on numbers of ruminal bacteria (Dehority et al. 1989). Although some researchers have isolated and identified bacteria from the feces of Creole goats (Draksler et al. 2004), no previous reports have enumerated or isolated rumen bacteria from the rumen of Creole goats. The aim of this study was to enumerate the predominant functional groups of bacteria involved in ruminal fermentation of native forage shrubs. Furthermore, we established how the nutritional composition of the diet affects the concentrations of these ruminal bacteria groups in Creole goats. The *roll tubes* method increased the possibility of isolating and 16S rDNA gene sequencing allowed definitive identification of some bacterial species involved in the ruminal fermentation of native forage shrubs. This information will be useful in evaluating whether ruminal fermentation can be manipulated to optimize the utilization of plant fiber by Creole goats.

Materials and methods

The trials were conducted in the Scientific and Technologic Center, located in Mendoza City. The techniques and procedures employed were in agreement with the provisions of the Guide for Care and Use of Agricultural Animals in Research and Teaching (Federal Animal Science Society 2010). Four female Creole goats, each fitted with a rumen fistula and weighing about 40 kg, were examined. The goats were 3-year-old and never had an offspring. The functional groups of rumen bacteria were evaluated by comparing total viable bacterial counts (TVBC), total anaerobic bacterial count (TABC), cellulolytic bacterial count (CBC), hemicellulolytic bacterial count (HBC), and amylolytic bacterial count (ABC) from rumen content of fistulated goats fed two different diets. Alfalfa hay and corn grain in a 80:20 ratio were the ingredients of traditional diet, which was used as control diet in this study. *Atriplex lampa* (fam. Amaranthaceae) and *Prosopis flexuosa* (fam. Fabaceae) were the ingredients of the native forage diet. The branches of these shrubs were lopped, weighed, and offered within the corral. Four fistulated goats were routinely fed with alfalfa and corn diet for a period of 90 days. The four goats were fed simultaneously at 1.0× the maintenance level of metabolizable energy content (National Research Council (NRC) 2007) once daily at 0900 hours and had free access to water. Then, four goats were switched to the native forage diet for a period of 30 days. The goats were fed ad libitum and had free access to water. The nutritional composition of the dietary components was determined according to the procedures described in full by the Association of Official Analytical Chemists (2006). The nutritional components measured were dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), and starch. The metabolizable energy (ME) was calculated from the digestibility as recommended by NRC (1985). The dry matter intake (DMI) was calculated from food offered and rejected. The nutrient intake was calculated from the relationship between the amount of DMI and the nutrient content of the food offered.

Leedle et al. (1982) recorded the maximum viable bacterial counts after 16 h of the intake of a high-forage diet (alfalfa and corn grain in a 77:23 ratio), from fistulated steers. Therefore, on the last day of each feeding diet, rumen samples were obtained 16 h after feeding, from fistulated goats. Samples of the whole-rumen contents contain approximately equal proportions of solids and liquid. The rumen contents were homogenized under a CO_2 atmosphere and filtered through two layers of gauze. The strained samples were diluted in a decimal series (10^{-1} to 10^{-10}) with anaerobic mineral solution (Bryant and Robinson 1961). Viable bacteria counts (CBC, HBC, and ABC) were estimated by the MPN procedure using a basal medium with cellulose (filter paper), xylan (birchwood xylan), or soluble starch, as the only source of saccharides, respectively. The composition of the basal medium was similar to medium 10 reported by Caldwell and Bryant (1966), but without agar or saccharides. The methods of media preparation and sterilization and procedures for media tubing, reduction, and inoculation were essentially those described by Bryant and Robinson (1961). After incubation at 39 °C during 7 days, HBC was estimated from the change in the pH of the xylan medium and ABC was measured using Lugol's iodine reaction to determine starch digestion (Persia et al. 2002). After incubation at 39 °C during 15 days, CBC was determined by observing the disappearance of the filter paper. The TVBC were calculated from the sum of CBC, HBC, and ABC.

For isolation, 10^{-6} to 10^{-10} dilutions were inoculated into medium 10 (M10), according to the procedure described by Grubb and Dehority (1976), based on the roll tubes procedure of Hungate (1966). The composition of the M10 was similar to medium previously reported by Caldwell and Bryant (1966). The inoculated roll tubes were incubated at 39 °C during 5 days and the colony-forming units (CFU) were counted under a dissecting microscope and used to determine TABC. Well-isolated colonies from the roll tubes with the highest dilution of bacteria showing growth were stabbed on slant M10 medium. The strains obtained were subsequently re-isolated and tested for growth in M10 medium. Organisms from cultures showing growth were

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microscopically examined for morphology, motility, and homogeneity and were also Gram stained.

The pure isolates were cultivated in basal medium, without ruminal fluid plus 0.5 % glucose and 7 % VFA mixture reported by Caldwell and Bryant (1966). The bacterial strains were cultured in this medium overnight at 38 °C. The bacterial culture was pelleted by centrifugation (5,000×*g*, 5 min), suspended in 3 mL of 5 mmol Tris–EDTA, centrifuged (5,000×*g*, 5 min), and stored at −20 °C. Bacterial DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, USA). Purified DNA was eluted from the silica membrane in a concentrated form and it was stored at −20 °C. The DNA concentrations were measured at 260 nm with a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). The DNA used for these experiments had an A_{260}/A_{280} ratio greater than 1.8. A 1,500-bp 16S rDNA fragment was amplified in a reaction volume of 20:10.5 μL of molecular-grade water, 4 μL of reaction buffer, 1.6 μL of dNTP mix, 1.5 μL of each forward (27F 5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse (1540R 5′-AGAAAGGAGGTGATCCAGCC-3′) primers (Tajima et al. 1999), 0.4 μL of $MgCl_2$, 0.1 μL of DNA Polymerase (GoTaq DNA Polymerase Kit, Promega, USA), and 1 μL of extracted DNA. The PCR conditions were as follows: 30 s at 94 °C for denaturing, 30 s at 50 °C for annealing, and 2 min at 72 °C for extension (20 cycles). In the first cycle, 5 min was used for denaturing and at the last one 10 min was used for extension. The amplified products were separated by electrophoresis on agarose gel (1.5 %) and stained with ethidium bromide. Products were excised from the gel and purified with illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, UK) in accordance with the manufacturer's recommendations. Purified PCR products were directly transferred into the TA Cloning Kit with One Shot TOP10 competent cells (Invitrogen, USA) according to the protocol of the manufacturer. The recombinant plasmids were then extracted and purified by the illustra plasmidPrep Mini Spin kit (GE Healthcare, UK) as stated by manufacturer's protocol. Purified plasmids were sequenced with the *Taq* Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Germany) as stated by manufacturer's protocol.

After editing, 16S rDNA sequences were compared with published sequences of related bacteria from the EMBL and GenBank nucleotide databases. The sequences were aligned with MUSCLE configured for the highest accuracy (Edgar 2004). Gblocks program was used to eliminate poorly aligned positions and divergent regions. The phylogenetic tree was constructed using the neighbor-joining method implemented in Neighbor from the PHYLIP package (Felsenstein 1989). The number of bootstrap was set at 1,000. Branches with a bootstrap support under 50 % were collapsed. Distances were calculated using FastDist using the K2P substitution model (Elias and Lagergren 2007). Graphical representation and edition of the phylogenetic tree have been performed with TreeDyn (Chevenet et al. 2006). The nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession numbers JN619348 (*Pseudobutyrivibrio ruminis*) and JQ673415 (*Pseudobutyrivibrio xylanivorans*).

The data for all parameters were calculated from duplicate determinations and then averaged across the replicate experiments for each animal on each diet. The term "replicated experiments" indicates a different sample of ruminal contents, from four goats on the same day. Mean values±SE were presented. Data were analyzed by Infostat statistical program (Di Rienzo et al. 2011). The bacterial counts were transformed to log10 for further ANOVA, followed by Tukey's HSD procedure ($P<0.05$). The DMI and nutrient intake of the diets were compared by ANOVA followed by Fisher's LSD procedure ($P<0.05$).

Results

The nutritional composition of the dietary components is reported in Table 1. The CP content in the three forages did not differ significantly, but the content of NDF and ADF of *P. flexuosa* were significantly higher ($P<0.05$) than *A. lampa* and alfalfa hay. *A. lampa* provided a significantly lower ($P<0.05$) content of LAD to diets compared with *P.*

Table 1 Nutritional composition (percent DM) of the dietary components of Creole goats

	Dietary components			
	P. flexuosa	*A. lampa*	Alfalfa hay	Corn
DM, %	40	31	80	88
ME, MJ/kg	6.15±0.4 a	8.07±0.4 b	8.20±0.4 b	12.09±0.4 c
CP	10.5±1.7 ab	11.1±1.1 ab	14.6±1.1 a	7.9±0.4 b
NDF	61.9±0.6 a	45.1±2.2 b	44.1±3.1 b	8.2±0.3 c
ADF	42.2±4.3 a	25.0±0.1 b	32.8±2.8 b	2.9±0.1 c
ADL	6.3±0.1 a	1.9±0.6 b	5.5±1.5 a	0.8±0.1 b
Starch	1.0±0.1 a	0.7±0.2 a	2.3±0.1 b	62.9±0.4 c

Within a row, means±SE without a common letter indicate difference ($P<0.05$)

DM dry matter, *ME* metabolizable energy, *CP* crude protein, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *ADL* acid detergent lignin

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flexuosa and alfalfa hay. The corn was dietary component that contributed significantly higher ($P<0.05$) content of starch and ME to diets. Table 2 shows the daily intake of DM, ME, and nutrients by Creole goats fed two different diets. The intake of DM, CP, and ME of both diets was sufficient to meet energy and protein requirements for goats at maintenance (National Research Council 2007). There were no differences in DMI and ADL intake between the two diets. Goats fed traditional diet had a greater ($P<0.05$) intake of CP, starch, and ME compared with goats fed native forage diet. The native forage diet was greater ($P<0.05$) in NDF and ADF. The intake of starch in traditional diet was almost 17 times greater ($P<0.05$) than intake of starch with native forage diet, which is the main difference between diets consumed by these goats. Table 3 shows the concentration of viable and total anaerobic bacteria from rumen of Creole goats fed two different diets. All counts of viable bacteria decreased ($P<0.05$) when goats were fed native forage diet. Predominant bacteria in goats fed traditional diet were hemicellulolytic and amylolytic bacteria, which represented 49 and 50 %, respectively, of the total viable bacteria cultured from the ruminal contents of these goats, whereas cellulolytic bacteria represented less than 1 % of the total viable bacteria. The total population density of viable bacteria cultured in the rumen of goats fed native forage diet was only 9 % of the total population density in traditional diet. The combined population density of fiber-digesting species, those digesting cellulose and xylan, was $815\pm15\times10^8$ cells per mL in traditional diet and $138\pm15\times10^8$ cells per mL in native forage diet. These values represent 49 and 96 % of the total population density of bacteria cultured in traditional and native forage diets, respectively. The hemicellulolytic bacteria group represented the predominant bacterial group in rumen of goats fed native forage diet. The TABC revealed an even greater difference between two diets (Table 3). In goats fed native forage diet, TABC were significantly lower ($P<0.05$) compared with corresponding counts for traditional diet.

Table 2 Nutrients intake and ME of the two different diets fed to Creole goats

	Native forages	Alfalfa hay–corn
DMI (g per day)	1,360±13 a	1,450±86 a
CP (g DM per day)	146±10 a	191±3 b
NDF (g DM per day)	731±5 a	532±10 b
ADF (g DM per day)	457±10 a	385±4 b
ADL (g DM per day)	57±1 a	64±6 a
Starch (g DM per day)	12±1 a	209±12 b
ME (MJ/kg)	9.6±0.4 a	13.0±0.8 b

Within a row, means±SE without a common letter indicate difference ($P<0.05$)

DM dry matter, *DMI* dry matter intake, *CP* crude protein, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *ADL* acid detergent lignin, *ME* metabolizable energy

Table 3 Concentration of viable (10^8 cells per mL) and total anaerobic (10^8 CFU/mL) bacteria in the rumen contents from Creole goats fed two different diets

	Native forage diet	Traditional diet
TABC	4.7±0.3 a	2630±68 b
TVBC	143±13.9 a	1650±94 b
CBC	0.003±0.001 a	9±0.4 b
HBC	138±14.7 a	806±15 b
ABC	4.8±0.9 a	835±58 b

TABC total anaerobic bacteria counts, *TVBC* total viable bacterial counts, *CBC* cellulolytic bacterial counts, *HBC* hemicellulolytic bacterial counts, *ABC* amylolytic bacterial counts

Within a row, means±SE without a common letter indicate difference ($P<0.05$)

Most colonies isolated from the rumen of goats fed traditional diet were classified as Gram-positive, strict anaerobe cocci; according to the morphology and Gram stain. Several colonies from the rumen of goats fed native forage diet were picked from roll tubes with the highest dilution showing growth. Material from well-isolated colonies was picked and was stab-inoculated into RGCA slant medium. Colonies were selected from roll tubes as randomly as possible, and as many colonies as possible were picked from the same *roll tube*. A total of nine isolates were chosen on the basis of morphology and the Gram stain for further study. Seven cultures either were lost or impure. Two cultures that grew well (strains 2 and 153) were Gram-negative, strict anaerobe, slightly curved rods, and did not produce spores. Phylogenetic analysis (Fig. 1) shows strains 2 and 153 to fall within XIVa cluster of the Gram-positive bacteria (Collins et al. 1994). The dendrogram, based on 16S rDNA sequence comparisons, shows that strains 2 and 153 are most closely related to *P. xylanivorans* and *P. ruminis*, respectively.

Discussion

Goats fed native forage diet had lower counts of total anaerobic bacteria and fibrolytic and amylolytic functional groups. The reduced starch intake by goats fed with this diet may explain these results. A variety of ruminal bacteria have the ability to

Fig. 1 Phylogenetic tree showing relationships of 16S rDNA sequences of different isolates of *Butyrivibrio* spp. and *Pseudobutyrivibrio* spp. *Bar*, three substitutions for each 100 nucleotides. The tree was constructed using neighbor-joining analysis of a distance matrix obtained from a multiple-sequence alignment. *Clostridium oroticum* was used as an outgroup sequence. *Asterisk:* Code of the individual strain. *Double asterisk:* Access number of the nucleotide sequences 16S rDNA ▶

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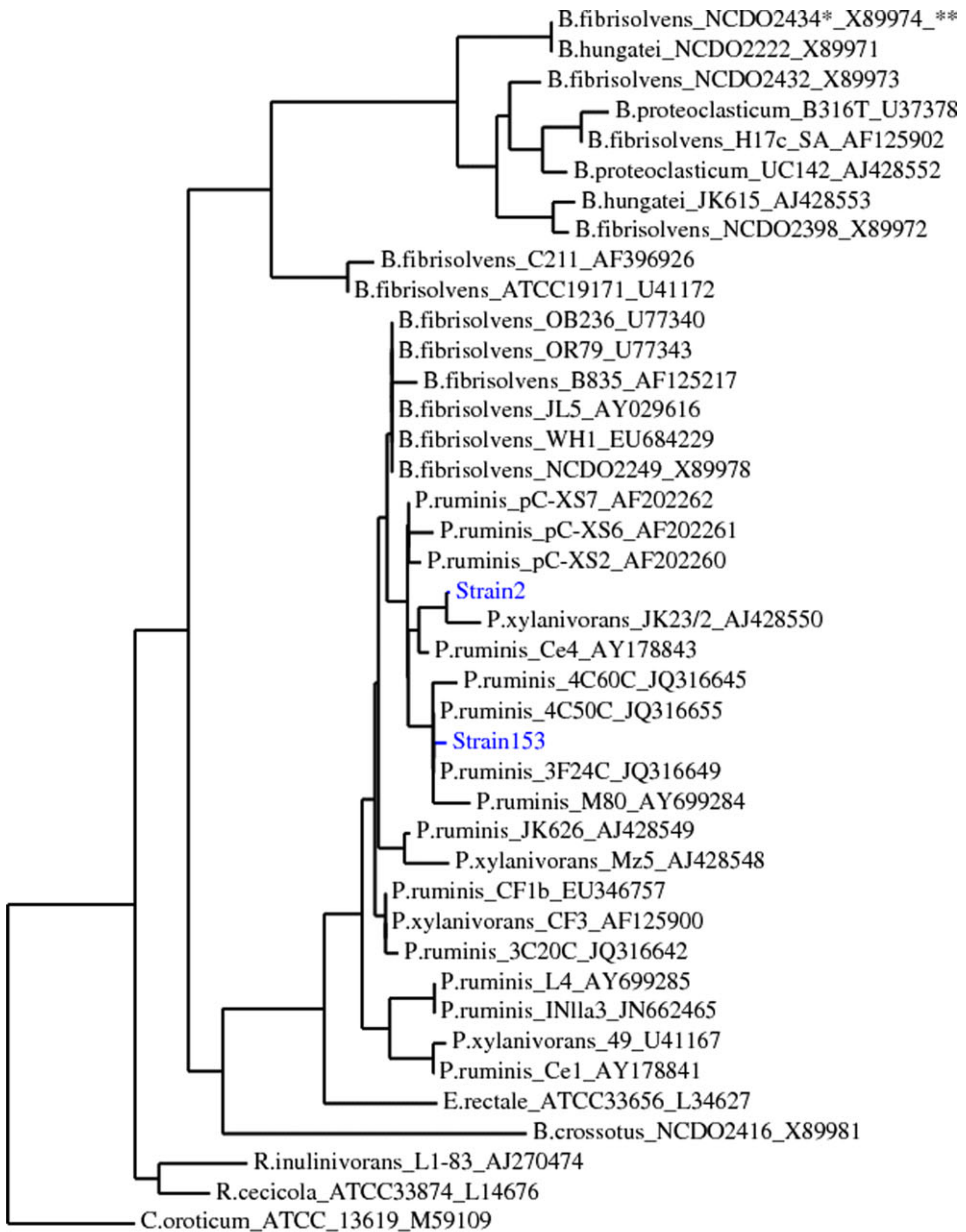

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utilize starch and other soluble saccharides as a growth substrate and are also involved in the digestion of cellulose and hemicellulose (Cotta 1988). The presence of tannins and other anti-nutritional factors in native forage shrubs can affect bacterial populations (Frutos et al. 2004). *A. lampa* possesses high salt and oxalic acid content, not palatable and symbiosis with alkalitolerant *Bacillus patagoniensis. P. flexuosa* contains high in phenols and tannins. This could explain the lower counts of rumen bacteria with native forage diet, but this effect has not been studied in this work. The present results cannot be directly compared to ruminal bacteria counts obtained by many other workers because of host species differences and type and amount of feed as well as differences in microbiological techniques and components in the media. The bacterial groups that predominated in goats fed traditional diet were hemicellulolytic and amylolytic bacteria, whereas cellulolytic bacteria represented a very low proportion of the total viable bacteria. Hungate et al. (1952) observed significant changes in the ruminal microbiota when sheep fed on alfalfa hay and corn diet. A rapid increase in the numbers of amylolytic bacteria and a decrease in the numbers of other kinds of bacteria were noted in diets containing grains. The greatest ABC coincided with the predominance of Gram-positive cocci obtained from the rumen of goats fed traditional diet. *Streptococcus bovis* would be included in the group of Gram-positive cocci which predominate in ruminants fed high starch diets (Hungate et al. 1952; Orpin et al. 1985), since *S. bovis* is involved in the fermentation of starch and other soluble saccharides in the rumen (Hungate 1966).

The hemicellulolytic bacteria group represented a major part of total bacteria in goats fed native forage diet. The roll tubes method allowed isolation of pure cultures of two ruminal bacteria, strains 2 and 153, from the rumen of goats fed diet based on native forage shrubs. In combination with phenotypical data, the 16S rDNA sequence data support the designation of strain 2 as *P. xylanivorans* and strain 153 as *P. ruminis.* The rumen bacteria *P. xylanivorans* and *P. ruminis* exhibit an enzyme system specialized in the degradation of hemicellulose within the rumen ecosystem (Dalrymple et al. 1999; Čepeljnik et al. 2006). This could indicate a possible role of *P. xylanivorans* 2 and *P. ruminis* 153 in the digestion of plant fiber in native forage diet. Therefore, we cannot exclude the presence of other rumen microorganisms involved in the digestion of fiber from the rumen of goats fed diet based on native forage shrubs.

Native forage diet allows the isolation of strains related to *Butyrivibrio fibrisolvens*, such as *P. ruminis* (van Gylswyk et al. 1996) and *P. xylanivorans* (Kopečný et al. 2003). *Butyrivibrio*-like microorganisms represent a significant proportion of culturable rumen bacteria in domestic and wild ruminants fed poor-quality forage diets (Orpin et al. 1985; Forster et al. 1996). These organisms ferment a wide variety of plant saccharides allowing maintaining high enzymatic activity under conditions of low nutrient intake (Orpin et al. 1985; Cheng et al. 1993). The identification of bacterial strains involved functionally in the rumen fermentation helps to explain the ability of these animals to digest fiber plant cell wall contained in native forage species. The strains of *P. ruminis* and *P. xylanivorans* possess many traits that could potentially have beneficial effects in rumen (Dalrymple et al. 1999; Zorec et al. 2001; Čepeljnik et al. 2003). For these reasons, strains of *B. fibrisolvens* and *P. ruminis* have been targeted to improve its ability to degrade plant fiber prior to reintroduction into the rumen (Dalrymple and McSweeney 1998; Dalrymple et al. 1999). *P. xylanivorans* has a potent xylanolytic enzymatic system (Čepeljnik et al. 2004), which favors its use as probiotic bacterium (Čepeljnik et al. 2003).

We conclude that species composition of the rumen microbial community changed under different feeding regimens, particularly when traditional diet was switched to native forage shrub diet. The hemicellulolytic bacteria group represented the major part of total bacteria in goats fed native forage diet. The strains of *P. ruminis* and *P. xylanivorans* could have a functional role in the digestion of hemicellulose, although further studies will be required to increase knowledge of the characteristics of the strains isolated from the rumen of Creole goats. This information will be useful in evaluating whether ruminal fermentation can be manipulated to optimize the utilization of plant fiber in Creole goats. This, in turn, should improve the efficiency of meat and milk production by these ruminants in arid systems.

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