

INFORME BREVE

Detection of fiber-digesting bacteria in the forestomach contents of llamas (*Lama glama*) by PCR

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Abstract

The high fibrolytic activity and large biomass of strictly-anaerobic bacteria that inhabit the rumen makes them primarily responsible for the degradation of the forage consumed by ruminants. Llamas feed mainly on low quality fibrous roughages that are digested by an active and diverse microflora. The products of this fermentation are volatile fatty acids and microbial biomass, which will be used by the animals. The aim of this study was to detect the three major fiber-digesting anaerobic bacteria in the forestomach contents of llamas by PCR. In this study, we detected *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* in the forestomach contents of eight native llamas from Argentina.

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PALABRAS CLAVE

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Detección de bacterias que digieren fibra en el contenido preestomacal de llamas (*Lama glama*) por PCR

Resumen

La alta actividad fibrolítica y la gran biomasa de las bacterias anaerobias estrictas que habitan el rumen las hacen las principales responsables de la degradación del forraje consumido por los rumiantes. Las llamas se alimentan sobre todo de forrajes fibrosos de baja calidad, que son digeridos por una activa y diversa microflora. Los productos de esta fermentación son ácidos grasos volátiles y biomasa bacteriana, los cuales serán utilizados por el animal. El objetivo de este estudio fue detectar las tres principales bacterias anaerobias que digieren fibra en el contenido del preestómago de llamas por PCR. En este estudio, detectamos *Ruminococcus albus*, *Ruminococcus flavefaciens* y *Fibrobacter succinogenes* en el contenido del preestómago de ocho llamas nativas de Argentina.

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The llama (*Lama glama*) is one of the two domestic species of South American Camelids (SACs) together with the alpaca (*Lama pacos*), while the guanaco (*Lama guanicoe*) and the vicuña (*Vicugna vicugna*) are wild species. Similarly to Old World Camels (*Camelus dromedarius* and *Camelus bactrianus*), SACs have a complex three-compartment stomach (C-1, C-2 and C-3), which comprises 83, 11 and 6% of the total volume of the stomach respectively; they are classified as pseudo-ruminants or false ruminants⁵. Camelids regurgitate and re-chew ingested forage as true ruminants. However, camelids are more efficient in extracting protein and energy from poor-quality forages⁴. In addition, the presence of glandular sacs in the stomach allows an efficient mashing, mixing and absorption of digesta. Like in ruminants, the forestomach of SACs have a highly complex microbial community, which comprises an undetermined number of species of protozoa, fungi, archaea and bacteria that combines to breakdown plant material. This community changes according to the health, age, the season of the year, the use of therapeutic and promoting antibiotics and, most importantly, to the diet of the host animal. Great efforts toward the isolation of rumen anaerobic bacteria were made and molecular techniques have allowed recognizing a predominance of uncultured bacteria in the rumen. The forestomach contents of SAC in general and of llama in particular are poorly described. Few reports have been published on the detection or bacterial isolation of the digestive tract of llama². Previous studies have determined the distribution of the rumen bacterial population based on 16S rRNA clone libraries from different ruminants, 55-71% belong to Firmicutes, 25-42% to Bacteroidetes and 0-10% to other phyla^{3,9,11}. The majority (77%) of fiber-associated community members are uncultured bacteria, while 17% of cloned bacterial 16S rRNA gene sequences were classified as known fibrolytic species such as *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens*⁷. Our current research focuses on three species of bacteria, which are considered to be principal fibrolytic bacteria in the rumen of ruminants: *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*⁶. Because of the complexity involved in the isolation of fiber-digesting bacteria, we used a molecular approach based on PCR to study fibrolytic microorganisms.

The llamas were sampled by trained personnel and specialized veterinarians following INTA's Animal welfare protocols and international guidelines on the care and use of farm animals in research, teaching and testing¹.

In this study, the forestomach contents (compartment C-1) were obtained from three adult male llamas slaughtered (estimated body weight, ~ 120 kg) during an annual sanitary program in the community of Cieneguillas, Jujuy, 22°08'15''S, 65°08'12''W (3800 m altitude) in the Altiplano in northern Argentina. In addition, the forestomach contents from five adult male llamas (estimated body weight, ~ 100 kg) were collected by esophageal tube in Buenos Aires, 34°36'12''S, 58°40'32''W (43 m altitude). Llamas from Jujuy fed on native plants based mainly in *Festuca argentinensis ad libitum* whereas llamas from Buenos Aires fed on alfalfa hay (2% body weight). The forestomach content sample (40 ml) was filtered through a double layer of gauze to remove particulate matter, was immediately frozen using dry ice and then kept at 80 °C until processing. Total DNA extraction was performed with the QIAamp DNA Stool Kit (Qiagen, Germany). Species-specific primer sets that amplify 16S rRNA of *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* are available to detect these species in gut microbial ecosystems^{6,10}. The PCR mixture was performed using 1X PCR buffer (60 mM Tris-SO₄ pH 8.9, 18 mM ammonium sulphate), 0.25 mM each dNTPs, 2 mM MgSO₄, 0.2 mM each primer, 1U of Platinum Taq High Fidelity (Invitrogen, USA), 20 ng of genomic DNA and DNA/RNA free water adjusted to a total volume of 50 µl. The PCR condition was 95 °C 5 min followed by 30 cycles of 94 °C 30 sec for denaturing, annealing at different temperatures (Table 1) for 30 sec and finally 68 °C 45 sec for elongation, using a PxE 0.2 thermal cycler (Thermo electron corporation, USA). The PCR products were separated by 2% agarose gel electrophoresis using the molecular weight marker 100 bp Ladder (Promega USA), stained with SYBR Safe (Invitrogen, USA) and the image was captured with a gel image analyzer (Uvitec, Cambridge, UK).

The DNA fragments of the expected size (Table 1) were amplified from all the samples tested, irrespective of the diet and the geographical location. A representative image of the amplification after gel electrophoresis is shown in Figure 1.

To confirm the specificity of the amplification, the PCR products were purified and three clone libraries were constructed in *Escherichia coli* using the vector TOPO TA cloning Kit (Invitrogen, USA). Plasmid DNA from several clones of each library was purified, sequenced and analyzed using BLAST (<http://blast.ncbi.nlm.nih.gov>). As expected,

Table 1 Species-specific primers sequences for 16S RNA genes used in this study

Bacterium	Primer name	Sequence (5' - 3')	Annealing temp. (°C)	Product size (bp)	Ref.
<i>Ruminococcus albus</i>	Ra1281 f	CCCTAAAGCAGTCTTAGTTCG	60	175	6
	Ra1439 r	CCTCCTTGCGGTTAGAACA			
<i>Ruminococcus flavefaciens</i>	Rf154 f	TCTGGAACGGATGGTA	60	295	6
	Rf425 r	CCTTTAAGACAGGAGTTTACAA			
<i>Fibrobacter succinogenes</i>	Fs219 f	GGTATGGGATGAGCTTGC	62	446	10
	Fs654 r	GCCTGCCCTGAACATC			

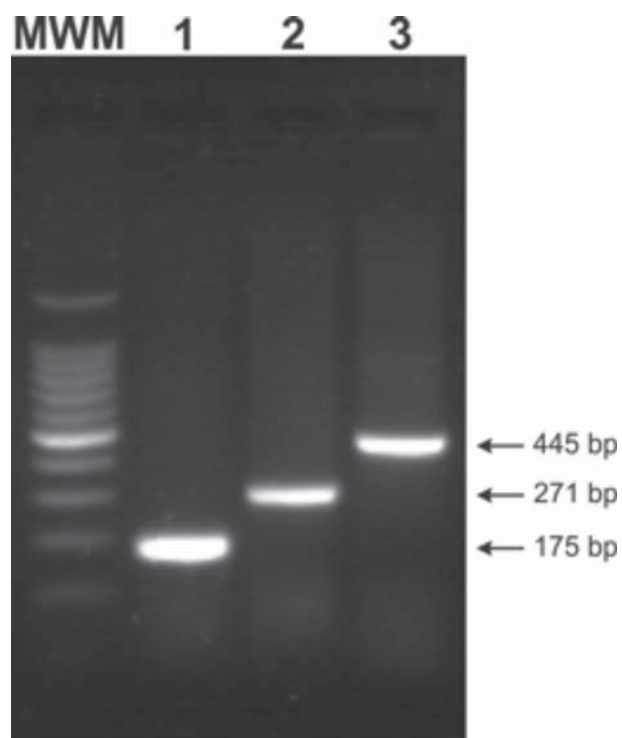


Figure 1 PCR detection of fibrolytic bacteria from the forestomach contents of llamas using species-specific primers for 1: *Ruminococcus albus*; 2: *Ruminococcus flavefaciens*; 3: *Fibrobacter succinogenes*. MWM: 100 bp ladder molecular weight marker.

BLAST hits confirmed the specificity of the amplification (data not shown).

In previous studies, fibrolytic bacteria have been isolated or detected from the gastrointestinal tract or feces of ruminants, horses, pigs, rats, rabbits, gorillas and ostriches⁸. The present study extends the host range of the habitat of these bacteria showing that *Fibrobacter* sp. and *Ruminococcus* sp. are common constituents in the anaerobic environments of the forestomach contents of llamas herein mentioned.

Like rumen, the forestomach houses a complex ecosystem that includes a high genetic microbial diversity with a vast array of metabolic functions. Fiber-digesting bacteria such as those described in this study are rich in enzymes capable of degrading complex polysaccharides present in the diet as cellulose, hemicellulose and pectin. The characterization of cellulolytic microorganisms for advanced ethanol production from lignocellulosic materials has the potential to provide a sustainable alternative to the global energy crisis. Thus, these bacteria could be a source of new hydrolytic enzymes for the biofuel industry. We conclude that the three major fiber-digesting anaerobic bacterial species are present in the forestomach contents of llamas. To the best of our knowledge, this is the first report on the presence of *Fibrobacter* sp. and *Ruminococcus* sp. in the forestomach contents of SACs.

This initial characterization provides evidence that the isolation of these bacteria from the contents of C-1

compartment of llamas is feasible, which will allow a phenotypic and genetic characterization.

Currently, we are analyzing the diversity of the amplified DNA through cloning and sequencing in order to define the presence of new phylotypes of these fibrolytic bacteria detected in llamas. Attempts to obtain the isolates are also under way.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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