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Quantitative analysis of rumen microbial populations by qPCR in heifers fed on *Leucaena leucocephala* in the Colombian Tropical Dry Forest

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ABSTRACT. Rumen fermentation and methanogenesis are vital metabolic processes in cattle and are carried out by microbial populations that are affected by dietary factors such as secondary metabolites, nutritional composition and degradability. The aim of this study was to monitor populations of total bacteria, total methanogens and *Butirivibrio fibrisolvens* in the rumen of Lucerne heifers fed on diets typical of intensive silvopastoral systems (ISS) or of a traditional (control) system. Rumen contents (RC) were collected orally from eight heifers consuming 100% *Cynodon plectostachyus* (control) and 76% *C. plectostachyus* + 24% *Leucaena leucocephala* (ISS) following a crossover design and DNA was extracted and quantified by quantitative PCR. Populations [Log10 (ng g^{-1} RC)] were 5.6 and 5.8 for total bacteria (p = 0.5343), 3.6 and 3.5 for *B. fibrisolvens* (p = 0.4742) and 5.0 and 5.3 for total methanogens (p = 0.2661) respectively in control and ISS diets. However, when measured in a separate parallel study, enteric methane emissions (g kg^{-1} of fermented dry matter) were significantly reduced with the inclusion of *L. leucocephala*. This fact indicated the importance of investigating the structure, function and interactions of populations beyond quantitative analysis to determine how diet affects rumen microbial populations and their function.

Keywords: bacteria, diversity, legume, methanogens, Real Time Polymerase Chain Reaction (RT-PCR).

A análise quantitativa de populações microbianas ruminais por qPCR em novilhas alimentadas com *Leucaena leucocephala* na Colômbia Tropical Dry Forest

RESUMO. A fermentação ruminal e metanogênisis são processos metabólicos vitais para os rumianteso e são realizados por populações microbianas afetados por fatores dietéticos, como a presença de metabólitos secundários, composição nutricional e degradabilidade. O objetivo deste estudo foi monitorar as populações de bactérias totais, metanogênicas e *Butyrivibrio fibrisolvens* no rúmen de novilhas Lucerna alimentadas com dietas típicas de sistemas silvopastoris intensivos (SSI) ou de um sistema tradicional (controle). Conteúdo do rúmen (CR) foram recolhidos por via oral de oito novilhas consumindo 100% *Cynodon plectostachyus* (controle) e 76% *C. plectostachyus* + 24% *Leucaena leucocephala* (SSI), após um estudo cruzado o DNA foi extraído e quantificado por PCR quantitativo. Populações [Log10 (ng g^{-1} CR)] foram de 5,6 e 5,8 para as bactérias totais (p = 0,5343), 3,6 e 3,5 para *B. fibrisolvens* (p = 0,4742) e 5,0 e 5,3 para o total metanogênicas (p = 0,2661) para o controle e dietas ISS, respectivamente. No entanto, quando medida em um estudo paralelo, as emissões de metano entérico (g kg^{-1} de matéria seca fermentada) foram significativamente reduzidas com a inclusão de leucena. Isso indica a importância de investigar a estrutura, função e interações de populações além da análise quantitativa para determinar como a dieta afeta populações microbianas no rúmen e sua função.

Palavras-chave: bactéria, diversidade, leguminosa, metanigênisis, Tempo real Reação em Cadeia da Polimerase (RT-PCR).

Introduction

The sustainability of livestock production demands the use and evaluation of strategies that increase its energetic efficiency, reduce its environmental impact and increase its contribution towards food security (Chará et al., 2011). Intensive silvopastoral systems (ISS) have recently become one of the most promising strategies to naturally intensify the Colombian cattle industry. Besides its production potential, ISS may mitigate the effects of climate change and make livestock agroecosystems more adapted to this phenomenon (Cuartas et al., 2014; Murgueitio et al., 2011). A successful ISS system for tropical conditions includes the use of

high density (5000-40000 shrubs ha^{-1}) of the forage shrub *L. leucocephala*, associated with grasses such as *C. plectostachyus* (stargrass) under a rotational grazing scheme with 12 - 24-hour periods of occupancy (Chará et al., 2011). The ISS system has shown great potential for the production of meat and milk, for the increase in energy efficiency and the provision of environmental benefits in terms of biodiversity, water availability (Calle et al., 2013), carbon sequestration from 4.4 to 22.4 ton CO_2 eq Ha^{-1} yr^{-1} (Calle et al., 2013) and, according to Naranjo et al. (2012), ISS removal of GHG from the atmosphere in amounts ranging between 8.8 and 26.6 ton CO_2 eq Ha^{-1} yr^{-1}, alone or associated with timber trees, respectively.

Rumen fermentation is a major source of greenhouse gases and represents energy losses of 2 to 12% of gross feed energy (Johnson & Johnson, 1995). Rumen methanogenesis, a feed fermentation process associated with methanogenic microorganisms, depends on complex interactions with other microbial populations (bacteria, fungi and protozoa), diet and animal intrinsic factors such as retention time of particles in the rumen and host feed efficiency (Morgavi et al., 2010). Due to its high nutritional quality, palatability, ability to fix nitrogen and tolerance to drought and grazing, Leucaena is considered an appropriate species for sustainable intensification of tropical livestock production (Garcia et al., 1996). Further, the dietary inclusion of Leucaena, *in vitro* (Molina et al., 2013) and *in vivo* (Abdalla et al., 2012; Possenti et al., 2008), has shown its potential to reduce methane emissions in cattle, which is attributable to a modulating effect on rumen microbial populations. Huang et al. (2011) suggested a direct effect of condensed tannins from Leucaena with higher molecular weight on CH_4 production with anti-methanogenic activity. On the other hand, when availability of nutrients increases and there are secondary metabolites, such as condensed tannins, methane production will be reduced (Galindo et al., 2008; Moreira et al., 2013).

Exploring changes in the structure, function and diversity of the rumen's microbial populations in response to dietary changes is important within the integral assessment of strategies to mitigate and adapt to climate change (Kittelmann et al., 2013). The employment of phylogenetic analysis tools is one way to accomplish this end. They are derived from the genomic sequencing of the 16s rDNA region which enables the characterization of microbial communities in complex ecosystems such as the rumen (McSweeney et al., 2007).

Among the Colombian native and crossbreed cattle, the Lucerna cattle have received attention due to their milk production and superior reproductive performance. However, few studies have been carried out to describe their ruminal microbial populations. The current study monitored through quantitative PCR (qPCR) the changes in the populations of total bacteria, total methanogens and the proteolytic bacteria *B. fibrisolvens* in the rumen of Lucerne heifers which received diets with and without the inclusion of *L. leucocephala*.

Material and methods

Location

The study was conducted at the Natural Reserve "El Hatico" in the municipality of Cerrito, Valle del Cauca, Colombia, at 3° 27' N and 76° 32' W. According to Holdridge, its ecological classification corresponds to Bs-T (Espinal, 1992). The reserve is located at 1000 m.a.s.l. and its average temperature, relative humidity and annual rainfall are 24°C, 75% and 750 mm, respectively.

Animals and diet

Eight Lucerne breed heifers with an initial weight of 218 ± 18 kg and 19 months old were included in the study following a crossover design with two treatments and two replicates in time. The animals were housed in 3 x 3 m individual wooden pens.

The diets evaluated were: 100% stargrass (*C. plectostachyus*) and 76% stargrass (*C. plectostachyus*) plus 24% Leucaena (*L. leucocephala*), mineralized salt and water were available *ad libitum*, Table 1 shows the nutritional composition of the diets. Adaptation to the diet comprised 10 days for each period.

Table 1. Nutritional composition of the diets offered in the four experimental periods.

Analysis	Treatment 1*	Treatment 2
Protein, %	10.78 ± 1.13	13.90 ± 0.90
NDF, %	74.60 ± 1.53	64.85 ± 1.43
ADF, %	42.95 ± 0.25	41.90 ± 2.04
Fat, %	1.16 ± 0.23	1.17 ± 0.09
Calorific value, Cal g^{-1}	4282 ± 47	4366 ± 23
Ash, %	10.18 ± 0.48	9.63 ± 0.50
Calcium, %	0.37 ± 0.05	0.42 ± 0.06
Phosphorus, %	0.33 ± 0.03	0.33 ± 0.05

*Treatment 1 = 100% C. plectostachyus; Treatment 2 = 76% C. plectostachyus + 24% L. leucocephala.

Forages were harvested at a regrowth age of 45 days. The stargrass was harvested from different paddocks, with and without legumes, depending on treatment. Diet was provided in four daily meals offered at 8.00, 11.00 am, 2.30 and 6.00 pm. Dry matter intake (DMI) was determined as the difference between feed offered and rejected, which were weighed daily; nutrient intake was estimated by taking into account DMI and feed nutrient content.

Sampling of rumen contents

Samples of rumen contents were collected by a ruminal probe at the end of each experimental period (Martín et al., 2005) to analyze quantitatively microbial populations by real-time PCR (qPCR). Samples were stored at -20°C for subsequent lyophilization and total DNA extraction.

DNA extraction

Total DNA was extracted with methodology described by McSweeney et al. (2007) and DNA isolation was carried out with a modified method by Denman et al. (2007). Briefly, an aliquot (1·5 mL) from the preserved sample was placed in the 2 mL capacity screw-capped microcentrifuge tube containing 1 g of zirconium beads (1 mm; BioSpec Products, Inc., Roth, Karlsruhe, Germany). One milliliter of lysis buffer (2% SDS, 100 mM-Tris-HCl, 5 mM-EDTA, 200 mM-NaCl) was added and the tubes were placed in liquid nitrogen (-196°C) for 1 min. The sample tubes were then thawed at room temperature and vortexed for 2 min. The freeze–thawing step was repeated twice to enhance the efficiency of DNA extraction. Thereafter, the samples were incubated at 48°C for 10 min. and then centrifuged at 14 000 *g* for 15 min. at 48°C. The supernatants (0·3 mL in duplicate) were transferred to 1.5 mL-Eppendorf tubes and an equal volume of the glass milk (5 g silica in 30 mL guanidine isothio- cyanate (3M), pH 6·0-6·5) was added. The tubes were shaken gently for 5 min. to allow DNA bonding. The glass milk suspension was then centrifuged at 10 000 *g* for 1 min. and then washed with aqueous cold ethanol (70:30, ethanol:nanopore water, v v^{-1}). DNA was finally eluted with 100 mL of PCR grade water (Sigma) and stored at -20°C until analysis. The purity and concentration of DNA in the extracted sample was determined with a nanospectrophotometer A260/A280.

Quantitative analysis of populations by qPCR

Microbial DNA was amplified from total DNA with primers specific for each population. In the case of methanogens, the mrcA (methyl coenzyme-M reductase; Denman et al. (2007) gene was used, whereas 16S rRNA was employed for total bacteria (McSweeney et al., 2007) and *B. fibrisolvens* (Klieve et al., 2003), as described in Table 2.

The different microbial groups, such as total bacteria, total methanogens and *B. fibrisolvens*, were determined in the samples by SYBR green quantitative PCR assay. The PCR machine used was from Biorad (Hercules, CA, USA; iQ5 Real-Time PCR detection system) with a 96-well plate. The PCR conditions and primers for different communities used were from Denman et al. (2007) and Klieve et al. (2003) (Table 2). The reaction mixture (25 mL) comprised SYBR green mix (12.5 mL), primers (forward and reverse, 0.71 mL each), PCR grade water (8.58 mL) and template (2.5 mL). Total microbial rumen DNA was diluted to a concentration of 20 ng mL^{-1} before use in the quantitative PCR assays. The assay was conducted with the following cycle conditions: one cycle at 50°C for 2 min. and at 95°C for 2 min. for initial denaturation; 40 cycles at 95°C for 15 s and at 60°C for 1 min. for primer annealing and product elongation. The dissociation curve analysis of PCR end products was performed with 71 cycles at 95°C for 1 min., followed by 60°C for 10 s. A negative blank (without the DNA template) was also run for each primer pair.

Table 2. Primer sequences used to quantify total bacteria, *B. fibrisolvens* and total methanogens by qPCR.

White	*Forward primer*	*Reverse primer*	Amplicon	Reference
Total Bacteria	5'CGGCAACGAGCGCAACCC3 '	5'CCATTGTAGCACGTGTGTAGCC3 '	130 bp	Denman et al. (2007)
Total methanogens	5'TTCGGTGGATCDCARAGRGC3 '	5'GBARGTCGWAWCCGTAGAATCC3 '	128 bp	Denman et al. (2007)
B. fibrisolvens	5'ACACACCGCCCGTCACA3 '	5'TCCTTACGGTTGGGTCACAGA3 '	90 bp	Klieve et al. (2003)

bp: base pairs.

Absolute quantification was carried out by generating calibration curves using serial dilutions (20 to 0.0002 ng mL^{-1}) of reference DNA, with the equation:

$$\log_{10} ng\ ADN = \frac{\text{SQmeans x C x DV}}{\text{S x P}}$$

where:

SQmeans is the average number of copies quantified during the amplification;

C is the DNA sample concentration;

DV is the dilution volume of extracted DNA;

S is the quantity of DNA (ng) used in the analysis;

P is the amount (mg) of rumen contents used in the extraction.

Statistical Analysis

Experimental design corresponded to that of a crossover in which all animals received all treatments. The model is described as:

$$Y_{ijk} = \mu + T_i + V_i + P_k + E_{ijk,}$$

where:

μ is the overall mean;

T_i is the treatment effect, with i1 = 100% stargrass; i2 = 76% stargrass, 24% Leucaena;

V_j is the effect of the j^{th} experimental unit, with j = 2 heifers per treatment;

P_k is the effect of the k^{th} period, with k = 2 periods;

E_{ijk} is the experimental error of the effect of time and treatment.

Data were analyzed by analysis of variance (ANOVA). When significant differences between treatments were found, a comparison test of means was performed using the Tukey's procedure at an alpha of 0.05, by SAS (2004) statistical package, version 9.2.

Results and discussion

Dry matter intake (DMI, kg day^{-1}) was 1.2 times greater in the Leucaena diet than in the 100% stargrass diet ($p < 0.05$, Table 3). Specifically, the average intake (kg per^{-1} day^{-1}) was 23.68 and 18.98 for the leucaena and 100% stargrass diet, respectively ($p < 0.05$, data not shown).

Heifers consumed 1.5, 1.22 and 1.18 times more protein, energy and fat when fed on Leucaena diet than when given 100% *C. plectostachyus* diet ($p < 0.05$, Table 3). Although DMI was different, there were no differences in NDF intake between the two diets, perhaps due to the low content of NDF in *L. leucocephala*, reported to be around 32% of DM (Cuartas et al., 2014).

Table 3. Intake of dry matter and nutrients by heifers fed on stargrass with and without *L. leucocephala*.

Daily intake	Treatment 1*	Treatment 2
C. plectostachyus, kg DM	4.71	4.27
L. leucocephala, kg DM		1.34
Total intake, kg DM	4.71 [b]	5.61 [a]
Protein, g	504 [b]	755 [a]
FDN, kg	3.51	3.72
FDA, kg	2.02 [b]	2.39 [a]
Fat, g	55.0 [b]	65.3 [a]
Gross energy, Mcal	20.2 [b]	24.6 [a]

*Treatment 1 = 100% C. plectostachyus; Treatment 2 = 76% C. plectostachyus + 24% L. leucocephala [a,b]. Means across rows with different letters are statistically different ($p < 0.05$).

Although not quantified in the current study, *L. leucocephala* contains up to 10% of condensed tannins (Barahona et al., 2003), and this is relevant in terms of DMI, rumen fermentation and methane emissions. Several *in vitro* studies have reported that *L. leucocephala* is a substrate of high degradability but low gas production in proportion to the amount of substrate degraded (Molina et al., 2013). During the ruminal fermentation of the substrates, greater microbial biomass, more efficient microbial protein synthesis and lower VFA production are reported. They are actually factors that lead to greater DMI and reduced methane emissions (Blümmel et al., 1997; Molina et al., 2013).

Real-time PCR quantification of total bacteria, total methanogens and *B. fibrisolvens* is shown in Table 4. Microbial populations were not significantly affected by the dietary inclusion of Leucaena, with 5.8, 3.5 and 5.3 ng DNA g^{-1} rumen content for total bacteria, methanogens and *B. fibrisolvens*, respectively.

Table 4. Microbial populations in rumen samples from heifers fed on stargrass with and without *L. leucocephala*.

Parameters evaluated	Treatment 1*	Treatment 2	P value
Total bacteria Log [ng DNA g^{-1} CR]	5.6	5.8	0.53
B. fibrisolvens Log [ng DNA g^{-1} CR]	3.66	3.57	0.47
Total methanogens Log [ng DNA g^{-1} CR]	5.01	5.35	0.27

*Treatment 1 = 100% C. plectostachyus; Treatment 2 = 76% C. plectostachyus + 24% L. leucocephala.

There were no differences in total bacteria (5.6 to 5.8 ng of DNA) when the legume was present, despite the increased nutrient (protein and energy) intake observed with this treatment (Table 3) which could generate an increase of proteolytic populations. Considering diet composition, future studies should consider monitoring the presence of

Synergistes jonesii, a bacteria capable of degrading 3,4 dihydroxypyridine (3,4 DHP), a degradation product of the non-protein amino acid mimosine present in different leucaena species and resulting from the adaptation to leucaena intake (Hammond, 1995).

Our results are similar to those of Galindo et al. (2008), who carried out *in vitro* incubations of stargrass and leucaena in proportions similar to those evaluated in our study (100% and 70 stargrass: 30% legume). Using traditional quantification techniques, these authors did not report any changes in colony-forming units in the presence of Leucaena. Differences may arise from variances in the methods used for estimating the growth of rumen bacteria. In fact, it is one of the main reasons for increasing use and development of molecular markers tools for monitoring rumen microbial populations (Tajima et al., 2001). Moreover, *in vivo* experiments evaluating supplementation with pelleted Leucaena leaves at inclusion levels of 150, 300 and 450 g day^{-1} showed increased populations of total bacteria and of cellulolytic species such as *R. albus*, *R. flavefaciens* and *F. succinogenes* as supplement and DM intake increased (Hung et al., 2013).

The similarity of the populations of *B. fibrisolvens* in the animals consuming the two diets is consistent with reports that this microbe grows well in forage or high fiber diets, but its growth is significantly reduced (up to 20 times less DNA was quantified by qPCR; Fernando et al. (2010) when soluble carbohydrates are added to the diet. Studies monitoring 16s rRNA has shown that the microorganism is polyphyletic with high metabolic variability (Forster et al., 1997). The metabolic versatility of *B. fibrisolvens* has been reported for proteolytic (Cotta & Hespell, 1986), cellulolytic (Krause & Russell, 1996) and biohydrogenation (McKain et al., 2010) activities, making it an organism of interest for the current work in which the inclusion of Leucaena affects the amount of structural carbohydrates and crude protein entering the rumen (Table 3). Biohydrogenation is not only associated with the production of precursors of linoleic acid in the rumen (McKain et al., 2010), but is also considered an alternative metabolic pathway for hydrogen produced during the fermentation and therefore, an efficient strategy to reduce ruminal methanogenic activity (Alemu et al., 2011; Cieślak et al., 2006).

As for the quantification of microorganisms, the amount of methanogens was not different when animals consumed Leucaena (Table 4). These data are consistent with previous studies that monitored the total population of methanogens in high and low feed efficiency animals receiving diets with high and low energy content ((Zhou & Hernandez-Sanabria, 2010).

In a parallel investigation using closed chambers (poly-tunnel), it has been observed that, despite greater DMI with Leucaena, there was no increase in overall methane production. The animals emitted less methane (liters of CH_4 kg^{-1} DMI) when consuming Leucaena versus 100% *C. plectostachyus* diet 37.7 versus 43.5, respectively (com pers). It is evident that changes in methanogenesis cannot be explained solely by a reduction in the abundance of methanogens due to the diversity of this community in terms of composition and function, and different molecular tool should be used to monitor the other factors (Mosoni et al., 2011).

Previous reports have suggested that microbial populations have a tendency to increase even if *in vivo* methane emissions are reduced (Hook et al., 2010), suggesting that the size of the population is not the sole factor associated with enteric methane production (Zhou & Hernandez-Sanabria, 2010). Methane emissions may be more influenced by metabolic activity and community composition of methanogens (McSweeney & Mackie, 2012). Likewise, if DMI increases simultaneously with crude protein intake, it may be expected that the degradation of the substrates and their rate of passage will increase with a decrease in methane production due to the dynamics of production and utilization of CO_2 and H_2 in the rumen (Janssen, 2010).

In this experiment, no significant differences were observed in rumen pH in animals consuming both diets ($p = 0.4641$), as it was 7.02 for 100% stargrass diet and 7.12 for legume diet. They are within physiological limits, usually between 5.5 and 7.0 (Araujo & Vergara, 2007). Rumen pH is a function indicator in terms of concentration of volatile fatty acids (VFA), ammonia production and buffer capacity. Galindo et al. (2008) reported that pH increased as greater proportions of Leucaena were included to stargrass in the diet, which may be associated with the greater calcium content in Leucaena when compared to the grass (Table 1).

High nutrition forages, with rapid ruminal degradation, may lead to low rumen pH (Kolver & De Veth, 2002). According to Janssen (2010), rumen pH is associated with rates of passage, methane formation and rumen VFA proportions. Moreover, both *in vivo and in vitro*, a high correlation has been reported between the rumen's pH and the acetic: propionic acid ratio ($R^2 = 0.82$) and ruminal ammonium ($R^2 = 0.65$) (Lana et al., 1998). Thus,

even in diets composed exclusively of forages, this rumen function parameter should be monitored. Increased pH in diets including Leucaena could be associated with greater ammonium concentrations and possibly with lower concentrations of VFA, a response consistent with increased dietary protein. The amphipathic nature of proteins may increase the rumen's pH, explaining the slight increase of rumen pH observed in animals consuming Leucaena. The production of VFA and ammonia nitrogen was not determined in this study and future studies should monitor these two factors to obtain a better understanding of enteric methane production.

Conclusion

In the current study, the total number of bacteria and methanogens, and the population of *B. fibrisolvens* in terms of ng of DNA per gram of rumen contents were not affected by the introduction of Leucaena in the diet.

Since the animals in this experiment had been previously exposed to Leucaena, the microbial populations in these animals should have been stable. However, it would be advisable to perform another experiment with a slightly longer adaptation period or to ensure that all changes in microbial populations take place in the animals consuming only *C. plectostachyus.* For future rumen microbiological studies associated with the adoption of silvopastoral systems, it is recommended to investigate population changes of other bacterial species or of microbial groups such as anaerobic fungi and protozoa. Further, it would be desirable to employ methodologies that yield information on diversity (genomics, proteomics and metabolomics). Exploring the structure of rumen microbial populations and their metabolism through metagenomic methodologies is becoming more feasible and necessary to improve the integration of animal response to rumen microbial behavior.

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