Determination of the effect of *Morus alba* cv. Cubana on the population of methanogens within the rumen liquor of water buffaloes, using the Denaturing Gradient Gel Electrophoresis (DGGE). Technical note

Niurca González [1], Juana Galindo [1], A. Aparecido Navarrete[2], Adibe Luiz Abdalla[3] and Siu Mui Tsai[2]

[1]*Instituto de Ciencia Animal. Apartado Postal 24. San José de las Lajas, Mayabeque, Cuba*

[2]*Laboratorio de Biología Celular y Molecular. Centro de Energía Nuclear en la Agricultura. USP. São Paulo. Brasil*

[3]*Laboratorio de Nutrición Animal. Centro de Energía Nuclear en la Agricultura. USP. São Paulo. Brasil.*

Email: ngonzalez@ica.co.cu

The effect of mulberry (*Morus alba* cv. Cubana) on the population of methanogens within the rumen liquor of water buffaloes was determined using the Denaturing Gradient Gel Electrophoresis (DGGE). For this, an *in vitro* fermentation was made and five treatments were evaluated, which corresponded to different inclusion levels of mulberry into the diet based on star grass (SG) (*Cynodon nlemfuensis*): (1) 100 % of SG (control); (2) SG + 15 % *M. alba* cv. Cubana; (3) SG + 20 % *M. alba* cv. Cubana; (4) SG + 25 % *M. alba* cv. Cubana and (5) SG + 30 % *M. alba* cv. Cubana. Before the analysis of samples, a standardization of the DGGE was carried out. After 12 h of fermentation, samples of the rumen liquor were taken, from which the DNA was extracted and the DGGE was performed. The gel was scanned, images were analyzed by the Bionumerics software and dendrograms of similarities were performed. As a result, the levels of inclusion of *M. alba* cv. Cubana did not produced marked variations in the composition of ruminant methanogens regarding the control, grouping all treatments in a cluster with 81 % of similarity. It can be concluded that *M. alba* cv. Cubana has no marked effect on the population of methanogens within rumen liquor of water buffaloes.

Key words: *mulberry, methanogenic Archaea, DGGE, rumen, water buffaloes*

The methanogenic Archaea are the microorganisms responsible for the formation of methane within the rumen (Poulsen *et al.* 2012). Therefore, in order to reduce the production of this gas, so harmful for environment and animal production, many strategies have been evaluated, and the direct inhibition of methanogens is one of them (Attwood and McSweeney, 2008).

Previous studies, in which *M. alba* cv. Cubana was evaluated as an strategy for controlling ruminant methanogenesis, confirmed that this plant managed to reduce *in vitro* methane production (González *et al.* 2010), but it did not decreased the amount of methanogens (González *et al.* 2011). This result led to state that the reduction of methane production could not be related to the direct inhibition of these microorganisms. Considering that traditional crop techniques were used in these researches and methanogens are difficult to cultivate, there is an important need of performing studies with more modern techniques in order to determine effectively if this plant has a direct effect on the methanogenic population.

The DGGE is one of the molecular methods that could be used for this purpose (Sigler *et al.* 2004). Therefore, the objective of this study was to determine effect of mulberry (*Morus alba* cv. Cubana) on the population of methanogens within the rumen liquor of water buffaloes was determined using the DGGE.

A fermentation was carried out using the *in vitro* gas production technique described by Theodorou *et al.* (1994).

As donors of rumen liquor, two adult male water buffaloes (Bufalipsos crossbreds) were used, with a simple cannula in rumen and mean weight of 453 kg. They were kept in individual paddocks, under the shadows and with free access to water and food. They all were offered forage of star grass (*Cynodon nlemfuensis*), without supplementation.

Five treatments were evaluated, which corresponded to different inclusion levels of mulberry into the diet based on star grass (SG): (1) 100 % of SG (control); (2) SG + 15 % *M. alba* cv. Cubana; (3) SG + 20 % *M. alba* cv. Cubana; (4) SG + 25 % *M. alba* cv. Cubana and (5) SG + 30 % *M. alba* cv. Cubana.

Mulberry plants came from a plantation of three years, located in the Estación Experimental de Pastos y Forrajes "Indio Hatuey", Matanzas, Cuba. These plants were found in red ferrallitic soils and fertilized with poultry manure. Leaves with young stems and petioles were manually cut, simulating animal selection. Star grass was also manually cut, at a height of 10 cm from the soil, in grazing areas from the Instituto de Ciencia Animal, located in Mayabeque, Cuba.

All plant material was dried in an oven, at 60 °C for 48 h. Later, it was grounded up to the size of a 1mm particle.

Chemical composition of a sample from experimental diets (table 1) was determined according to techniques described by AOAC (1995). Fibrous fractions were analyzed by the procedure of Goering and van Soest (1970).

The rumen liquor of fasting animals was extracted using the cannula, with help of a thermos. This liquor was put into a hermetic vacuum flask for guaranteeing temperature conditions (39 °C) and anaerobiosis during

Table.1. Bromatological composition of experimental diets (%).

Treatment	Ash	Ca	P	CP	NDF	ADF	Lig.	Cel.
SG (Control)	11.33	0.57	0.33	9.00	68.78	39.22	6.56	29.58
SG + 15 % *M. alba* cv. Cubana	10.84	0.77	0.32	11.38	62.64	35.79	6.33	26.82
SG + 20 % *M. alba* cv. Cubana	10.67	0.84	0.32	12.18	60.60	34.65	6.25	25.9
SG + 25 % *M. alba* cv. Cubana	10.51	0.90	0.32	12.97	58.56	33.50	6.17	24.98
SG + 30 % *M. alba* cv. Cubana	10.34	0.97	0.32	13.76	56.52	32.36	6.09	24.06

the transportation to the laboratory.

Ruminant content of both animals was mixed and filtered with muslin. A small portion of buffersolution of Menke and Steingass (1988) was added to the resulting solid, and it was shaken for a few seconds in a domestic blender for separating the microorganisms attached to the fiber. Later, the filtrate of this portion was added to the liquor fraction. The rumen liquor was kept in a CO_2 atmosphere all the time. Experimental units consisted on glass bottles of 100 mL, which contained 0.5 g of the food to be evaluated. Each bottle was added 50 mL of a mixture of rumen liquor and buffer solution of Menke and Steingass (1988), in a 1:3 (v/v) proportion, and they were sealed with a butyl and agrafe stopper. Every bottle was randomly located in a controlled temperature water bath, at 39 ºC.

After 12 h of fermentation, samples of rumen liquor from the experimental units were taken and the DNA was extracted for a later performance of DGGE. This experiment was carried out four times.

For extracting DNA, the technique described by Goel *et al.* (2009) was used, in which the rupture of cells was achieved after repeated freezing and defrosting with liquid nitrogen, in presence of zirconium pearls. DNA was obtained through the use of a matrix (glassmilk), which united to the DNA and later it was released, when the matrix was dissolved in bi-distilled water.

DNA concentration and purity of samples (DO 260nm/ DO 280nm) were determined using a spectrophotometer Ultrospec 1100 pro, Amersham, Biosiences. Quality of the extracted DNA was confirmed through electrophoresis in gel of azarosa (1 %, p/v).

Before the analysis of samples, a standardization of DGGE was carried out. Specific indicators for amplifying DNA of methanogens were validated for this purpose. Two previous reactions of amplification were carried out through a polymerase chain reaction (PCR). A total of 40 DNA samples, extracted from rumen liquor, were amplified. Later, the DGGE was performed.

In the first amplification, the following indicators, designed by Denman *et al.* (2007), were used: Meth F5'- TTC GGT GGA TCD CAR AGR GC-3' and Meth R5'-GBA RGT CGW AWC CGT AGA ATC C-3', with a concentration of 5 pmoles•μL^{-1}. The final reaction volume was 25 µL.

The second reaction of amplification by PCR was carried out with the product of the first reaction, which was dissolved 50 times. The following indicators were used: Meth R5'-GBA RGT CGW AWC CGT AGA ATC C-3' and Meth FGC 5'-CGC CCG CCG CGC GCG GGC GGG GCG. GGG GCA CGG GGG TTC GGT GGA TCD CAR AGR GC-3' (Denman *et al.* 2007), with a concentration of 5 pmoles•μL^{-1}. The final reaction volume was 50 µL.

Both amplification reactions were performed in thermocycler Gene Amp, PCR System 9700, Applied Biosystems, with the following program: 1 cycle of 95 ºC 5 min, 30 cycles of 95 ºC 30 s, 60 ºC 1 min, 72 ºC 1 min and 1 cycle of 72 ºC 10 min. An electrophoresis in gel of agarosa 2 % (p/v) was carried out to visualize the amplification.

The equipment INGENY phor U (Holanda) was used for running the DGGE. Products of the second reaction of PCR were directly applied in a gel of polyacrilamide of denaturing gradient 40-60 %. The running time was 16 h, at 60 ºC, and a constant voltaje of 80 V. at the end of running, the gels were dyed with a silver nitrate solution at 0.2 %, and they were scanned.

For the analysis of DGGE samples, the same procedure of the standardization was used. Gel analysis and similarity dendograms were performed using Bionumerics software, version 5.1 (Applied Maths, Austin, TX).

This study took into account that DGGE has some technical problems, like difficulty in establishing chemical gradients in polyachrilamide, which implies that not always the gel conditions can be exactly reproduced (Soto 2011). Sometimes, a proper resolution or chain separation can be difficult to obtain. Due to this fact, it is important to select good fatteners and, in adequate moments, to value the possibility of using more than one marked fattener, mention the microbial group to be study, or perform a PCR with several pairs of fatteners (Schütte *et al.* 2008). That is why the optimal standardization of the process can minimize the already mentioned problems, and contribute to improve the reproducibility of analysis.

Regarding the previous standardization of the DGGE carried out in this study, the specific indicators of methanogens, which were validated in each reaction of PCR, could amplified all DNAs. This allowed that these indicators could be used later in the performance of DGGE, under the running conditions, which were

also standardized and effective.

The DGGE is one of the methods that allow to study the structure and diversity of microbial communities (Tan *et al.* 2012). It is widely used for systemic analysis of population changes, and the microbial populations of rumen among them (Firkins 2010), and their results can be related to nutrition studies (Guan *et al.* 2008 and Firkins *et al.* 2008). The band patterns, obtained in the DGGE, represent the higher constituents of the microbial community in analysis because this technique allows the separation of DNA molecules that differ in a simple base (Dar *et al.* 2005).

Figure 1shows DGGE profiles, together with similarity dendograms, for each of the evaluated treatments. This figure shows that the band profiles obtained in the gel presented low differences among treatments, which allows to express that the different inclusion levels of *M. alba* cv. Cubana (15, 20, 25 and 30%) did not produced great variations in the composition of methanogens within the rumen liquor of water buffaloes, regarding the control treatment. The previous information can be confirmed with the cluster analysis, where all the treatments were gathered into one, with of 81 % similarity. These results were similar to those obtained by Longo (2006), who compared, through the DGGE, the effect of different legumes (*Styzolobium aterrimum*, *Styzolobium deeringianum*, *Leucaena leucocephala* and *Mimosa caesalpiniaefolia*) on the population of methanogens regarding a control treatment of Cynodon, and found similarity among treatments when the cluster analysis carried out.

The results of this study also coincide with those obtained by Tan et al (2012), who evaluated the effect of supplementation with pellets of mulberry leaf on meat cattle, and found out that diversity of methanogens was slightly different among treatments. However, they obtained only seven clusters, unlike the present study, in which fourteen were obtained.

This may be related to the exposed by Zhou *et al.* (2011), who stated that the differences among the populations of methanogens are the results of hosts located in different geographic regions. For instance, studies carried out by Wrigth *et al.* (2007) and Hristov *et al.* (2009) report between nine and ten cluster in milking cows and fourteen clusters in sheeps, respectively.

As the objective of this study was to analyze the population of methanogenic Archaea at a general level, species of methanogens within the rumen liquor of water buffaloes were not determined for the different diets. Further studies could be carried out with the objective of knowing these species, taking into account that DGGE allows to identify individual species that are part of a microbial community through dividing, cloning and sequencing of bands (Zhou *et al.* 2011).

The methanogenic Archaea are microorganisms responsible for the formation of methane within the rumen (Poulsen *et al.* 2012). Therefore, their decrease could bring about a reduction in the ruminal methanogenesis. González *et al.* (2012) evaluated the same inclusion levels of *M. alba* cv. Cubana in the production of ruminal methane, and found that this plant managed to decrease the methane formation in

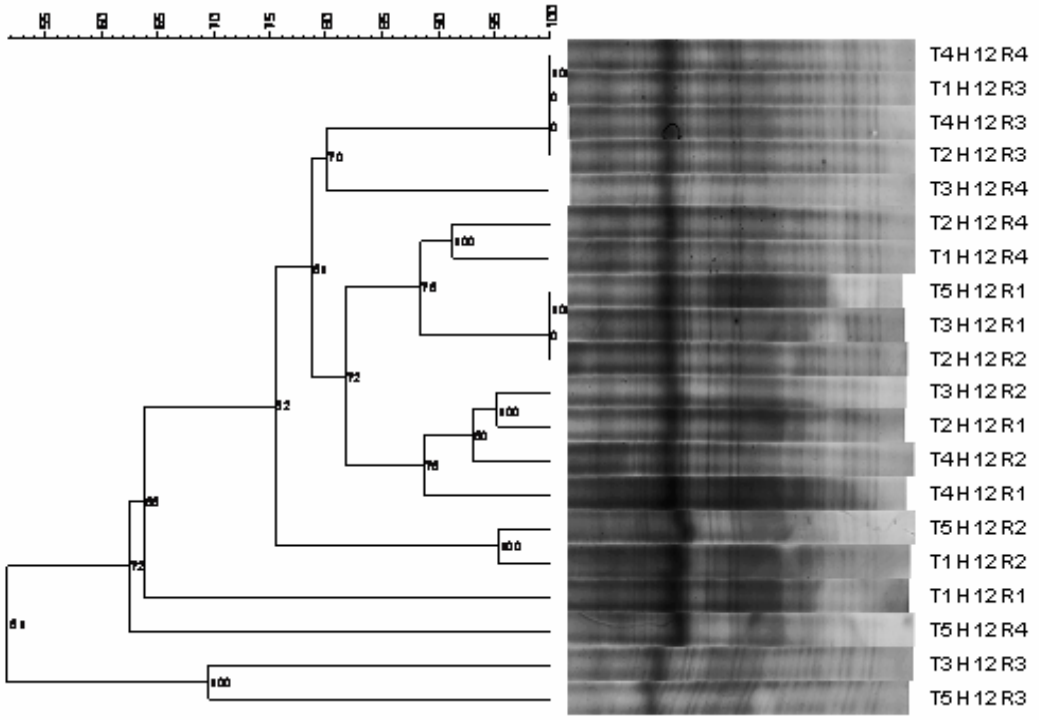

Figure 1. Profile of bands obtained through the use of Denaturing Gradient Gel Electrophoresis (DGGE) and cluster analysis, corresponding to ruminant methanogens population for the different treatments after 12 h of fermentation. T: Treatment, H: Hour, R: Repetition.

the rumen. That is why this study had to wait for an affectation in the population of methanogens, and this should be the cause for the decrease in the methane production found by these authors, but it did not happened this way.

This demonstrates the stated by other authors, about that the decrease in the methane production does not necessarily implies changes in the community of methanogens (Hook *et al.* 2009), but it could be due to changes in the metabolic routes involved in gas production within the rumen. Future researches are suggested to quantify this microbial population using molecular techniques, and to study biochemical routes that take part of the ruminal methanogenesis. This would allow to confirm the previous considerations and results.

Results of this study allow to share the criteria of different researchers on the molecular techniques, which could be successfully used to study microbial communities of rumen. They also support the criterion of the effect that could have the modifications of the diet consumed by the animals on these communities.

It can be concluded that the inclusion levels of 15, 20, 25 and 30 % of *M. alba* cv.. Cubana had no marked effect on the population of methanogens within rumen liquor of water buffaloes.

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