Effect of different inclusion levels of *Morus alba* Linn cv. Cubana on the methane fermentation and production under *in vitro* conditions with rumen liquor from river buffaloes (*Bubalus bubalis*)

Niurca González, Juana Galindo, Ana Irma Aldana, Onidia Moreira and Lucía Sarduy

Instituto de Ciencia Animal, Apartado Postal 24, San José de las Lajas, Mayabeque, Cuba
Email: ngonzalez@ica.co.cu

In order to evaluate the effect of different inclusion levels of mulberry (*M. alba* Linn.) cv. Cubana on the fermentation and reduction of the rumen methanogenesis in river buffaloes (*Bubalus bubalis*), an *in vitro* fermentation was performed and five levels of inclusion of *M. alba* L. cv. Cubana (0, 15, 20, 25 y 30 %) were assessed in a diet based on star grass (*Cynodon nlemfuensis*). The volume of gas and methane accumulated was determined at 4, 8, 12, and 24 h of fermentation, as well as the concentration of total and individual short chain fatty acids (SCFA), the ammonia (NH_3), and the pH at 0, 4, 8, 12, and 24 h of incubation. The inclusion of *M. alba* L. cv. Cubana increased the production of gas accumulated at 24 h of fermentation, compared with the treatment containing only star grass. The different mulberry inclusion levels reduced the methane production in the rumen, compared with the control. The 30 % was the level that reduced it the most. The concentration of total and individual SCFA was not affected, nor that of NH_3. It was concluded that the inclusion levels of 15, 20, 25, and 30 % of *M. alba* L. cv. Cubana did not affect the end products of the fermentation and reduced the *in vitro* rumen methane production. The 30 % was the level that reduced the most the rumen methanogenesis.

Key words: *rumen methanogenesis, rumen, mulberry, end products of the fermentation, in vitro gas production.*

Methane is a noxious gas, which contributes to the greenhouse effect and, thus, participates significantly in the environmental contamination. It is estimated that its potential damage to the environment is twenty-one times higher than that of the carbon dioxide (IPCC 2001 and Rasmussen and Harrison 2011). Approximately, 800 million tons of this gas are produced annually in the atmosphere. Out of them, 30 % come from ruminants (McAllister *et al*. 1996, Sharma 2005 and Beauchemin *et al*. 2008).

Methane from ruminants is formed, primarily, from the fermentation of the diet carbohydrates in the rumen (Attwood and McSweeney 2008 and McGinn *et al*. 2008), thus, it is considered a normal product of the rumen fermentation. The formation of this gas, besides provoking great damages to the environment, represents an energy loss of the feed consumed by the animals (Mirzaei-Aghsaghali and Maheri-Sis 2011). Therefore, the control of the rumen methanogenesis, besides reducing the global methane releases (Wood *et al*. 2009), could make more efficient the digestion of carbohydrates in the rumen.

At present, strategies are looked for to reduce the methane formation in the rumen and its release to the environment. Among them, those of nutritional nature are the most studied, including the utilization of trees and shrubs. Studies of González *et al*. (2010) evaluated the inclusion of 30 % of different mulberry varieties (*Morus alba* L.) in the rumen methane production. These authors reported that the Cuban variety was the most promising. However, they did not determine the levels of inclusion of this mulberry variety that could reduce the productions of this gas in the rumen.

The objective of this research was to evaluate the effect of different levels of inclusion of mulberry (*M. alba* Linn.) cv.. Cubana on the methane fermentation and production under *in vitro* conditions with rumen liquor from river buffaloes (*Bubalus bubalis*).

Materials and Methods

The *in vitro* gas production technique was applied, as described by Theodorou *et al*. (1994) and modified by Rodríguez *et al*. (2008).

Animals and diet. Two upgraded Bufalipso river buffaloes were used as donors of the rumen liquor. They were adult males, fitted with simple cannula in the rumen, and average weight of 453 kg. They were allocated in individual pens, under shade and ad libitum water and feed. They were all given fodder of star grass (*Cynodon nlemfuensis*) and not supplementation.

Treatments. Five treatments were assessed with different levels of inclusion of *M. alba* L. cv. Cubana: 100 % of star grass (*Cynodon nlemfuensis*) as control, and star grass being added 15, 20, 25 and 30 % of *M. alba* L. cv. Cubana.

The mulberry plants had three years of establishment. They came from a plantation of the Experimental Station of Pastures and Forages "Indio Hatuey", located in the Matanzas province, Cuba. They were on lixiviated and poultry litter fertilized red ferrallitic soils. The leaves were cut manually with the petioles and young stalks, at 60 d of regrowth and in the rainy season, to simulate the animal browsing. Star grass was collected in grasslands of the Institute of Animal Science, Mayabeque province, Cuba. It was cut manually, at a height of 10 cm above the soil. All the plant material was dried in an oven at 60ºC for 48 h. Later, it was ground up to particle size of

Table1. Chemical composition of the feeds under study (%)

Tratamiento	DM	Ashes	Ca	P	CP	NDF	ADF	Lignin	Cellulose
Star grass (Control)	90.12	11.33	0.57	0.33	9.00	68.78	39.22	6.56	29.58
Star grass + 15 % *M. alba* L. cv. Cubana	89.94	10.84	0.77	0.32	11.38	62.64	35.79	6.33	26.82
Star Grass + 20 % *M. alba* L. cv. Cubana	89.89	10.67	0.84	0.32	12.18	60.60	34.65	6.25	25.9
Star grass + 25 % *M. alba* L. cv. Cubana	89.83	10.51	0.90	0.32	12.97	58.56	33.50	6.17	24.98
Star grass + 30 % *M. alba* L. cv. Cubana	89.78	10.34	0.97	0.32	13.76	56.52	32.36	6.09	24.06

1mm. The chemical composition was determined in the treatments under study (table 1).

Experimental procedure. A total of 0.5 g were weighed from each of the treatments and added to glass bottles of 100 mL. The animals under fast were extracted rumen liquor through the cannula, with the help of a vacuum pump. The liquor was stored in thermo with hermetic seal to guarantee the temperature (39° C) and anaerobiosis conditions during the transfer to the laboratory. The rumen content from both animals was mixed and filtered through muslin. The resulting solid was added a small fraction of incubation broth of Menke and Steingass (1988). It was shaken for a few seconds in a domestic blender to remove the fiber-adhered microorganisms. Later, the filter of this portion was added to the liquid fraction. The rumen liquor was kept in CO_2 atmosphere. Fifty milliliters of a mixture of rumen liquor and incubation broth of Menke and Steingass (1988) were added to each bottle of 50 mL at a ratio of 1:3 (v/v). The bottles were sealed with butyl stopper and metal seal. As blank, bottles without substrate were included to correct the supply of rumen liquor to the volumes of gas produced. All the bottles were randomly put in a water bath at controlled temperature of 39° C.

The gas was measured by the displacement of the piston of the syringe of 10 mL, after the puncture of the stopper. It was determined the volume of gas accumulated at 4, 8, 12, and 24 h of fermentation.

The pH was measured, and the concentration of ammonia (NH_3) was determined, as well as the total and individual SCFA, at 0, 4, 8, 12, and 24 h of incubation.

The methane production was calculated through the molar proportion of the different SCFA, according to the program of Stuart (2010) and according to the stoichiometric balance of the rumen fermentation.

Chemical analysis. The analysis of the chemical composition of the treatments was performed according to the techniques of AOAC (1995). The fibrous fractions were analyzed by the procedure of Goering and van Soest (1970).

The total and individual SCFA concentrations were determined by gas chromatography. Dani chromatographer was used, with column DN-FFAP 30 m, 0.32 ID, 25µm and FID detector. Nitrogen was used as carrier gas. The temperature of the oven and the injector was of 200 °C, and that of the detector was of 240 °C.

The determination of the NH_3 concentration was performed according to the technique of Conway (1957). The pH was determined through the reading in digital pH meter, Sartorius brand.

Statistical design and analysis. A completely randomized design was applied with measures repeated in time. Four repetitions were performed per treatment.

A multivariate analysis of variance was used for processing the results. When there was interaction between the treatments and the sampling times, a split plot model was used. The main plots were the treatments, and the subplots, the sampling times. When there was not interaction, a model linear was used for the effects of the treatments and the sampling times. When necessary, Duncan's test was applied for $P < 0.05$ (Duncan 1955).

A correlation was performed between the methane production and the different indicators of the chemical composition of the plant material, as well as of the end products from the rumen fermentation.

In all the analyses, the statistical program INFOSTAT was applied, as proposed by Balzarini *et al*. (2001).

Results and Discussion

There was interaction between the treatments and the sampling times, only for the accumulated gas and methane production.

The inclusion of *M. alba* L. cv. Cubana in the ration increased the gas production accumulated at 24 h of fermentation, compared with the treatment containing only star grass (table 2). The analysis of the performance of this indicator in time evidenced that, during the first 12 h of incubation, the control diet and that including 15 % of mulberry produced equal gas volumes. The effect provoked by the treatments with mulberry inclusion was manifested since the 8 h, for the levels of inclusion of 20, 25, and 30 %.

Table 2. Effect of different levels of inclusion of *M. alba* L. cv. Cubana on the accumulated gas production (mL of gas g of DM incubated^{-1})

Fermentation hour	Treatment, levels of *M. alba* L. cv. Cubana, %				
	0	15	20	25	30
4	1.67^{a}	2.36^{a}	4.03^{a}	4.18^{a}	5.29^{abc}
8	3.19^{a}	4.87^{a}	8.07^{bcd}	10.99^{cd}	11.14^{cd}
12	3.19^{a}	4.87^{a}	8.07^{bcd}	12.11^{d}	12.26^{d}
24	21.36^{e}	29.19^{f}	28.93^{f}	36.32^{g}	40.39^{g}

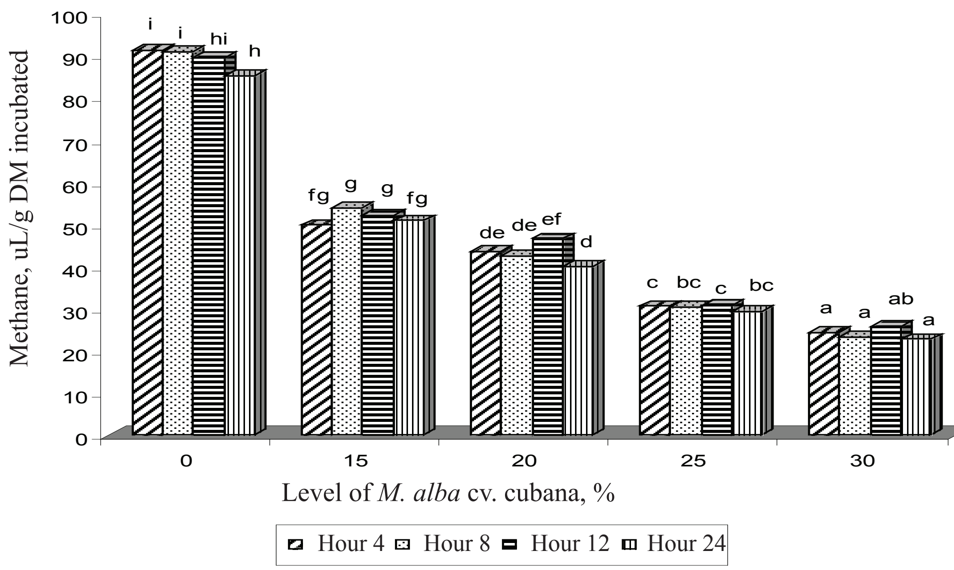
Different letters show differences ($P < 0.05$)
SE = ± 2.56 for the hours at the same level of the treatments
SE = ± 1.16 for the treatments at the same or different level of the hours

Figure 1 shows that the different levels of inclusion of *M. alba* L. cv. Cubana reduced the rumen methane production compared with the control. Since the four hours of fermentation, the treatments that included this mulberry variety started to decline the volume of rumen methane. The fall in the methane production with 25 % of inclusion coincided with the results of Delgado *et al*. (2007). These authors evaluated this level of mulberry inclusion in a basic diet of *Pennisetum sp.* and noted decline of the amount of methane in mLg^{-1} of fermented DM. At 24 h of fermentation, the levels of mulberry inclusion reduced the rumen methane production. The level of 30 % was the one that produced less methane, and represented a production of this gas 3. 8 times lower than the control.

The feed consumed by the animals are fermented in the rumen through the activity of a group of microorganisms. Among the end products, SCFAs are obtained (Kumar *et al*. 2007), as well as NH_3. The fermentation of *M. alba* L. cv. Cubana did not affect the concentration of SCFA (total and individual) or of NH_3 (tables 3 and 4).

In a study of Agarwal *et al*. (2008), the production of SCFA was higher in the alfalfa (*Medicago sativa*) hay than in that of corn (*Zea mays*), which was attributed to higher degradability and availability of the cell contents in the alfalfa hay. This suggests that in the treatments including mulberry, the cell contents were degraded in the same magnitude as in that in which it was not included.

Hoover *et al*. (2006) and Wanapat *et al*. (2009) proved that the ratio of SCFA (acetic, propionic and butyric) in the rumen liquid from buffaloes is not affected by the levels of non-structural carbohydrates, and that in these animals there is special fermentation compared with cattle. This research, by using river buffaloes as animals donating rumen liquor, corroborated the previous criteria, because the high contents of readily degradable carbohydrates present in mulberry (García 2003) did not take part in the



abcdefg Different letters show differences at P < 0. 05, according to Duncan (1955)
SE = ± 2.16 for the hours at the same level of the treatments
SE = ± 2.15 for the treatments at the same or different level of the hours

Figure 1. Effect of different levels of inclusion of *M. alba* L. cv. Cubana on the production of rumen methane ($P < 0.001$)

Table 3. Effect of different levels of *M. alba* L. cv. Cubana on the concentration of total and individual SCFA in the rumen (mmol L^{-1})

Indicator	Treatments, levels of *M. alba* L, %					SE ±
	0	15	20	25	30	
Acetic	25.30	20.95	20.55	19.12	17.82	2.06
Propionic	5.34	5.06	4.56	4.28	4.53	0.43
Butyric	0.67	0.73	0.56	0.52	0.55	0.08
SCFAt	31.44	26.90	25.68	23.75	22.24	2.59

Table 4. Effect of different levels of inclusion of *M. alba* L. cv. Cubana, on the pH and the concentration of rumen ammonia

Indicator	Treatments, levels of *M. alba* L., %					SE ±
	0	15	20	25	30	
pH	6.89[c]	6.77[b]	6.76[b]	6.66[a]	6.64[a]	0.03***
NH_3 (mmol•L^{-1})	10.00	10.62	10.24	13.40	14.02	1.36
N-NH_3 (mg•100 mL^{-1})	14.01	14.87	14.33	18.76	19.63	1.90

[abcdefg]Different letters show differences (P < 0. 05) according to Duncan (1955).
*** P < 0.001

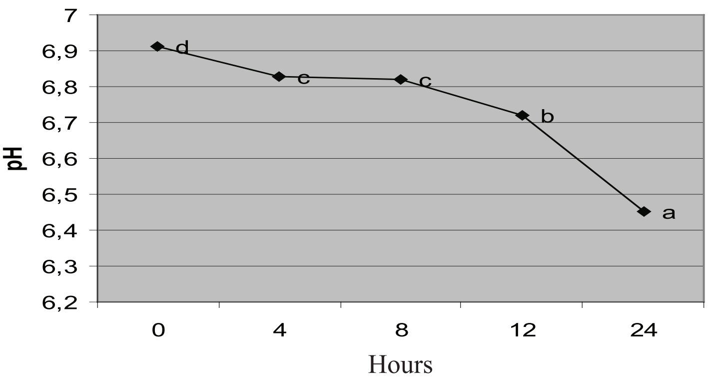
concentration of SCFA.

According to Russel (1998) and Lila *et al*. (2005), the methane production was related to the acetic-propionic ratio in the rumen. The highest methane productions are associated with a trend to higher acetic-propionic ratio in the rumen and vice versa (McAllister and Newbold 2008). The acetic-propionic ratio was of 4.74, 4.14, 4.51, 4.47, and 3.93 for the levels of inclusion of *M. alba* L. cv. Cubana of 0, 15, 20, 25, and 30 %, respectively, that is, the lowest value corresponded to 30 % of inclusion of mulberry. This accounts for the lowest amount of methane produced in this level. McGinn *et al*. (2004) obtained similar results, when determining the effect of the addition of sunflower oil in the rumen methane production. These authors reported decline in the methane concentration, as response to the lower acetic-propionic ratio.

The pH (table 4) decreased with the inclusion of mulberry and showed the lowest values for the levels of 25 and 30 % of inclusion. Figure 2 represents that this indicator also decreases in time, regardless the treatment.

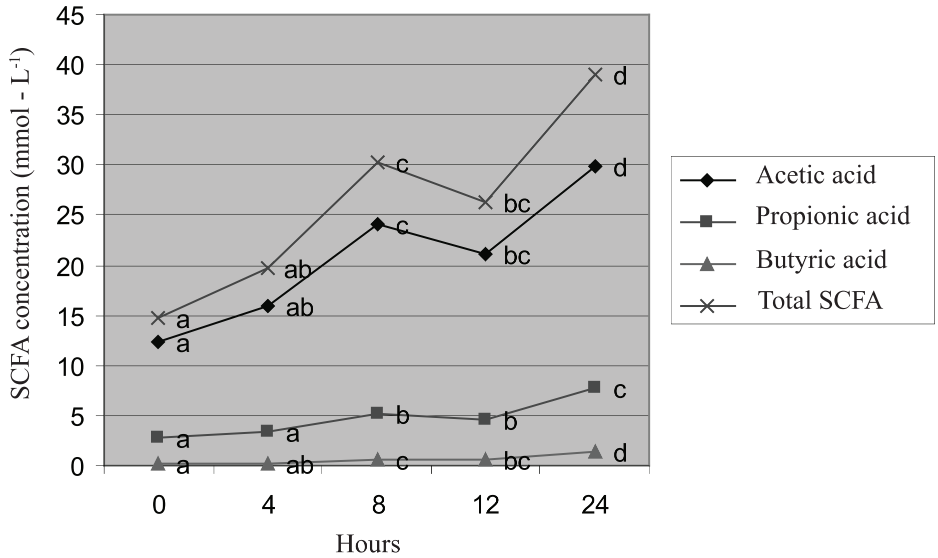
The pH decrease in time is the result from the accumulation of organic acids (Valdez-Vázquez and Poggi-Varaldo 2009). The concentration of individual SCFA, as that of total SCFA, was increased in time (figure 3). The experiment was performed under *in vitro* conditions, having no input or output of substances, which favored the accumulation of some compounds such as the SCFA. This is reflected in the pH decline.

The values of N-NH_3 obtained for the different levels of inclusion of mulberry (table 4) were in correspondence with Wanapat and Pimpa (1999). These authors reported that a concentration of N-NH_3 higher than 13.6 mg.dL^{-1} in buffaloes is optimum for the process of fiber degradation.



SE = ± 0.03. (P < 0.001)
[abcd]Different letters differ at P < 0.05, according to Duncan (1955)

Figure 2. Performance of the pH in time



[abcd]Different letters differ at P < 0.05, according to Duncan (1955)
Acetic acid, SE±2.06. Propionic acid, SE±0.43.
Butyric acid, SE±0.08. Total SCFA, SE±2.59.

Figure 3. SCFA performance in time (P < 0.001).

The NH_3 concentrations were not affected by the inclusion of 15, 20, 25, and 30 % of *M. alba* L. cv. Cubana, which agreed with the results of González *et al*. (2010). These authors evaluated the effect of 30 % of inclusion of different mulberry varieties on the rumen microbial populations and on the products of the rumen fermentation. This confirmed the criterion of González *et al*. (2010) about the fact that the highest degradation of the protein present in mulberry could be in the lower parts of the gastrointestinal tract. This propitiated higher utilization of the protein by the animal and justified the good productive results obtained by Benavides (2000), González and Milera (2000) and Ba *et al*. (2005) with the utilization of this plant in the feeding of ruminants.

If it is considered that the protein is one of the limiting nutrients in the diet for animals in developing countries (Sarwar *et al*. 2002), the supplementation of the diets for ruminants with non-degradable protein in the rumen increases the efficiency of utilization of nitrogen through the increment of the flow of nitrogen and amino acids to the small intestine (Titgemeyer *et al*. 1989).

Table 5 shows the results of the multiple correlation between the methane production and the different indicators of the chemical composition of the plant material, and between the methane and the end products of the rumen fermentation. The high positive correlation between the NDF and the cellulose with the methane production was proved and, at the same time, the negative correlation between the CP and the methane.

Also, there was high positive correlation between the concentration of acetic acid and the methane formation. This is logical, considering that hydrogen is released in the pathway of formation of acetic acid, which is used by the methanogens to form methane (Attwood and McSweeney 2008, Cheng *et al*. 2009 and Kobayashi 2010). Therefore, at lower formation of acetic acid, there is lower production of this gas.

Although there were not statistical differences in the concentration of acetic acid for the treatments under study, it decreased as the level of inclusion of *M. alba* L. cv.. Cubana was increased up to 30 %. This was reflected in the reduction of methane production.

Table 5. Matrix of multiple correlation between chemical indicators of the plant material and end products of the rumen microbial fermentation

	CP	NDF	Cellulose	Ammonia	Acetic acid	Methane
CP	1.0					
NDF	-1.0***	1.0				
Cellulose	-1.0***	1.0***	1.0			
Ammonia	-0.98***	0.98***	0.98***	1.0		
Acetic acid	-0.99***	0.99***	0.99***	0.98***	1.0	
Methane	-1.0***	1.0***	1.0***	0.98***	0.99***	1.0

*P < 0.05 **P < 0.01 ***P < 0.001

The levels of inclusion of 15, 20, 25, and 30 % of *M. alba* L. cv. Cubana did not affect the concentrations of the end products of the fermentation (NH_3 and SCFA) and reduced the methane production in the el rumen. The 30 % was the level that reduced the most the rumen methanogenesis.

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