Effect of four mulberry (*Morus alba* Linn.) varieties on microbial population and fermentative products with rumen liquid from river buffaloes (*Bubalus bubalis*) under *in vitro* conditions

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The effect of four mulberry (*Morus alba* Linn) varieties on the microbial population and fermentative products was determined under *in vitro* conditions, using rumen liquid from river buffaloes (*Bubalus bubalis*). An *in vitro* fermentation was developed to evaluate five treatments: 1) 100 % of star grass (SG) (*Cynodon nlemfuensis*) (control), 2) SG + 30 % Cuban variety, 3) SG + 30 % Acorazonada variety, 4) SG + 30 % Tigreada variety and 5) SG + 30 % Indonesia variety. The culture and count of total viable, cellulolytic, proteolytic, amylolytic, and methanogenic bacteria and fungi were performed. Also, the count of protozoa was conducted, as well as the pH measurement and the determination of the ammonia concentration. A completely randomized design was applied with 5 x 3 factorial fit for the counts of bacteria and fungi, and with 5 x 5 for the indicators pH, ammonia concentration, and count of protozoa. The inclusion of the different mulberry varieties did not affect the populations of total viable, proteolytic, and celullolytic bacteria and fungi. At eight hours of fermentation, the treatment with the Acorazonada variety showed the lowest counts of methanogens (1.96×10^9 cfu.mL^{-1}) compared with those including the Cuban, Tigreada, and Indonesia varieties (3.75, 3.99 and 3.70×10^9 cfu•mL^{-1}, respectively). The counts of protozoa were superior for the Cuban variety (1.04×10^4 cel•mL^{-1}). The pH declined, regardless the treatment, with the fermentation time, although it was always closed to neutrality. The concentration of ammonia was similar for all the treatments. It was concluded that the inclusion of 30 % of the mulberry varieties: Acorazonada, Cuban, Tigreada, and Indonesia did not affect the fiber-degrading populations or the end products of the rumen fermentation.

Key words: *rumen microorganisms, methanogens, river buffaloes, mulberry.*

The use of tree and shrub species is one of the most used strategies in ruminant nutrition to control rumen methane production for being considered a practical and simple application (Sharma 2005). Works of Delgado *et al*. (2007) and González *et al*. (2010) proved their efficiency in evaluations of mulberry (*Morus alba* Linn).

The decrease in rumen methane formation can be attained through several mechanisms of action. The direct inhibition of methanogens is one of them (McAllister and Newbold 2008). The strategies to control rumen methanogenesis are effective when reducing the rumen methane formation, without affecting the fiber-degrading microorganism populations (Soliva *et al*. 2003).

In studies of González *et al*. (2010) it was assessed the effect of four mulberry varieties (Acorazonada, Cuban, Tigreada, and Indonesia) on the rumen methane formation, but it was not determined their action on the rumen microorganisms or on the products formed as a result of their activity, aspects needed to account for the forms intervening in the methane decline. For being mulberry a plant used in ruminant feeding with excellent productive results, it is noteworthy to control its action on rumen populations. However, physiological studies accounting for this effect are not available.

This work had as objective to determine the action of four mulberry varieties (*M. alba* Linn) on the microbial populations and fermentative products under *in vitro* conditions with rumen liquid from river buffaloes (*Bubalus bubalis*).

Materials and Methods

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

Animals and diet. Two river buffaloes (Bufalipso crossbred) were used as rumen liquid donors. They were two adult males, fitted with simple rumen cannula and having average weight of 453 kg. They were allocated in individual pens, under shade and with free access to water and feeds. They were all given star grass forage (*Cynodon nlemfuensis*) without supplementation.

Treatments. Five treatments were evaluated including different mulberry varieties: 1) 100 % of star grass (SG) (*Cynodon nlemfuensis*) (control), 2) SG + Cuban, 3) SG + Acorazonada, 4) SG + Tigreada, and 5) SG + Indonesia.

The inclusion level for all the mulberry varieties was 30 %. It was the one applied for cow milk production in researches developed at the "Indio Hatuey" Experimental Station of Pastures and Forages, in Matanzas, Cuba, (Ojeda 2006, personal communication).

The mulberry varieties (*Morus alba* Linn.) came from a plantation with three years of establishment. It was from the "Indio Hatuey" Experimental Station of Pastures and Forages, in Matanzas, Cuba. They were sown on lixiviated red ferrallitic soil and they were fertilized with poultry litter. At 60 d after sprout and in the rainy season, the leaves with the petioles and the young stems were cut manually, to simulate the animal selection. Star grass (*Cynodon nlemfuensis*) was collected from grasslands at the Institute of Animal Science, located in the Mayabeque province. The cut

was performed manually, at a height of 10 cm from the soil.

The plant material was dried in an oven at 60 ºC for 48 h. Later, it was ground until reaching 1-mm particle size. The chemical composition of the treatments is shown in table 1.

Table 1. Chemcial composition of the treatments, %

Treatment	Ash	Ca	P	CP	NDF	ADF	Lig	Cel
Star grass	11.33	0.57	0.33	9.00	68.78	39.22	6.56	29.58
SG + *M. alba* L. cv. Cuban	10.34	0.97	0.32	13.76	56.52	32.36	6.09	24.06
SG + *M. alba* L. cv acorazonada	11.24	0.97	0.30	13.58	57.45	33.13	6.31	24.58
SG + *M. alba* L. cv. tigreada	11.68	1.01	0.32	16.79	56.27	32.85	7.10	24.11
SG + *M. alba* L. cv. indonesia	11.85	1.22	0.31	10.82	57.03	32.75	6.79	24.09

Experimental procedure. The material (0.5 g) corresponding to each treatment was weighed and added to 100 mL glass bottles.

The fasting animals were extracted rumen liquid through the cannula with a vacuum pump. It was kept in a hermetically sealed thermos 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 portion of buffering solution of Menke and Steingass (1988) and it was agitated for a few seconds in a domestic blender to separate the microorganisms adhered to the fiber. Later, the filter of this portion was incorporated to the liquid fraction. The rumen fluid was kept in CO_2 atmosphere.

Fifty milliliters of mixture of rumen liquid and buffering solution of Menke and Steingass (1988) were added to each bottle in 1:3 (v/v) ratio. The bottles were sealed with tap of butyl and agrafe. Later, they were randomly put in water bath at 39 °C of temperature.

The culture and count of total viable, cellulolytic, proteolytic and methanogenic bacteria and cellulolytic fungi were performed at 0, 4, and 8 h of fermentation. The count of protozoa, the pH measurement and the determination of the ammonia concentration were conducted at 0, 4, 8, 12, and 24 h of incubation.

Chemical analysis. It was performed according to the techniques of AOAC (1995). The fibrous fractions were analyzed by the procedure of Goering and van Soest (1970). The concentration of NH_3 was determined according to Conway (1957) and the pH was measured through the reading of digital Sartorius pHmeter.

Microbiological analysis. The culture technique of Hungate (1970) was used in roll tubes and under strict anaerobiosis conditions.

The culture of total viable, cellulolytic and proteolytic bacteria was performed in the culture media of Caldwell and Bryant (1966), modified by Elías (1971). For the proteolytic bacteria, 10 % of skim milk was added, according to Galindo *et al*. (1984). The culture medium of Joblin (1981) was used for determining the fungi population. The methanogenic microorganisms were grown on the culture medium of Anderson and Horn (1987), with influence of hydrogen and carbon dioxide mixture (60:40).

Three dilutions were used for the inoculations. Each was replicated three times.

For the count of total viable, cellulolytic, proteolytic, and methanogenic bacteria and fungi, the roll tubes were put under a magnifying glass. The colonies having digestion hale were counted. The results were expressed in colony forming units (cfu) in bacteria, and in thallus forming units (tfu) in fungi.

The protozoa were preserved in formol at 10 %. Later, they were counted directly in the optic microscope in Neubauer chamber, after staining them with Gentian violet solution at 0.01 % in glacial acetic acid.

Experimental design and mathematical analysis. A complete random design was used with 5 x 3 factorial fit (five treatments and three sampling hours) for the measures of microbial populations (bacteria and fungi) and with 5 x 5 (five treatments, five sampling hours) for the indicators pH, ammonia concentration, and protozoa counts. Four repetitions were performed in time. In order to examine the results, multivariate analysis of variance was applied. When there was interaction between the treatments and the sampling times, a split plot model was applied, the principal plot corresponding to the treatments, and the subplot to the sampling times. When there was not interaction, a linear model was used for the effects of treatment and sampling times. Duncan's (1955) multiple range test was applied for $P < 0.05$ when necessary. The statistical software INFOSTAT proposed by por Balzarini *et al*. (2001) was used.

Results and Discussion

There was interaction between the treatments and the sampling times, only for the count of methanogenic bacteria and the pH.

Table 2 shows the counts of rumen methanogenic bacteria for the five treatments under study. In time, the number of methanogens did not vary for the different

treatments, except when including 30 % of mulberry cv. Acorazonada, declining in this population of microorganisms at eight hours of fermentation. At this time, the treatments including the different mulberry varieties were compared, and none differed from the control. However, the Acorazonada variety showed the lowest counts of methanogenic bacteria.

of methanogens (Tavendale *et a*l. 2005). González *et al*. (2010) made the determination of condensed tannins in mulberry varieties, and found that none had estimable amounts of condensed tannins. Therefore, this type of secondary metabolite does not seem to have action on the population of methanogens, which helps to account for the few variations between the treatments in the

Table 2. Effect of four mulberry (*Morus alba* L.) varieties on the methanogen population of the buffalo rumen (x 10^9 cfu • mL^{-1})

Treatment	Fermentation time, hours		
	0	4	8
Star grass	3.28^{bc} (33.18)	3.15^{abc} (26.44)	3.19^{abc} (36.04)
M. alba L.cv. Cuban	2.82^{abc} (19.38)	3.31^{bc} (29.58)	3.75^{bc} (47.64)
M. alba L. cv. acorazonada	3.41^{bc} (35.06)	3.87^{c} (54.10)	1.96^{a} (13.46)
M. alba L. cv. tigreada	3.15^{abc} (26.58)	2.84^{abc} (19.13)	3.99^{c} (84.72)
M. alba L. cv. indonesia	2.52^{ab} (17.38)	3.45^{bc} (35.20)	3.70^{bc} (60.20)

Data transformed according to ln. Original means between parentheses.
SE ±=0.56 the hours level of the treatments.
SE ±=0.46 the treatments at the same level of the hours or different.
Means with different letters differ at $P < 0.05$ (Duncan 1955)
**$P < 0.01$

The methanogens are the microorganisms in charge of the methane formation in the rumen (Attwood *et al*. 2008). When considering the methane volumes obtained by González *et al*. (2010), in assessing the same treatments for the control of the rumen methanogenesis, and finding that the Cuban variety was the one producing less methane, it was, then, expected that it were this, and not the Acorazonada, the one decreasing the number of methanogens. In this instance, the fall in methane production does not seem to be by direct effect on the methanogens.

According to Cook *et al*. (2008), the methanogens are compulsory anaerobic, hard to cultívate in the laboratory. At present, it is recognized that the cultivable species do not represent the diversity of the native rumen methanogenic population, which could lead to an underestimation of the real amounts in the rumen. In this experiment, the counts of methanogenic microorganisms were performed through the utilization of the traditional culture methods, thus, it is also possible that many of the methanogens did not grow on this medium. This consideration could also account for the decline of these microorganisms, observed in the Acorazonada variety.

In some plants such as legumes, condensed tannins proved their toxicity for the methanogens (Hess *et al*. 2003). However, few reports were available about the inhibiting effects of the tannins on pure crops cultures

rumen population.

The count of the rest of rumen populations is shown in table 3. The inclusion of 30 % in the ration of *M. alba* L. cv. Cuban, and in the Acorazonada, the Tigreada and Indonesia, did not produce variations in the populations of total viable, celullolytic, proteolytic bacteria and celullolytic fungi, compared with the control of star grass. However, the counts of protozoa were higher for the Cuban.

From the point of view of the fibrous fraction degradation in the rumen, it is favorable that no negative effects can be produced in the populations of celullolytic bacteria and fungi, when including 30 % of any of the four mulberry varieties. Thus, if any of these varieties of *M. alba* L. manages to reduce the rumen methane production, it may be selected as alternative to control the rumen methanogenesis because it would fulfill with the statement of Soliva *et al*. (2003). This means that the strategies used to decrease the rumen methane production should be performed in a way that they do not affect the microoganisms involved in the fiber degradation process.

Protozoa establish ectosymbiotic and endosymbiotic relations with the rumen methanogens (Finlay *et al*. 1994 and Ohene-Adjei *et al*. 2007), which favors the methane formation in the rumen. When connecting the protozoa counts in this work with the methane volumes reported by González *et al*. (2010), who evaluated 30 % of these

Table 3. Effect of including four mulberry varieties (*M. alba* L.) on some populations of buffalo rumen microorganisms

Microbial population	Treatments					SE ±
	Star grass	*M. alba* L.cv. Cuban	*M. alba* L. cv. acorazonada	*M. alba* L.cv. tigreada	*M. alba* L.cv. indonesia	
Total viable bacteria (10^{11}cfu•mL^{-1})	3.02 (32.27)	3.54 (41.95)	3.61 (52.62)	3.46 (40.48)	3.69 (48.09)	0.21
Cellulolytic bacteria (10^{4}cfu•mL^{-1})	2.85 (19.80)	2.86 (20.64)	2.91 (22.81)	3.12 (25.59)	3.07 (21.70)	0.16
Proteolytic bacteria (10^{6}cfu•mL^{-1})	3.04 (31.10)	3.46 (47.98)	3.55 (41.37)	3.33 (33.34)	2.93 (26.40)	0.24
Hongos celulolíticos (10^{3}uft•mL^{-1})	2.28 (14.54)	2.47 (18.75)	2.72 (27.63)	2.53 (17.48)	2.75 (21.82)	0.26
Protozoa (10^{4}cel•mL^{-1})	0.05[a] (1.06)	1.04[b] (4.29)	0.30[a] (1.75)	0.16[a] (1.34)	0.14[a] (1.63)	0.19**

cfu, colony forming units
tfu thallus forming units
cel.mL cells/mL
Data transformed according to ln
Original means between parentheses
Means with different letters differ at $P < 0.05$ (Duncan 1955)
** $P < 0.01$

same mulberry varieties, it should be expected that the Cuban, which produced the lower methane volumes, had also the lower counts of protozoa. However, the opposite happened. This confirmed the criterion that the lower methane formation, with 30 % of this variety, does not seem to be the result from the direct inhibition of methanogens or protozoa in the rumen, but from other factors that should be further studied.

The defaunation or reduction of protozoa is one of the ways to decrease the methanogens and the methane production in the rumen.

In several laboratories, studies have been developed with large amount of plants and their secondary compounds, such as saponins and tannins (Galindo *et al*. 2001 and Patra *et al*. 2006). The supressing effect on the methane production exerted by the saponins seem to be related to the direct action against the rumen microorganisms involved in the methane formation, such as the methanogens and the protozoa (Hu *et al*. 2005). In this work, the presence of saponins was not determined, but not finding declines in the protozoa counts when including in the ration different mulberry varieties suggests that these plants do not contain this type of secondary metabolite. This evaluation would be in correspondence with results from previous studies, conducted by Maldonado *et al*. (2000) and García (2003), proving the presence of saponins in *M. alba* L.

The pH values (table 4) diminished in time for all the treatments, but they were always next to neutrality. This decrease is expected, because the experiment was performed under *in vitro* conditions, where there is accumulation of end products from the fermentation. According to Valdez-Vázquez and Poggi-Varaldo (2009), under *in vitro* systems, the decline in the pH is, among other factors, due to the accumulation of organic acids.

Most of the methanogens grow under narrow pH values (6-8) (Valdez-Vazquez and Poggi-Varaldo 2009). In this experiment, the pH values were not lower than six, thus, it is considered that this indicator did not exert either suppressing effect on the methanogen population. Although the cellulolytic activity was not determined, it was not considered either that the pH had negative effect on the fiber degradation because it could have been reflected in the cellulolytic populations, which did not show either variation for any of the treatments.

The concentrations of rumen ammonia did not have differences for the treatments under study (table 5). It is known that non-protein nitrogen and the soluble fractions of the proteins are usually degraded in the rumen (Kandilys *et al*. 2009), and one of the end products from the fermentation of these compounds is the ammonia. Wohlt *et al*. (1973) and Kretovich (1986) reported that the feeds composed mainly of albumins and globulins have higher solubility in water and saline solutions compared with the proteins formed primarily by prolamins and glutelins. If the protein fractionation of mulberry by Kandylis *et al*. (2009) is considered, finding the higher protein fraction was of prolamins and albumins (44.1 and 11.1 % of the total true protein, respectively), and the NPN values were moderate, it could be explained why the mulberry inclusion did not no provoke variations in the ammonia concentration.

The highest proportion of insoluble proteins that could be present in mulberry suggests that the highest protein degradation in this plant occurs in the lower parts of the gastrointestinal tract. This could explain the excellent productive results found by Benavides

Table. 4. Effect of the inclusion of four varieties of *M. alba* L. on the *in vitro* rumen pH

Treatment	Fermentation hours				
	0	4	8	12	24
Star grass	7.07^{h}	7.09^{h}	7.06^{h}	6.68^{cdefg}	6.38^{abc}
M. alba L. cv. Cuban	7.00^{gh}	6.97^{fgh}	6.74^{defgh}	6.53^{abcd}	6.35^{abc}
M. alba L. cv. acorazonada	7.05^{h}	6.88^{defgh}	6.88^{defgh}	6.60^{cde}	6.30^{ab}
M. alba L. cv. tigreada	6.98^{gh}	6.93^{efgh}	6.83^{defgh}	6.62^{bcdef}	6.27^{ab}
M. alba L. cv. indonesia	6.99^{gh}	6.83^{defgh}	6.82^{defgh}	6.58^{bcde}	6.19^{a}

SE ± =0.14 the hours level of the treatments.
SE ± =0.14 the treatments at the same level of the hours or different.
Means with different letters differ at $P < 0.05$ (Duncan 1955)
**$P < 0.01$

Table 5. Effect of the inclusion of four varieties of *M. alba* L. on the concentration of buffalo rumen ammonia ($mmol.L^{-1}$)

Indicator	Treatments					SE ±
	Star grass	*M. alba* L.cv. Cuban	*M. alba* L.cv. acorazonada	*M. alba* L.cv. tigreada	*M. alba* L.cv. indonesia	
NH_3	10.35	12.39	13.07	12.92	10.81	0.83

(2000), González and Milera (2000) and Ba *et al*. (2005), when giving *M. alba* L. to growing sheep and goats. The similar concentration of rumen ammonia for all the treatments was in correspondence with the counts of proteolytic bacteria that, as known, are responsible for degrading the proteins to end products in the rumen, such as the NH3.

It was concluded that the mulberry varieties: Acorazonada, Cuban, Tigreada, and Indonesia, when included at 30 % did not have negative effects on the populations of fiber degrading microorganisms, or did not affect either the pH or the rumen ammonia concentration.

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