Effect of tropical plant foliage on the control of methane production and *in vitro* ruminal protozoa population

Juana Galindo, Niurca González, Yoandra Marrero, Areadne Sosa, T. Ruiz, G. Febles, Verena Torres, Ana I. Aldana, G. Achang, Onidia Moreira, Lucía Sarduy and Aida C. Noda

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

In order to evaluate the effect of tropical plant foliage on the control of methane production and protozoa population, an experiment under *in vitro* conditions was carried out, where the foliage of 12 trees was used and compared to the control of Star grass (*Cynodon nlemfuensis*). Plants evaluated were: *Leucaena leucocephala*, *Gliricidia sepium*, *Samanea saman*, *Albizia lebbeck*, *Azadirachta indica*, *Moringa oleifera*, *Pithecellobium dulce*, *Cordia alba*, *Guazuma ulmifolia*, *Enterolobium cyclocarpum*, *Tithonia diversifolia* plant material (pm) 10 and *Tithonia diversifolia* pm 23. Methane production and protozoa population were determined. Regarding the ability of producing methane, plants were divided into four groups: (1) control, (2) plants able to reduce, at a great extent, methane production in the rumen, (3) group integrated by *Enterolobium cyclocarpum*, and (4) plants that can be used for reducing the ruminal methanogenesis, but at a low extent. All plants reduced the protozoa population and its populations were: 4.5; 3.7; 4.5; 4.6; 4.6; 4.5; 4, 4,6; 6; 6; 5; 6 and 9 x 10^6 cells.mL^{-1} for *S. saman*, *A lebbeck*, *T. diversifolia* pm 23, *C. alba*, *L. leucocephala*, *P. dulce*, *M. oleifera*, *G. sepium*, *G. ulmifolia*, *T. diversifolia* pm 10, *E. cyclocarpum* and *C. nlemfuensis*, respectively. It can be concluded that the evaluated plants can be used in the animal diet for reducing methane production and ruminal protozoa population.

Key words: *fermentation, rumen, trees, secondary metabolites*

The use of trees and shrubs of legumes or other species as a supplement for ruminant diets has been very important during the last 10 years. These plants have some characteristics, like the presence of secondary metabolites, which make them very valuable (Kamra *et al.* 2006). Secondary metabolites can modify the speed of degradation and path of nutrients through whole the gastrointestinal tract (Pedraza 2000, Rodríguez 2010, La O *et al.* 2011, Galindo *et al.* 2012 and Galindo *et al.* 2013) as a result of a direct effect on the ruminal ecology.

Cunningham (1997) has indicated that protozoa ingest great volumes of bacteria and maintain constant their population within the rumen, so that animal extinction means the disappearance of ecological relations (predation and competence) which affect the type, generic distribution and metabolic activity of fungi and bacteria population of the ruminal ecosystem. The A type protozoa, like *Polyplastrom multivesiculatum*, act as predators of cellulolitic bacteria, specifically of *Butyrivibrio fibrisolvens*, *Ruminococcus flavefaciens*, regarding the aminolitic bacteria like *Selenomonas ruminantium*, *Streptococcus bovis*, or the acidophilic species like *Megasphaera elsdenii*. However, B type protozoa, which include the cellulolitic protozoa like *Epidinium ecaudatum*, *Eremoplastron bovis* y *Eudiplodinium maggii*, are also predators of cellulolitic bacteria, although their process is slower.

The reduction of protozoa population favors the increase of the population of cellulolitic microorganisms, the stabilization of pH within the rumen, the decrease of free ammoniac level, the reduction of methanogenesis and the increase of efficiency of digestive use of different diets, mainly the fiber diets (Makkar 2005). The objective of this study was to evaluate the effect of tropical plant foliage on the control of methane production and *in vitro* protozoa population

Materials and Methods

The experiment was performed under *in vitro* conditions, using the technique of Theodorou *et al.* (1994).

Experimental treatments. Treatments were the plants to be evaluated: *Leucaena leucocephala*, *Gliricidia sepium*, *Samanea saman*, *Albizia lebbeck*, *Azadirachta indica*, *Moringa oleifera*, *Pithecelobium dulce*, *Cordia alba*, *Guazuma ulmifolia*, *Enterolobium cyclocarpum*, *Tithonia diversifolia* pm 10 and *Tithonia diversifolia* pm 23.

Trees were part of the arboretum from the Institute of Animal Science, located in San José de las Lajas municipality, Mayabeque province, Cuba, at 92 m o.s.l., 22º53' N and 82º02' W. The soil is fersialitic undulating, with 4.84 % of organic matter, 0.26 % of total nitrogen, 40.59 ppm of phosphorus, 4.60 % of calcium, 0.46 % of magnesium and 6.34 of pH. Trees were around seven years old.

The fractions leaves + petiole + green pods were collected from the trees selected to develop the study, resembling the browsing made by animals. Once they were collected, they were spread over an asphalt plate in order to dry them under the sun for three consecutive days. Later, trees and star grass samples were ground in a mill until reaching the size of a particle with 1mm.

Star grass was obtained in an area of the Institute, and it had three years of establishment, with 45 d of rest. For preparing it, leaves with petioles were collected,

resembling the bite of an animal. The sample was dried in an oven at 60 ºC for 48 h.

The chemical composition of the plants was determined using the methodology proposed by AOAC (1995). The fiber fraction was quantified, according to the protocol described by van Soest and Robertson (1991). Minerals were determined by an atomic absorption spectroscopy, with SP-9 Pey Unicam equipment.

The phyto-chemical sieving, for determining the qualitative content of secondary metabolites, was carried out according to the method of Rondina and Cussio (1969), described by Alfonso *et al.* (2000). For the description of assays, the system of crosses was used to specify the presence or absence of metabolites in the analysis.

Experimental procedure. A total of 34 sealed bottles, of 100 mL each, was used to incubate the food samples into rumen liquor and a buffer environment. An amount of 30 mL of a mixture of rumen liquor and buffer solution was poured into each bottle, at a rate of one part of rumen liquor and two parts of buffer solution. The feed was included at a rate of 0.3 g treatment^{-1}. Out of that amount, 30 % corresponded to the plant to be evaluated, and the rest corresponded to *C. nlemfuensis*.

Animal donors of rumen liquor. Two adult male water buffaloes (Bufalipso crossbred), with a simple cannula in rumen and mean weight of 453 kg, were used as donors of rumen liquor. The animals were kept into individual cages, in the shadow and with free access to water and feed. They received forage of star grass (*C. nlemfuensis*) and were supplemented with 1 kg/d of commercial feedstuff.

Procedure for extracting rumen liquor. The rumen liquor was extracted of fasting animals through the cannula, with the help of a vacuum pump. It was kept into thermos for guaranteeing the temperature conditions (39 ºC) and anaerobiosis during the transportation to the lab. Once the sample was in the lab, it was filtered with muslin. A rumen liquor pool of both buffaloes was used to create the mixture to be fermented, with the purpose of eliminating the animal effect. The production of *in vitro* CH_4 and protozoa counting was determined.

Methane determination. Methane was determined by a gas chromatography, using a chromatograph Philips PU-4400, with capillary column of 25 meters and estacionary phase DB^{-1}. A FID detector and H_2 (1mL•min^{-1}), as carrier gas, were used. The temperature of the detector and of the injector was of 200 ºC, and the temperature of the column was 60 ºC. An amount of 1 mL of gas contained in the syringe was injected and calculation of methane concentration was carried out using the equation obtained from the calibration curve: $y = 0.0001x + 2.8515$ ($R^2 = 0.99$).

Protozoa count. The Neubauer chamber was used for counting protozoa. For that purpose, the protozoa were dyed with a solution of gentian violet 0.01 %, in glacial acetic acid at 1 %.

Protozoa counts were transformed according to Log N, to guarantee the normal conditions in the growth curve. For the analysis, the applied formula was (K+N).10x, where:

K is the constant that represents the logarithm of the dilution in which the microorganisms were counted,

N is the counting logarithm determined as cells x mL^{-1},

10 X is the dilution in which the inoculation was carried out.

Samplings. Samplings were performed at 4, 8, 12 and 24 h of incubation. Four replications were carried out in a row.

Experimental design and statistical analysis. A completely randomized experimental design was used, in a 13x4 factorial arrangement (13 treatments or plants and four sampling moments). The statistical treatment of experimental results was made according to the experimental design used, with the purpose of identifying the interaction between treatments (plants) and time of fermentation (hours of sampling). For the methane production indicator, a Cluster analysis was performed. The test of Duncan (1955) at $P < 0.05$ was used for the necessary cases. The statistical analyses were carried out with the statistical package SPSS+ (Visauta 1998).

Results and Discussion

Table 1 shows the chemical composition of the evaluated plants. The content of CP is between 14.25 % in *Azadirachta indica* and 29.47 % in *Leucaena leucocephala*. All the plants had a high protein value. These results coincide with those reported by Rodríguez (2010), La O *et al.* (2011) and La O *et al.* (2012).

Results obtained by Ku Vera *et al.* (2000), Ku Vera (2005), García *et al.* (2008), Hart *et al.* (2008) and Ku Vera (2013) have demonstrated that foliage of tropical trees has a high nutritional value, which is very good as cattle feed, and that its use can contribute to the reduction of production costs.

Azadirachta indica was included in the evaluation only because of its content of secondary metabolites, which could have effect on the reduction of methane production and, strategically, be a source of the referred metabolites for their use as additive. Every plant showed substantial and moderate amounts of secondary metabolites. Table 2 shows the phyto-chemical sieving of them.

With the qualitative tests of phyto-chemical sieving of these trees and shrubs, secondary metabolites indicated a significant presence (+++) of tanins in *A. indica*, *L. leucocephala* and *E. cyclocarpum* and moderate amounts of this metabolite in *S. saman*, *G. ulmifolia*, *M. oleifera*, as well as in two evaluated plant material of *T. diversifolia*.

No plant was highlighted due to the presence of high amounts of saponins. The most remarkable were

Table. 1. Chemical composition of the evaluated protein plants, % of DM

Plant	CP	NDF	ADF	Ash	Ca	P
Samanea saman	18.15	41.45	29.51	6.92	1.26	0.32
Albizia lebbeck	17.18	49.86	34.69	6.54	1.17	0.20
Azadirachta indica	14.25	41.00	28.92	3.92	2.28	0.28
Tithonia diversifolia pm 23	23.95	33.43	29.54	5.81	2.14	0.35
Cordia alba	18.78	38.43	29.16	5.65	1.32	0.23
Leucaena leucocephala	29.47	39.96	19.26	8.91	1.18	0.28
Pithecelobium dulce	19.15	37.42	28.15	7.12	2.23	0.45
Moringa oleifera	22.60	35.73	25.54	9.57	1.28	0.24
Gliricidia sepium	23.83	42.83	24.96	11.39	1.48	0.34
Guazuma ulmifolia	19.36	52.65	41.12	9.74	2.10	0.41
Tithonia diversifolia pm 10	24.20	35.30	30.40	5.94	2.3	0.38
Enterolobium cyclocarpum	15.59	63.94	42.99	11.80	0.97	0.20
Cynodon nlemfuensis	11.90	41.06	39.95	12.87	0.54	0.08

Table 2. Phyto-chemical sieving of the plant evaluated in the research

Plant	Tannins	Flavonoids	Saponins	Triterpenes	Steroids	Anthocyanidins	Reducers	Alkaloids
Samanea saman	++	+	+	++	++	+	+	+++
Albizia lebbeck	+	+	++	+	+	+	++	+++
Azadirachta indica	+++	+	+	+	+	+	++	+++
Tithonia diversifolia pm 23	++	++	+	+	++	+	+++	++
Leucaena leucocephala	+++	+	++	++	++	+	+++	+++
Pithecelobium dulce	+	-	+	+	+	-	+	++
Moringa oleifera	++	-	+	+++	-	-	+	++
Gliricidia sepium	+	+	++	+	+	+	ND	-
Guazuma ulmifolia	++	-	+	+++	-	-	+	++
Tithonia diversifolia pm 10	++	-	++	++	++	+	+++	++
Enterolobium cyclocarpum	+++	+	++	+	+	-	++	++

(+++) High; (++) Moderate; (+) Low; (-)No presence; ND- not detected

A. lebbeck, *L. leucocephala*, *G. sepium*, *T. diversifolia* pm.10 and *E. cyclocarpum*, which presented a moderate response (++), although there was low (+) presence of this metabolite in the rest of the plants. The presence of reducers (+), flavonoids, triterpenes, steroides, alkaloids and anthocyanidins was variable among the different plants, while in *M. oleifera*, there was no presence (-) of steroids, anthocyanidins and flavonoids.

There was no significant interaction between time of fermentation and methane production of the plants evaluated in this study. Therefore, results of the treatments are presented independently.

The study demonstrated that evaluated plants, except *E. cyclocarpum*, in association with star grass (*C. nlemfuensis*), are able to reduce the methane production within the rumen. *S. saman*, *A. lebbeck*, *A. indica* and *T. diversifolia* pm 23 are considered among the most promising, although *C. alba*, *L. leucocephala*, *P. dulce* and *M. oleifera* (table 3) are also very important.

Different groups of researchers develop strategies for reducing methane production using microorganisms from the Archaea, commonly known as ruminal methanogens (Agarwal *et al.* 2008 and Beauchemin *et al.* 2008), highlighting the antibiotics, methane halogens, chemical products and lipids. However, the abilities of tropical shrubs and trees for reducing methane production have been demonstrated in the last decade (González 2010).

As figure 1 shows, at the end of the evaluation of the effect of fermentation time on the ruminal methane production, the highest production of gas within the rumen was reached after eight hours (39.59 μL. gDM^{-1}), and its concentration was reduced at 12 and 24 h (30.91 and 29.85 μL.gDM^{-1}, respectively).

Table 3. Effect of the foliage of different protein plants on ruminal methane production, µL. gDM^{-1}

Plant	CH_4
Samanea saman	4.3[a]
Albizia lebbeck	5.73[a]
Azadirachta indica	8.59[a]
Tithonia diversifolia pm 23	9.2[a]
Cordia alba	11.76[a]
Leucaena leucocephala	16.38[a]
Pithecelobium dulce	20.03[a]
Moringa oleifera	25.33[a]
Gliricidia sepium	29.02[ab]
Guazuma ulmifolia	37.98[ab]
Tithonia diversifolia pm 10	47.15[ab]
Enterolobium cyclocarpum	64.71[b]
Cynodon nlemfuensis	65.15[b]
SE ±	1.20***

[a, b] Means with different letters differ *** P < 0.001

It was evident that microorganisms of the rumen started to have their best development in substances contained in plants after the eight hour because they require a specific amount of time for colonization. Once the feed start their fermentative process, final products are obtained, like methane in this case. This is demonstrated because after four hours of initiated the fermentation, the highest amount of methane has not been produced (9.66 µL.gDM^{-1}).

A Cluster analysis was carried out using the results obtained from the methane production of different plants. The dendrogram shows the formation of four main groups (figure 2).

The groups are described in table 4. Group 1 was formed by the control plant, the grass *C. nlemfuensis* (star grass). Group 2 included plants capable of reducing, in a large amount, methane production within the rumen (*T. diversifolia* pm 23, *A. indica*, *L. leucocephala*, *A. lebbeck*, *S. saman*, *P. dulce* and *C. alba*). Group

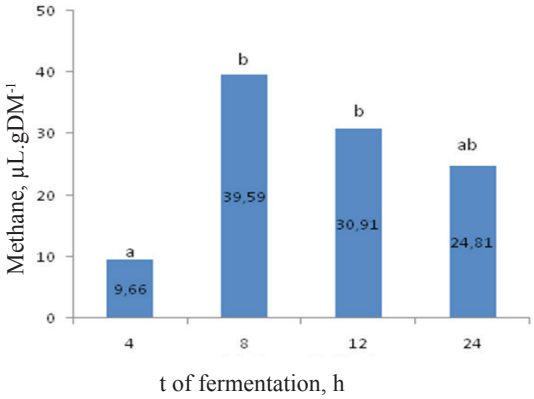

Figure 1. Effect of fermentation time on the ruminal methane production (µL.gDM^{-1}), SE± 0.31***

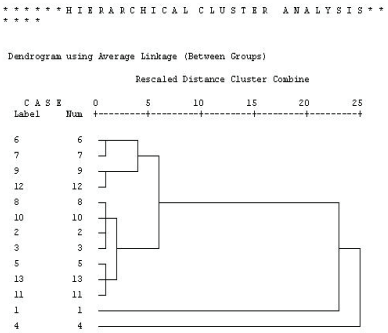

Figure 2. Dendrogram of ruminal methane production values for evaluated plants

3 was integrated by *E.cyclocarpum*, which produces ruminal concentrations of methane similar to control, but, apparently, it shows different characteristics. The explanation of this aspect was not properly clarified, so further studies are required. Group 4 is formed by plants that can be used for reducing ruminal methanogenesis, even though their extent is not so highlighted as those included in group 2 (*G. sepium*, *M. oleifera*; *G. ulmifolia* and *T. diversifolia* pm 10).

One of the most remarkable results of this study refers to the effect of tree foliage on ruminal protozoa population. Figure 3 shows the protozoa population

Table 4. Groups of plants using the Cluster Analysis

Groups	Plants
1	*Cynodon nlemfuensis*
2	*Tithonia diversifolia* pm. 23 *Azadirachta indica* *Leucaena leucocephala* *Albizia lebbeck* *Samanea saman* *Pithecelobium dulce* *Cordia alba*
3	*Enterolobium cyclocarpum*
4	*Gliricidia sepium* *Moringa oleifera* *Guazuma ulmifolia* *Tithonia diversifolia* pm 10

(10^5 cells mL^{-1}) in the rumen of animals fed with different tropical plants. As it was confirmed, all plants reduce protozoa population, regarding the control of star grass. AAlbizia, Samanea, Azadirachta, Moringa, Pithecelobium, Cordia, Leucaena, Gliricidia, Enterolobium, and the two plant materials of *T. diversifolia* are the most remarkable.

Results obtained by Galindo *et al.* (2001a and 2001b) indicated that ruminal protozoa decrease in presence of leucaena, gliricidia and titonia. Recent reports of Galindo *et al.* (2011) and Galindo *et al.* (2012) state that *S. saman* and *A. lebbeck* reduce the cited microbial groups.

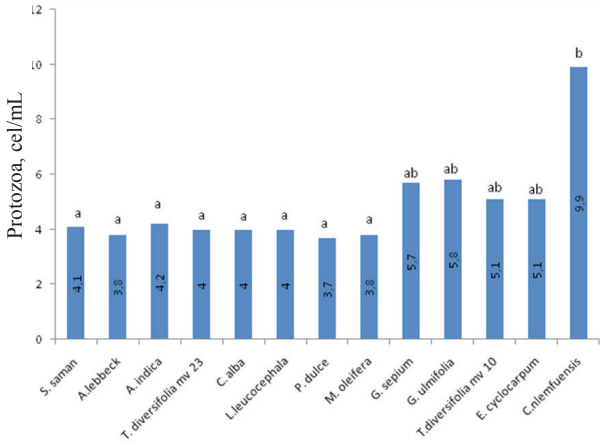

Figure 3. Effect of foliage of different tropical plants on the population of ruminal protozoa. SE ± 0.25***

Joblin (2004) has informed that methanogens have a symbiotic relation with ruminal protozoa. Any exogenous factor, able of diminishing the protozoa population within the organ, will reduce the amount of methanogens and, consequently, the methane production (Kobayashi 2010).

It can be concluded that tropical plants like *S. saman*, *A. lebbeck*, *A. indica*, *T. diversifolia* pm 23, *C. alba*, *L. leucocephala*, *P. dulce*, *M. oleifera*, *G. sepium*, *G. ulmifolia*, *T. diversifolia* pm 10 and *E. cyclocarpum* can be used to reduce methane production and *in vitro* ruminal protozoa population.

References

Agarwal, N., Kamra, D.E., Chatterjee, P.N., Kumar, R. & Chaudhary, L.C. 2008. *In vitro* methanogenesis, microbial profile and fermentation of green forages with buffalo rumen liquor as influenced by 2 bromoethanesulphonic acid. Asian Austr. J. Anim. Sci. 21:818

Alfonso, M., Fernández, L., González, N. & Avilés, R. 2000. La Achira (*Canna edulis* Ker.) y su potencialidad en el control de plagas. XII Forum de Ciencia y Técnica. INIFAT. Ciudad de La Habana, Cuba. 11 p.

AOAC. 1995. Official Methods of Analysis (15th Edition). Assoc. Off. Anal. Chem. Artlington, V. A.

Beauchemin, K.A. Kreuzer, M., Mara, O. & McAllister. 2008. Nutritional management for enteric methane abatement´s review. Australian J. Experimental Agric. 48:21

Cunningham 1997. Fisiología veterinaria. Second edition. McGraw-Hill Interamericana. México

Duncan, D.E. 1955 Multiple range and multiple F. test. Biometrics 11:1

Galindo, J., González, N., Alberto, M., Abdalla, A.L., Marrero, Y., Sosa, A., Aldana, A.I., Moreira, O., Sarduy, L. & Herrera, M. 2013. Evaluación del efecto del fruto de *Sapindus saponaria* en la población de protozoos del rumen y su relación con la producción de metano en condiciones *in vitro*. XXIII Congreso Internacional de la ALPA, La Habana, Cuba

Galindo, J., González, N., Aldama, A.I. & Marrero, Y. 2001a. Effect of *Enterolobium cyclocarpum* on rumen microbial population and its activity under *in vitro* conditions. Cuban J. Agric. Sci. 35:229

Galindo, J., González, N., Aldama & A.I., Marrero, Y. 2001b. Effect of *Gliricidia sepium* on rumen protozoa population and cellulolytic organisms. Cuban J. Agric. Sci. 35:223

Galindo, J., González, N., Scull, I., Marrero, Y., Sosa, A., Aldana, A.I. Moreira, O., Delgado, D., Ruiz, T., Febles, G., Torres, V., La O, O., Sarduy, L., Noda, A. & Achang, O. 2012. Effect of *Samanea saman*, (Jacq.) Merr, *Albizia lebbeck* Benth and *Tithonia diversifolia* (Hemsl.) Gray (plant material 23) on the methanogen population and on the ruminal microbial ecology. Cuban J. Agric. Sci. 46:273

Galindo, J., González, N., Sosa, A., Ruiz, T., Torres, V., Aldana, A.I. Díaz, H., Moreira, O., Sarduy, L. & Noda, A.C. 2011. Effect of *Tithonia diversifolia* (Hemsl.) Gray (Giant Mexican Sunflower) on the population of rumen protozoa and methanogens under *in vitro* conditions. Cuban J. Agric. Sci. 45:33

García, D.E., Medina, M.G., Cova, L.J., Torres, A., Soca, M., Pizzani, P., Baldizán, A. & Domínguez, C.E. 2008. Preferencia de vacunos por el follaje de doce especies con potencial para sistemas agrosilvopastoriles en el Estado Trujillo, Venezuela. Pastos y Forrajes 31:3

González, N. 2010. Evaluación de morera (*Morus alba* Lin) en la fermentación y control de la metanogénesis ruminal de búfalos de río (*Bubalus bubalis*). PhD. Thesis. Instituto de Ciencia Animal, Mayabeque, Cuba

Hart, K. J., Yañez-Ruiz, D.R., Duval, S.M., Mc Ewans, N.R, & Newbold, C.J. 2008. Plant extracts to manipulate rumen fermentation. Animal Feed. Sci. Technol. 147:8

Joblin, K.N. 2004. Methanogenic Archaea. In: I Planning Meeting of Project Contract Research and Training Workshop "Development and Use of Rumen Molecular Techniques for Predicting and Enhancing Productivity". Brisbane

Kamra, D.E., Agarwal, N. & Chaudhary, L.C. 2006. Inhibition on ruminal methanogenesis by tropicals plants containing secondary compounds'. Int. Congress Ser. 1293: 156

Ku Vera, J. 2005. Uso de árboles para la alimentación animal en el estado de Yucatán, México. Memorias II Curso Internacional Silvopastoril Cuba- Colombia. Neiva-Bogotá-Valledupar

Ku Vera, J. 2013. Manipulación del metabolismo energético de los rumiantes en los trópicos: opciones para mejorar la producción y la calidad de la carne y leche. XXIII Congreso Internacional de ALPA. La Habana, Cuba

Ku Vera, J., Ramírez, C., Jiménez, G., Alayón, J. Ramírez L. 2000. Árboles y arbustos para la producción animal en el trópico mexicano. Available on: FAO.www.fao.org/wa [Consulted: July 8th, 2012]

La O.O., García, R., Ruiz, O., Castillo, Y., Muro, A., Rodríguez, C., Arzola, C., González, H. & Ortiz, B. 2011. *In vitro* ruminal fermentative potential of two trees (*Pithecellobium dulce* and *Tamarindus indica*) of importance for livestock rearing in fragile, saline ecosystems with high drought, located in the Eastern part of Cuba. Cuban J. Agric. Sci. 42:57

La O, O., González, H., Orozco, A., Castillo, Y., Ruiz, O., Estrada, A., Ríos, F., Gutiérrez, E., Bernal, H., Valenciaga, D., Castro, B. & Hernández, Y. 2012. Chemical composition, in situ rumen degradability, and *in vitro* digestibility of *Tithonia diversifolia* ecotypes of interest for ruminant feeding. Cuban J. Agric. Sci. 46:47

Makkar, H.P.S. 2005. *In vitro* gas method for evaluation of feed containing phychemicals. Anim. Feed. Sci. Technol.

123:291

Pedraza, R.M. 2000. Valoración nutritiva del follaje de *Gliricidia sepium* (Jacq) kunth ex walps y su efecto en el ambiente ruminal. PhD. Thesis. Instituto de Ciencia Animal. La Habana, Cuba

Rodríguez, R. 2010. Estudio *in vitro* del valor nutritivo y de los efectos antinutricionales de cuatro leguminosas arbóreas tropicales con potencialidades como suplementos del *Pennisetum purpureum* (vc CUBA CT-115). PhD. Thesis. Universidad de Zaragoza, España

Theodorou, M.K, Williams, B.A., Dhanoa, M.S., McAllan, A.B. & France, J. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminants feed. Anim. Feed. Sci. Tech. 48:185

Van Soest, P.J. & Robertson, J.L. 1991. Carbohydrate methodology metabolism and nutricional implications in dairy cattle, methods for dietary fiber, neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583

Visauta, B. 1998. Análisis estadístico con SPSS para Window. Estadística multivariada. Vol II. Mc Graw-Hill/ Interamericana de España, S.A.V. p. 358

Received: May 30, 2013