Scientific Paper

Ecosystem with *Leucaena leucocephala*: its effect on the rumen microbial population in fattening bulls

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Abstract

A trial was conducted in order to evaluate the effect of *Leucaena leucocephala* cv. Peru on the rumen microbial population of Zebu crossbred bulls, in the two stages of the fattening cycle. The system, integrated by five strips of grasses associated with *L. leucocephala* and five without association, consecutively alternated in 25 ha, allowed the fattening of 80 animals, from which eight were used for the microbiological studies. The samplings of rumen liquid were made bimonthly (February-March, April-May, June-July) and four-monthly (August-November). The cellulolytic bacteria reached values between 15,94 and 46,13 10^6 CFU.mL^{-1} when the bulls grazed *L. leucocephala* associated with grasses. The highest population of rumen cellulolytic fungi was found in the rumen of animals in the system with *L. leucocephala*, in the August-November samplings. The concentration of rumen ammonia had values of 8,76 and 24,09 mmol.L^{-1} in the system with grasses and the one of *L. leucocephala* associated with grasses, respectively ($p < 0,001$). The pH in rumen was significantly higher, the population of total viable bacteria was 2,9 times higher and the protozoans decreased when *L. leucocephala* participated in the ration. It is concluded that the system with *L. leucocephala* associated with grasses is advantageous, because it propitiates increases in the populations of total viable bacteria and fiber-degrading microorganisms; this, along with the depressing effect of protozoans, guarantees a favorable ecological environment for rumen cellulolysis, which would contribute in a sustained way to the productive increase of the animal husbandry system.

Keywords: bacterium, metabolites, protozoans

Introduction

L. leucocephala is one of the most widely utilized legumes in Cuba (Ruiz, 2005), and among the qualities that distinguish it is its high protein content (Ku-Vera, 2013). In works conducted by Galindo *et al*. (2007) it was proven that *L. leucocephala* can be included in 100 % of a grassland area, which increases rumen cellulolysis as consequence of a higher population of fibrolytic organisms. Likewise, Galindo (2013) reported that *L. leucocephala* reduces the methane production, mainly due to the presence of secondary metabolites. Prieto-Manrique (2017), when using 14-16 % of *L. leucocephala* in grazing cows, considered that these quantities were insufficient to reduce the methane production at rumen level.

Iraola *et al*. (2016), when implementing an agroecological strategy of transformation in a system of degraded pasturelands with a silvopastoral arrangement with *L. leucocephala* for cattle fattening, found increases in the plant biomass of 60 % and a mean daily gain (MDG) between 0,648 and 0,769 kg/animal/day, with which economically viable systems in Cuban pesos were achieved.

In previous studies conducted by Iraola *et al*. (2014) in a silvopastoral system with *L. leucocephala*, the effect of management or season on the indicators related to the rumen microbial ecology was not studied. For such reason, the objective of this study was to evaluate the effect of *Leucaena leucocephala* cv. Peru on the rumen microbial population of Zebu crossbred bulls, in the two stages of the fattening cycle.

Materials and Methods

The trial was conducted in the Ayala Feedlot of the Institute of Animal Science –Mayabeque, Cuba–, in an agroecosystem of pasturelands integrated by a mixture of natural and cultivated pastures with *L. leucocephala* cv. Peru, in a physical surface of 25 ha on a Brown soil with carbonates.

The design of the system corresponded to the strategy of ecological redesign for the recovery of degraded pastures in an agrosilvopastoral system, according to the report by Iraola *et al*. (2014), and its purpose was to improve the stocking rate capacity with fattening cattle. The system was structured according to the methodology proposed by Ruiz

(2005), with *L. leucocephala* densities between 5 000 and 5 700 trees per hectare. Once *L. leucocephala* reached between 1,00 and 1,20 m of height, it began to be utilized.

In the experiment 80 Zebu crossbred bulls were used, of 199 ± 3 kg of average live weight (LW). From them eight animals were randomly selected to conduct the rumen fermentation studies. All the animals grazed in a unique group and passed successively through the five strips of grasses associated with *L. leucocephala* and the five strips of grasses without association.

The samplings of rumen liquid were made with bimonthly (February-March, April-May, June-July) and four-monthly frequency (August-November) for finishing fattening, through the esophageal pathway and with the aid of a probe, which was introduced through the mouth to the rumen. The animals entered the system 15 days before the first sampling, that is, in the second fortnight of January, in order to guarantee the adequate adaptation period.

Microbiological and fermentative analyses. The culture technique proposed by Hungate (1950) was used, in roll tubes and under strict anaerobiosis conditions. The sowing of total viable bacteria, proteolytic and cellulolytic, was carried out in Caldwell and Bryant (1966) culture media; the fungi were analyzed through the technique suggested by Joblin (1981); and the protozoans were counted under microscope, in Neubauer chamber.

The pH was determined in an EIL model 22-A pH meter, and the ammonia concentration, according to Conway (1957). The analysis of the chemical composition of the pastures was made through the techniques described by AOAC (1995); while the fibrous fractions were analyzed through the procedure proposed by Goering and Van Soest (1970). The chemical composition of the species is shown in table 1.

The secondary metabolites against the qualitative tests of the phytochemical sieving of *L. leucocephala* indicated high presence (+++) of tannins, alkaloids and anthocyanidins; and moderate quantities (++) of saponins, reducers, flavonoids, triterpenes and steroids.

Experimental design and statistical analysis. Multifactorial variance analysis was carried out. The considered factors were: 1) strips or grazing in grass associated with *L. leucocephala* and in mixture of grasses, and 2) sampling bimester (February-March, April-May, June-July) and four-month period (August-November).

In the case of the indicators proteolytic, cellulolytic bacteria and cellulolytic fungi a split plot design was used, where the main plot was the strip (*L. leucocephala* or mixture of grasses), and the subplot, the sampling bimesters. In the case of the indicators total viable bacteria, protozoans, pH and NH_3 a linear model was used for the effects of the strips and sampling bimesters.

It was verified that the data fulfilled the assumptions of normal distribution and variance homogeneity. The counts of viable microorganisms were transformed according to Ln N, to guarantee the normality conditions. For the analysis the formula $(K+N).10^x$ was applied, where: K is the constant that represents the logarithm of the dilution in which the microorganism was inoculated; N is the logarithm of the count of colonies determined as CFU/mL, TFU/mL, or cells/mL; 10 is the basis of the logarithm; and x is the dilution at which the inoculation was carried out.

For the comparison between means Duncan's test ($p < 0,05$) was applied in the necessary cases. The statistical package INFOSTAT version 2001, by Balzarini *et al*. (2001) was used.

Results and Discussion

L. leucocephala acquires particular importance in agroforestry systems because, besides its good growth in tropical environments, it shows high contents of protein, minerals, vitamins and energy, which guarantees an adequate nutritional value when it is used in the diet of animals.

In this study significant interaction was found between the grazing system and the sampling moment on the populations of proteolytic, cellulolytic bacteria and fungi of the rumen (table 2). The proteolytic bacteria were more abundant when the animals grazed *L. leucocephala*. Such results confirm

Table 1. Chemical composition of the species that integrated the pastureland ecosystem (%).

Species	CP	NDF	ADF	Ash	Ca	P
L. leucocephala	29,47	39,96	19,26	8,91	1,18	0,08
Mixture of natural and cultivated pastures	11,90	91,06	39,95	12,87	0,54	0,00

Table 2. Effect of the silvopastoral system and sampling period on the population of total viable bacteria, protozoans and proteolytic bacteria.

Indicator	System	Sampling moment				SE ±
		Febrary-March	April-May	June-July	August-November	
Proteolytic bacteria, 10^6 CFU.mL^{-1}	*L. leucocephala*/grass	$2,86^{b}$ (20,46)	$2,63^{b}$ (14,15)	$2,64^{b}$ (14,27)	$2,55^{b}$ (14,96)	0,146**
	Grass	$2,77^{b}$ (17,4)	$1,79^{a}$ (6,04)	$1,76^{a}$ (6,27)	$2,45^{b}$ (11,67)	
Cellulolytic bacteria, 10^6 CFU.mL^{-1}	*L. leucocephala*/grass	$2,63^{cd}$ (15,94)	$3,17^{e}$ (24,33)	$2,98^{e}$ (21,11)	$3,64^{f}$ (46,13)	0,222***
	Grass	$2,49^{bc}$ (13,29)	$2,13^{ab}$ (8,61)	$1,78^{a}$ (6,02)	$1,91^{a}$ (6,88)	
Cellulolytic fungi, 10^5 TFU.mL^{-1}	*L. leucocephala*/grass	$2,23^{c}$ (13,25)	$2,95^{d}$ (20,38)	$2,81^{d}$ (18,23)	$3,77^{e}$ (43,83)	0,344**
	Grass	$2,38^{b}$ (11,27)	$1,83^{a}$ (6,54)	$1,79^{a}$ (6,13)	$1,49^{a}$ (4,5)	

Data transformed according to Ln X, original means between parentheses.
a, b, c, d, e: means with different letters within the same row differ at $p < 0,05$; $^{**}p < 0,01$; $^{***}p < 0,001$.
CFU.mL^{-1}: colony forming units per milliliter; TFU: colony forming units per talo.

the presence of factors that stimulate the growth of these microbial groups when *L. leucocephala* is present, due to its high content of protein and minerals (Rodríguez *et al*., 2013). However, when the animals grazed the grasses during the two-month period February-March and the four-month period August-November, there were no statistical differences in this indicator, aspect that should be object of study in later stages.

L. leucocephala produced a beneficial effect on the populations of cellulolytic bacteria and fungi of the rumen. In all the samplings higher population of cellulolytic bacteria was found when the animals grazed in the system with this plant, with the highest values ($p < 0,001$) in the samplings comprised between April and November, and within them, the four-month period August-November showed the highest populations. These high populations of cellulolytic organisms can lead to reach higher rumen cellulolysis, due to a higher population of microorganisms capable of excreting the complex of cellulase enzymes; likewise, provide the rumen of necessary metabolites for the synthesis of microbial proteins (Galindo *et al*., 2017); and, consequently, increases should be expected in the degradation of the fibrous fraction, given the interaction that appears at rumen level among the different microorganisms in the fiber degradation process (Gerber *et al*., 2013).

The experimental animals were exposed to better nutritional conditions in these months (four-month period August-November), because it has been reported that *L. leucocephala* is three times richer in protein (22,3-30,0 %) than tropical grasses in the same time of the year (Rivera *et al*., 2015).

No significant interaction was found among the treatments and the sampling moment on the population of total viable bacteria, protozoans, pH and ammonia concentration in the rumen.

When evaluating the effect of the system on pH and NH_3 (table 3), it was found that in the animals that grazed *L. leucocephala*/grass the ammonia concentration was higher, and, subsequently, also the rumen pH. This effect is due to the action exerted by the microbial deaminases excreted by proteolytic bacteria on the proteins contained in *L. leucocephala*.

In this regard, Rodríguez *et al*. (2014) stated that the protein contained in this plant is degraded between 56 and 59 % at rumen level; but, because its content is high, there will always be remarkable quantities of free ammonia. This effect can be attenuated with the use of energy supplementation to animals that graze *L. leucocephala*.

In works conducted by Iraola *et al*. (2017) it was reported that one of the elements which limit animal productivity in silvopastoral systems (SPS) with *L.*

Table 3. Effect of grazing on the silvopastoral system *L. leucocephala*/grass, on pH and ammonia concentration in the rumen of bulls.

Indicator	*L. leucocephala*/grass	Grass	SE ±
pH	7,44	6,81	0,06***
NH_3, mmol.L^{-1}	24,09	8,76	0,46***

*** $p < 0,001$

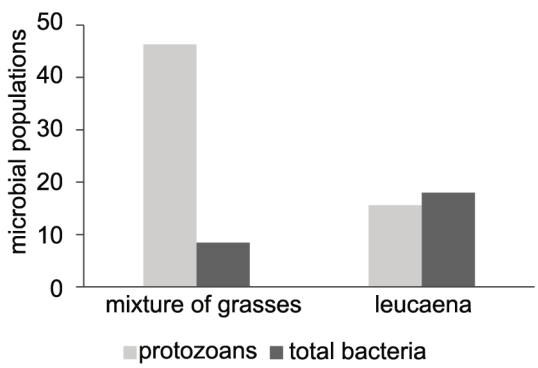
leucocephala is the contribution of energy and its synchronization with the available nitrogen sources for rumen microorganisms, limiting the microbial protein synthesis and the productive performance of fattening animals. On the other hand, *L. leucocephala* intake tends to increase the crude protein content in the rumen and can cause excesses in the nitrogen balance, situation that produces additional energy cost for its elimination as urea.

In this regard, there are different forms of manipulating the SPS with *L. leucocephala* and obtaining productive advantages; within them are the use of easily fermented carbon hydrates, such as the ones contributed by sugarcane (*Saccharum officinarum*), and the utilization of biologically activated beneficial microorganisms, produced in the Institute of Animal Science of Cuba (Elías, 2014). This product is composed by lactobacilli, yeasts and short-chain organic acids, and constitutes an activator of rumen fermentation (Garcia and Garcia-Curbelo, 2015). Its use as microbial additive in the finishing of cattle in silvopastoral system with *L. leucocephala*, complemented with sugarcane during the rainy season, generated a contrasting productive effect on the mean daily gain (MDG).

Figure 1 shows the effect of grazing in *L. leucocephala* associated with grasses or in the mixture of grasses, on the populations of total viable bacteria and protozoans of the rumen. As can be observed, when the animals grazed *L. leucocephala*, the populations of total viable bacteria were higher ($p < 0,001$); while the protozoans reduced their presence.

The reduction in the population of protozoans when the animals grazed *L. leucocephala* corroborates the anti-protozoan properties of this plant (Galindo, 2013); and represents an advantage that is ascribed to its high content in tannins (4,02 % DM) and other secondary metabolites, such as saponins, which exert similar effect at rumen level.

With regards to the role of saponins in the population of protozoans, Zhou *et al*. (2011) and Galindo *et al*. (2016) reported that this metabolite can cause depressive effect on the population of protozoans,



SE total bacteria = 0,145, p < 0,001; SE protozoans = 0,126, p < 0,001. Protozoans = 105 cells/mL; total viable bacteria = 1 011 colony forming units per milliliter.

Figure 1. Effect of grazing on grasses and *L. leucocephala* or on mixture of grasses, on the population of total viable bacteria and protozoans of the rumen of fattening bulls.

because changes occur in the permeability of the cell membrane, which can facilitate lysis at rumen level.

Summarizing, the presence of the above-mentioned antinutritional factors or secondary metabolites causes modifications in the microbial population of the rumen, and sometimes they could be toxic (McSweeney *et al*., 1999).

Likewise, it is important to consider that defauning is one of the likely ways to reduce the enteric emission of CH_4, due to the flow of microbial cells from the rumen and to the reduction in the acetate/propionate ratio (Leng, 2014).

The results explain the productive responses reported by Iraola *et al*. (2015a), between 0,682 and 0,769 kg/animal/day, when studying the effect of *L. leucocephala* on cattle fattening; as well as higher animal comfort given by the fauna and ecological development represented by the presence of trees and the increase of biomass production in the system (Iraola *et al*., 2015b). This last aspect is indirectly related to the population of microorganisms of the rumen, while it contributes to the accurate decision making about the rational use of technical and economic resources that guarantee higher sustainability and profitability of animal husbandry systems for beef production.

It is concluded that the management system with *L. leucocephala* associated with grasses is advantageous, because it propitiates increases in the populations of total viable bacteria and fiber-degrading microorganisms; which along with the protozoan depressive effect, guarantees a favorable ecological environment for rumen cellulolysis. Likewise, it is recommended to study, in later stages, the effect of the two-month period February-March and the four-month period August-November in the populations of rumen proteolytic bacteria.

Bibliographic references

AOAC. *Manual of official methods of analysis*. Washington, DC: Association of Official Analytical Chemists, 1995.

Balzarini, Mónica G.; Casanoves, F.; Di Rienzo, J. A.; González, Laura A. & Robledo, C. *Infostat sofware estadístico. Manual del usuario. Versión 1*. Córdoba, Argentina: Grupo InfoStat, FCA, Universidad Nacional de Córdoba, 2001.

Caldwell, D. R. & Bryant, M. P. Medium without fluid for non-selective enumeration and isolation of rumen bacteria. *Appl. Microbiol*. 14 (5):794-801, 1966.

Conway, E. J. *Microdiffusion analysis and volumetric error*. London: Crosby Lockwood Sons, Ltd, 1957.

Elías, A. Avances de los procesos biotecnológicos para incrementar el valor nutritivo de los alimentos. Impacto en la producción animal. *II Simposio de Zootecnia, Alimentación y Genética y XXIV Congreso Panamericano de Ciencias Veterinarias (PANVET).* La Habana: Asociación Consejo Científico Veterinario de Cuba. p. 35, 2014.

Galindo, Juana. *Evaluación de plantas con capacidad antiprotozoaria en la ecología microbiana ruminal y producción de leche en vacas*. Informe final de proyecto No. 003 Programa Nacional de Biotecnología Agropecuaria CITMA/GEPROP. Mayabeque, Cuba: Instituto de Ciencia Animal, 2013.

Galindo, Juana; García, C.; Marrero, Yoandra; Castillo, E.; Aldana, Ana I.; Torres, Verena *et al.* Effect of the composition of grassland of *Leucaena leucocephala* with grasses on the microbial rumen population in bulls. *Cuban J. Agric. Sci*. 41 (2):137, 2007.

Galindo, Juana; González, Niurca; Abdalla, A. L.; Alberto, Mariem; Lucas, R. C.; Dos Santos, K. C. *et al.* Effect of a raw saponins extract on ruminal microbial population and *in vitro* methane production with star grass (*Cynodon nlemfuensis*) substrate. *Cuban J. Agric. Sci*. 50 (1):77-88, 2016.

Galindo, Juana; González, Niurca; Scull, Idania; Marrero, Yoandra; Moreira, Onidia & Ruiz, T. E. *Tithonia diversifolia* (Hemsl.) A. Gray and its effect on the rumen population and microbial ecology. In: Lourdes L. Savón, Odilia Gutiérrez and G. Febles, eds. *Mulberry, moringa and tithonia in animal feeds and others. Results in Latin American and its Caribbean*. Cuba: FAO, ICA, EDICA. p. 251-256, 2017.

Garcia, Yaneisy & Garcia-Curbelo, Yanelys. Additives for animal feeding: The Institute of Animal Science on its 50 years. *Cuban J. Agric. Sci*. 49 (2):173-177, 2015.

Gerber, P. J.; Henderson, B. & Makkar, H. P. S., Eds. *Mitigación de las emisiones de gases de efecto invernadero en la producción ganadera. Una revisión de las opciones técnicas para la reducción de las emisiones de gases diferentes al CO_2*. Roma: FAO, 2013.

Goering, H. K. & Van Soest, P. J. *Forage fiber analyses (apparatus, reagents, procedures, and some applications)*. Agricultural Handbook No. 379. Washington, DC: US Agricultural Research Service, 1970.

Hungate, R. G. The anaerobic, mesophilic cellulolytic bacteria. *Bacteriol Rev.* 14 (1):1-49, 1950.

Iraola, J.; Elías, A.; Gutiérrez, D.; García, Yenny; Fraga, L. M.; Vázquez, Arelis *et al.* Efecto de microorganismos beneficiosos activados en la finalización de toros en silvopastoreo con leucaena, complementados con caña de azúcar. *Rev. Científ. FCV-LUZ*. 27 (6):403-410, 2017.

Iraola, J.; García, Yoleisy; Muñoz, E.; Fraga, L. M.; Barros-Rodríguez, M.; Hernández, J. L. *et al*. Modeling of live weight per age in fattening bovines under a silvopastoril system with *Leucaena leucocephala*. *Cuban J. Agric. Sci*. 49 (3):307-315, 2015a.

Iraola, J.; Muñoz, E.; García, Yenny & Hernández, J. L. Caracterización faunística en un sistema silvopastoril destinado al ganado de engorde. *Pastos y Forrajes*. 38 (4):418-424, 2015b.

Iraola, J.; Muñoz, E.; García, Yenny; Hernández, J. L. & Moreira, E. Estrategia agroecológica de transformación en un sistema de pastizales degradados con un arreglo silvopastoril. *LRRD*. 28 (7). http://www.lrrd.org/lrrd28/7/irao28120.html. [11/04/2017], 2016.

Iraola, J.; Muñoz, E.; García, Yenny; Hernández, J. L. & Moreira, E. Estrategia ecológica de rediseño para la recuperación de pasturas degradadas en un sistema agrosilvopastoril de engorde bovino. *Memorias de la III Convención Internacional Agrodesarrollo 2014*. Matanzas, Cuba: EEPF Indio Hatuey. p. 1040-1043, 2014.

Joblin, K. N. Isolation, enumeration and maintenance of rumen anaerobic fungi in roll tubes. *Appl. Environ. Microbiol*. 42 (6):1119-1122, 1981.

Ku-Vera, J. C. Manipulación del metabolismo energético de los rumiantes en los trópicos: opciones para mejorar la producción y la calidad de la carne y leche. *IV Congreso Internacional Producción Animal Tropical*. La Habana, 2013.

Leng, R. A. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. *Anim. Prod. Sci*. 54 (5):519-543, 2014.

McSweeney, C. S.; Palmer, B.; Bunch, R. & Krause, D. O. Isolation and characterization of proteolytic ruminal bacteria from sheep and goats fed the tannin-containing shrub legume *Calliandra calothyrsus*. *Appl. Environ. Microbiol*. 65 (7):3075-3083, 1999.

Prieto-Manrique, E. Efecto de la suplementación con aceites vegetales a vacas pastoreando con/sin sistema silvopastoril intensivo con leucaena sobre los ácidos grasos en la leche y la producción de metano *in vitro*. Sistemas silvopastoriles. Aportes a los objetivos de desarrollo sostenible. *IX Congreso Internacional de Sistemas Silvopastoriles*. Cali, Colombia: CIPAV, 2017.

Rivera, J. E.; Molina, C. H.; Molina, J. J.; Molina, E. J.; Chará, J. & Barahona, R. Determinación de las emisiones de gases de efecto invernadero (GEI) provenientes de la orina y el estiércol bovino en dos sistemas de pastoreo orientados a la producción de leche. *Actas del III Congreso Nacional de Sistemas Silvopastoriles, VII Congreso Internacional de Sistemas Agroforestales*. Misiones, Argentina: Ediciones INTA. p. 565-569, 2015.

Rodríguez, R.; de la Fuente, G.; Gómez, S. & Fondevila, M. Biological effect of tannins from different vegetal origin on microbial and fermentation traits *in vitro*. *Anim. Prod. Sci*. 54 (8):1039-1046 2013.

Rodríguez, R.; González, Niurca; Alonso, J.; Domínguez, Marbelis & Sarduy, Lucía. Nutritional value of foliage meal from four species of tropical trees for feeding ruminants. *Cuban J. Agric. Sci*. 48 (4):371-378, 2014.

Ruiz, T. E. Siembra, establecimiento y biomasa de *Leucaena leucocephala*. *II Taller Internacional Silvopastoril Cuba-Colombia*. Bogotá: CORPOICA, Instituto de Ciencia Animal. p. 115, 2005.

Zhou, Y. Y.; Mao, H. L.; Jiang, F.; Wang, J. K.; Liu, J. X. & McSweeney, C. S. Inhibition of rumen methanogenesis by tea saponins with reference to fermentation pattern and microbial communities in Hu sheep. *Anim. Feed Sci. Technol*. 166-167:93-100, 2011.

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