

Scientific Paper

Silvopastoral system with *Tithonia diversifolia* (Hemsl.) A. Gray: effect on the rumen microbial population of cows

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Abstract

A trial was conducted in order to evaluate the effect of a silvopastoral system with *Tithonia diversifolia* (Hemsl.) A. Gray, associated with a mixture of tropical grasses, on the rumen microbial population of dairy cows under production conditions. One hundred Holstein cows were used, which grazed in a leader-follower system, with permanence time of two days. The grazing in *T. diversifolia* occurred at night, and the animals remained from 5:00 p.m. until 5:00 a.m. For the rumen liquid collection six cows were randomly selected in each of the sampling times. The populations of total viable, amylolytic and cellulolytic bacteria were more numerous when the cows grazed on the SPS with *T. diversifolia*, with regards to grass grazing. The methanogen values were 9,83 and 19,33 10^{10} CFU mL^{-1} , and the protozoa values, of 4,67 and 7,67 10^5 cells mL^{-1} for SPS with *T. diversifolia* and grasses, respectively. The pH and NH_3 were higher in the SPS. The microbial biomass showed values of 196,9 and 192,3 g, and the organic matter that was fermented, of 547,2 and 506,4 g for SPS and grasses, respectively. It is concluded that the silvopastoral system with *T. diversifolia* improved the rumen ecosystem, by increasing the organisms that degrade fiber and reducing protozoa and methanogens; as well as making a higher quantity of fermented organic matter for the synthesis of microbial protein available for the animal.

Keywords: grazing, methanogenic bacterium, protozoa, rumen

Introduction

At present efforts are made aimed at diversifying the forage offer in traditional animal husbandry systems and the implementation of silvopastoral systems is promoted, among which *Tithonia diversifolia* (Hemsl.) A. Gray (Gallego-Castro *et al.*, 2017a) particularly stands out, which can constitute a good alternative in cattle feeding.

Among its nutritional characteristics the protein content, soluble carbohydrates and tannin content stand out, important indicators which should be analyzed, because these components can help improve the feeding balance regarding the energy and protein contribution in the diet of dairy cattle (Gallego-Castro *et al.*, 2017b; Mahecha-Ledesma and Angulo-Arizala, 2017).

In turn, the protein contribution can contribute to improve the rumen balance and increase the efficiency for the transformation of ammonia into microbial protein, which would imply lower energy costs due to losses of rumen ammonia, methane and CO_2 , which decrease the possible environmental contamination.

Regarding secondary metabolites, Galindo *et al.* (2017) reported that the presence of total phenols,

tannins, saponins, among other compounds, is variable in the plant. Likewise, Hess (2006) stated that tannins are associated with the reduction in methane production; this coincides with the report by Galindo *et al.* (2012), who found that the inclusion of 10 and 20 % of *T. diversifolia* caused reductions in the population of rumen methanogens.

Gallego-Castro (2016) proved that a silvopastoral system (SPS) of *T. diversifolia* associated with kikuyu grass increased milk production, as well as milk quality. In this sense, García-López *et al.* (2016) also found increases in production when they used a SPS with this species.

The nutritional characteristics of *T. diversifolia* exert an important effect on the productive yield of the animals; however, the studies related to its influence on rumen ecology have been conducted under *in vitro* and *in situ* conditions, and there are no reports of its effect under production conditions. Hence the objective of this study was to evaluate the effect of a silvopastoral system with *T. diversifolia*, associated with a mixture of tropical grasses, on the rumen microbial population in dairy cows under production conditions.

Materials and Methods

Location. The trial was conducted during 2012-2016, in the grazing areas of the dairy farm G-2, located between 22° 54' 44.94" LN and 82° 00' 38.34" LW and at 83 m.a.s.l.: such farm belongs to the Institute of Animal Science (ICA for its initials in Spanish), in the San José de las Lajas municipality –Mayabeque province, Cuba.

Edaphoclimatic characteristics. The experimental areas are located on a eutric Ferralic Red soil, of fast desiccation, clayey and deep on limestone, equivalent to the Rodic Ferralic Cambisol subtype (Hernández-Jiménez *et al.*, 2015). The mean annual temperature of the region was 24,9 °C. Maximum temperatures between 32,7 and 33,5 °C and minimum temperatures between 21,5 and 22,5 °C were recorded. The mean annual rainfall was 1 361 mm, and the average annual relative humidity, 80,9 % (Meteorological Station of the Institute of Animal Science, San José de las Lajas, Mayabeque).

Planting and establishment. The SPS system of *T. diversifolia* was planted in a stratified way, during 2011-2013, in a 9-ha area. Afterwards, 20,4 ha were planted during 2015 and 2016 until completing a total of 29,4 ha. As propagation material plant material from the ICA germplasm bank was used, according to the proposal made by Ruiz *et al.* (2014) to be used in grazing systems. The soil was conventionally prepared during the rainy season, in double rows at 0,5 m between them, with a spacing distance of 4 m. In the case of grasses, they were regenerated from the material present in the soil.

Floristic composition of the pastureland. During more than 15 years this area of the farm (53,6 ha) was covered with a mixture of tropical grasses integrated by *Cynodon nlemfuensis* (star grass), *Megathyrsus maximus* (Jacq.) (Guinea grass) and mixture of natural pastures, with predominance of *Dichanthium caricosum* (L.) and *Sporobolus indicus* (L.). The SPS was established with a density of 6 912 *T. diversifolia* plants ha⁻¹. The estimated proportion of the tree-grass stratum in one hectare was 20:80, respectively.

Animal management and grazing. One hundred Holstein cows were used, which grazed in a leader-follower system, with a time of permanence in the SPS paddock of two days. Grazing occurred at night and the animals remained from 5:00 p.m. to 5:00 a.m. In the morning they grazed the mixture of grasses. Each of the grazing cycles in the SPS lasted 1 520 days. At the end, the animals grazed

in the remaining grass area until completing 80-90 days of resting for the tree, and the stocking rate was 2,2 to 2,7 cows ha⁻¹.

Experimental procedure

The cows had a 10-day period of adaptation to the diet (SPS or grasses), before each of the rumen liquid samplings. In each sampling cycle the work was done with six cows.

The samplings of rumen liquid were performed after the morning milking, with an esophageal probe. The collected rumen liquid was stored in thermoses with airtight closing, to guarantee the temperature (39 °C) and anaerobic conditions during the transfer to the laboratory.

The chemical composition of *T. diversifolia* showed values of 24,2; 5,9; 2,3; 0,4; 35,3 and 30,4 % for CP, ash, calcium, phosphorus, neutral detergent fiber (NDF) and acid detergent fiber (ADF), respectively.

This plant in the qualitative essays of the phytochemical sieving showed moderate concentrations of tannins (++), saponins (++), triterpenes (++), steroids (++) and alkaloids (++); as well as high concentrations of reducers (+++) and low concentrations of anthocyanins (+).

Analyses and determinations

Cultivation of rumen microorganisms. The cultivation technique proposed by Hungate (1950) was used, in rolled tubes and under strict anaerobic conditions. The planting of total viable, cellulolytic and proteolytic bacteria was carried out in the Caldwell and Bryant (1966) culture media. In the case of proteolytic bacteria, 10 % of skimmed milk was added. For the determination of the fungi population, the cultivation medium of Joblin (1981) was used. The methanogenic microorganisms were cultivated by the same technique, with a mixture of the gases hydrogen and carbon dioxide in 60:40 ratio. The results were expressed in colony-forming units (CFU) for the bacteria and in thallus-forming units (TFU) for the fungi per milliliter of rumen liquid (mL) in the determined dilution.

The protozoa were directly counted under the optical microscope in Neubauer chamber, after staining them with a 0,01 % gentian violet solution in glacial acetic acid. Likewise, the counts were expressed as cells per milliliter of liquid rumen (mL) in the determined dilution.

To perform the protozoa counts they were preserved in a 10 % formaldehyde solution in 1:1 dilution (v/v).

The pH was determined through reading in Sartorius digital pH meter.

The NH_3 concentration was determined according to the technique described by Conway (1957). The concentration of short chain fatty acids (SCFA) and individual fatty acids were determined by gas chromatography.

Calculation of the stoichiometric balance of rumen fermentation. The program BALANCE-RU-METANO was used to estimate the stoichiometric balance of rumen fermentation and the contribution to the animal metabolism (Stuart, 2015). Likewise, the bacterial biomass (BB) and the organic matter fermented in rumen (OMf) were calculated

Statistical processing. The theoretical assumptions of the variance analysis for the studied variables were verified, from Shapiro Wilk (1965) tests for error normality and Levene's test (1960) for variance homogeneity; while the counts of viable microorganisms were transformed according to $\ln N$, in order to guarantee the conditions of normality in the growth curve. In the analysis the formula $(K + N) \cdot 10^x$, 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^{-1} , TFU mL^{-1} or cells mL^{-1} ; 10 is the basis of the logarithms and x is the dilution at which the inoculation was performed; the counts of microorganisms did not fulfill the theoretical assumptions of ANOVA, for which the non-parametric Kruskal Wallis test was used for the analysis. The statistical program InfoStat was utilized (Di Rienzo *et al.*, 2012).

Results

Table 1 shows the effect of grazing in the SPS with *T. diversifolia* on the populations of

rumen microorganisms, with regards to the grass grazing. The populations of total viable, amylolytic and cellulolytic bacteria were more numerous, with significant statistical differences when the cows grazed on the SPS with regards to the grass grazing. Likewise, in *T. diversifolia* the protozoa population in the rumen was reduced. In turn, there was no effect of the SPS on the populations of proteolytic bacteria and cellulolytic fungi of the rumen; while depressive effect was found, with significant differences, on rumen methanogens. When the cows grazed in *T. diversifolia*, a reduction was observed in approximately two times in the population of microorganisms that produce methane in the rumen.

With regards to the pH of rumen liquid, highly significant values ($p < 0,0001$) were found when the cows grazed in the SPS (table 2). This effect can be ascribed to the increase in the concentration of rumen ammonia, due to the high CP value of *T. diversifolia*.

Regarding the short chain fatty acids, higher concentration was observed in the total volatile fatty (SCFAt), acetic, propionic and isobutyric acids when the animals grazed in this system. In addition, the concentration of valeric acid was highly significant ($p < 0,0001$) in the animals that grazed in the SPS.

The quantity of microbial biomass in the rumen of the cows that grazed in the SPS and in the grasses was 196,9 and 192,3 g, respectively. In turn, the quantity of organic matter that was fermented had values of 547,2 and 506,4 g for SPS and grasses, respectively.

Table 3 shows the results obtained from the calculation of the stoichiometric balance of the rumen fermentation and fermentation patterns. The

Table 1. Effect of grazing in SPS with *T. diversifolia* on the microbial populations of the rumen in dairy cows.

Indicator	Treatment		P-Value
	SPS with <i>T. diversifolia</i>	Grasses	
Total viable bacteria, $10^{11} \text{ CFU mL}^{-1}$	9,25 (19,33) \pm 4,18	3,75 (10,83) \pm 3,60	P = 0,0065
Amylolytic bacteria, 10^6 CFU mL^{-1}	9,0 (7,33) \pm 3,83	4,0 (2,50) \pm 0,55	P = 0,0216
Proteolytic bacteria, 10^6 CFU mL^{-1}	5,0 (8,17) \pm 3,06	8,0 (11,67) \pm 4,97	P = 0,1580
Cellulolytic bacteria, 10^5 CFU mL^{-1}	8,75 (8,83) \pm 2,93	4,25 (5,0) \pm 2,45	P = 0,0303
Fungi, 10^6 TFU mL^{-1}	7,92 (8,33) \pm 2,25	5,08 (6,33) \pm 2,58	P = 0,1385
Methanogens, $10^{10} \text{ CFU mL}^{-1}$	2,29 (9,83) \pm 2,64	2,96 (19,33) \pm 2,64	P = 0,0022
Protozoa, $10^5 \text{ cells mL}^{-1}$	5,08 (4,67) \pm 1,51	7,92 (7,67) \pm 3,50	P = 0,0191

CFU: colony-forming units, TFU: thallus-forming units, SD: deviation, () original means

Table 2. Effect of grazing in SPS with *T. diversifolia*, on the pH and the final products of fermentation in dairy cows.

Indicator	Treatment		SE ±	P-Value
	SPS with <i>T. diversifolia</i>	Grasses		
pH	6,78	6,37	0,04	0,0001
NH ₃ , meq L ⁻¹	35,13	24,17	2,05	0,0036
SCFA t, mmol L ⁻¹	71,13	65,83	1,41	0,0244
Acetic acid, mmol L ⁻¹	45,60	40,67	1,16	0,0134
Propionic acid, mmol L ⁻¹	11,83	10,58	0,34	0,0278
Isobutyric acid, mmol L ⁻¹	0,46	0,36	0,02	0,0028
Butyric acid, mmol L ⁻¹	11,45	12,48	1,47	0,6301
Isovaleric acid, mmol L ⁻¹	1,20	0,99	0,07	0,0705
Valeric acid, mmol L ⁻¹	0,63	0,46	0,02	0,0001

SCFAt: Total short chain fatty acids .

Table 3. Effect of grazing in SPS with *T. diversifolia*, on the fermentation pattern and stoichiometric balance of the rumen in dairy cows.

Indicator	Treatment		SE ±	P-Value
	SPS with <i>T. diversifolia</i>	Grasses		
acetic, %	66,69	63,96	2,32	0,4267
propionic, %	17,43	16,49	0,37	0,1062
butyric, %	16,06	19,55	2,09	0,2642
Glucose, g	55,23	56,78	1,07	0,3329
CO ₂ , g	61,70	65,43	2,03	0,2240
Methane, g	36,93	37,63	0,28	0,1072
Water, mL	24,77	27,79	2,20	0,3545

fermentation pattern was acetic, as it is produced in the rumen of animals that consume fibrous diets, with molar percentages of 66,7 and 64,0 % for the animals that grazed in the SPS and in grasses, respectively, without differences between both grazing systems. No statistical differences were observed in the contribution in glucose, carbon dioxide, methane and water, calculated from the concentration of volatile fatty acids.

Discussion

T. diversifolia stands out due to its capacity for nitrogen accumulation (Ruiz *et al.*, 2017) and its nutritional characteristics, such as: protein content, soluble carbohydrates (Mejías-Díaz *et al.*, 2017) and tannin content (La O *et al.*, 2012); which can contribute to improve the nutritional balance in the animals, with regards to the energy and protein contribution in the diet of dairy cattle. This is in ad-

dition to the fact that studies conducted by Sauvante *et al.* (2011), from a compilation of 59 experiments, showed that the production of CH₄ per kilogram of digested OM linearly decreased when the CP content of the feedstuffs increased (CH₄, g/kg digestible OM = 40,1 – 0,32 × CP, DM percentage).

In this experiment a depressive effect on the population of protozoa was proven when the cows grazed on the SPS with *T. diversifolia*. In this regard, it has been shown that the secondary metabolites of these plants exert defauning effects, by reducing the population of rumen protozoa.

Galindo *et al.* (2017) indicated that such effect is direct on the protozoa population and indirect on the cellulolytic bacteria, because protozoa engulf huge quantities of these microbial groups during the day and, consequently, improve digestive efficiency. Likewise, they naturally protect protein, which reduces its fermentation in the rumen.

The effect found on the increase of the population of total viable bacteria could have had its explanation in the fact that defaunation increases the population of total viable bacteria in the rumen, according to reports by Galindo *et al.* (2014). The authors have indicated that protozoa ingest large volumes of bacteria and maintain their population constant in the rumen, so that defaunation implies the disappearance of the ecological relations (predation and competition) which affect the type, genus distribution and metabolic activity of the fungal and bacterial population of the rumen ecosystem.

Likewise, protozoa exert selective predator action on certain cellulolytic bacteria of the rumen (Leguizamón and Carreño, 2013). Type-A protozoa, among them *Polyplastrum multivesiculatum* act as predators of cellulolytic bacteria, such as *Butyrivibrio fibrisolvens* and *Ruminococcus flavefaciens*; while type-B protozoa, such as *Epidinium ecaudatum*, *Eremoplastron bovis* and *Eudiplodinium maggii*, act as predators of cellulolytic bacteria at a lower rate.

Among the most important advantages of defaunation are: increases in the population of cellulolytic microorganisms, stabilization of the rumen pH, decrease of free ammonia, reduction of methanogenesis, and increase in the digestive utilization efficiency of different diets, mainly the fibrous ones. These results coincide with compilation reports by Hristov (2013), in which it is asserted that the most outstanding contribution of protozoa reduction in the rumen is that it improves the energy metabolism and reduces the losses due to the production of methane, which is an environmental pollutant. Such effect was proven in this research.

Leng (2014) reported that defaunation reduces the enteric emission of CH₄, due to the flow of microbial cells from the rumen and to the reduction in the acetate/propionate ratio, events that are considered electron sinks. In this study, when calculating the relations among the different fatty acids in the rumen, it was found that the acetic/SCFAt ratio was 0,64 and 0,62 for the fermentation of *T. diversifolia* and the grasses, respectively; while the propionic/SCFAt ratio was 0,17 and 0,16 for the rumen of the animals which grazed *T. diversifolia* and grasses, respectively. On the other hand, the acetic/propionic ratio was 3,85 and 3,84 for the rumen when the animals grazed *T. diversifolia* and grasses, respectively.

Reports by Ramírez *et al.* (2014) assert that the existing relation between methanogens and protozoa constitutes an irrefutable example of

the transference of H₂ among rumen species. The presence of methanogens on the surface of ciliated protozoa of the rumen and as endosymbionts within them has been detected, and it has been estimated that such presence is responsible for 9-25 % of the methanogenesis in the rumen liquid. As in the rumen aerobic respiration is not possible, protozoa do not have mitochondria but intracellular organelles called hydrogenosomes, in which the H₂ produced during the pyruvate or malate oxidation is released. A close relation has been observed between methanogens and hydrogenosomes. The advantage of this cooperation for the protozoon is evident: the removal of H₂ allows the protozoon to ferment the organic matter to acetate and CO₂, which prevents the generation of reduced products such as ethanol and lactate, process that allows to obtain maximum ATP production.

The response to the use of SPS with *T. diversifolia* with regards to the depressive effect on the population of methanogens could be explained by the fact that they live in an endosymbiotic way on the rumen protozoa (Galindo *et al.*, 2012), and any effect that contributes to remove rumen protozoa will decisively contribute to reduce the methane-producing populations. It is known that methanogenic Archaea are strictly anaerobic microorganisms, which belong mainly to the order Methanobacteriales (Ramírez *et al.*, 2014). Either way, the microbial relations in the rumen are complex, and the fermentative pathways that use or produce H₂ are even much more complex, because microorganisms can change their fermentation patterns in response to small differences in the energy conservation, ceasing to use less thermodynamically favorable pathways.

The above stated facts could explain the response found in the concentration of isovaleric and valeric acid in the rumen when the cows consumed grasses, with regards to those that had access to grazing with *T. diversifolia*, because it has been proven that the increase in the partial pressure of hydrogen in the rumen also reduces the deamination of reduced amino acids, including the branched-chain ones.

The results of the relations among SCFAs in the rumen seem to indicate that the mechanism through which the foliage of this plant acts is very complex and implies other factors, such as degradability of protein in the rumen. Leng (2014) proposed a possible microorganism by which the soluble proteins propitiate higher volumes of methane in the rumen, while the less soluble ones can pass to the lower GIT.

On the other hand, Rodríguez *et al.* (2014) stated that when feeding is supplemented with tree or shrub foliage increases occur in the bacterial and bypass protein, protein degradation is reduced and protein flow to the duodenum is increased, which ensures higher nitrogen absorption. This changes importantly the evaluated concepts until the present, and a preponderant place is ascribed to protein metabolism in the rumen when the animals graze in silvopastoral systems.

Conclusions

The silvopastoral system with *T. diversifolia* improved the rumen ecosystem, by increasing fiber-degrading organisms and reducing methanogens and protozoa, important aspect that contributes to increase the nutrient utilization efficiency and minimize the energy losses in ruminants. Likewise, higher quantity of fermented organic matter becomes available for the animal for the synthesis of microbial protein.

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