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RUMINAL DEFAUNATION AND GREENHOUSE GAS MITIGATION WITH TREE FORAGE CROPS IN THE AMAZON

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Summary

The objective of the study was to evaluate the effect of forages *Gliricidia sepium* (T1), *Leucaena leucocephala* (T2)*), Moringa oleifera* (T3), *Morus alba* (T4), *Tithonia diversifolia* (T5) on rumen function. For each treatment, the nutritional quality, digestion, rumen degradability, CH 4 gas production, CO2, holotric and entodinomorphic protozoa were analyzed. It was found in T4 greater digestibility of DM (P = 0.0002) and MO (P<0.0001). The degradability of DM in the soluble fraction was higher (P<0.0001) in T4 and T5, as well as in the potential for degradation and effective degradability. Gas production was lower (P=0.0009) in T1, T3 and T5 with 249.6, 232.1 and 257.2 m L gas/0.5 g DM respectively, the lowest production of CH 4 (P=0.0049) was for T4 with 52.4 mL CH_4/0.5 g DM and for CO2 no significant differences were found (P>0.05). The count of holotric protozoa at 6 h was lower (P = 0.0206) in T3 and in Entodinomorphs in T3 and T4 (P = 0.0082), at 12 and 24 h no significant differences were observed (P>0.05). The efficiency in rumen function found in T4 and T5 were influenced by the levels of FDN and FAD.

Keywords: Carbon dioxide, Degradability, Methane, Protozoa.

Introduction

Livestock production systems cause global impacts, due to greenhouse gas (GHG) emissions, such as carbon dioxide ($_{CO2}$), methane (CH4) and nitrous oxide ($_{N2O}$), from respiration, enteric fermentation and stool handling (Beltrán et al. 2016). The CH 4 generated contributes to the increase of the greenhouse effect and reduces energy retention in animals, so it is important to know in equatorial areas, where animal feed is based on pastures and forages with high fiber content, theeffects of different diets and additives that allow to increase digestive efficiency and reduce CH_4 emissions (Ochoa and Noguera 2014).

Due to the fragility of tropical rainforest ecosystems, especially in the Ecuadorian Amazon Region, the intervention of cattle ranching must start with the implementation of production systems analogous to the forest such as agrosilvopastoral systems, protein bank, association of pastures with legumes (Alemán et al. 2018; Nieto and Caicedo 2012). Silvopastoral systems are promising alternative production systems in the tropics, because they incorporate trees and tree legumes rich in nutrients, particularly protein, and promote higher feed intake in ruminants, which increases livestock production and reduces dependence on external inputs (Villanueva et al. 2019).

Mitigation strategies seek to reduce GHG production, or increase capture mechanisms (sources of sinks) (Alayón 2018). In this context, an alternative to reduce GHG emissions from enteric fermentation is the handling of ruminant feed rations (Rendón et al. 2018). The use of leguminous and non-leguminous tree forages in ruminant feed can improve the rumen environment, increase dry matter (SS) digestibility and degradability, and modify the rumen protozoan population, as well as decrease GHG emissions (Galindo et al. 2005; Manotoa and Barros 2016). In rumen methanogenesis the defaunating effect of fodder shrubs is related to the presence of secondary metabolites and essential oils, which have effects on fermentation and rumen microorganisms (Cardona et al. 2017a; Ortiz et al. 2014). The identification of secondary metabolites in plants with antimethanogenic properties is an area of research, which offers multiple benefits especially to production systems with grazing ruminants (Vélez et al. 2014).

The rumen microbial ecosystem has a significant impact on digestive physiology and also provides a test bed for other microbial environments in the development and validation of technologies (Puniya et al. 2015). In the Ecuadorian Amazon Region, studies of forage shrubs and legumes, as a source for the formation of production systems, have been based on the evaluation of forage potential and nutritional quality (REF). However, there are few studies that evaluate the efficiency in terms of degradation kinetics and rumen digestibility, as well as the mitigation of greenhouse gases and rumen protozoan populations, in the agroecological conditions of the region. Therefore, the following hypothesis is proposed: *"Tree forages favor rumen digestion, mitigation of enteric greenhouse gases and reduce the population of protozoa in the rumen, and therefore maximize the use of energy in the animal."*

Materials and methods

The phytomass was collected in the grassland and forage germplasm bank of the Central Amazonian Experimental Station (EECA) of the Agricultural Research Institute (INIAP), located at 285 meters above sea level. (6°52'35.87" W, 0°21'20.63" S,) in Orellana Province, Ecuador.

The study area corresponds to a tropical humid forest-bhT (Holdridge 1987), with an average annual rainfall of 3217 mm, heliophany of 1418.2 light hours, average annual temperature of 24 °C and relative humidity of 91.5%.

Forage bushes were pruned 1 m off the ground with a Felco 630 traction curved cutting saw to stimulate uniform regrowth (Geraldine and Simon 2001; Pinot et al. 2019). The biomass (organic matter) of forage shrubs was cut from 6 plants grown under similar soil and management conditions, at an age of 60 days (Grijalva et al. 2011; Peters et al. 2011; Ramos et al. 2016). In the center of each plot (15 x 15 m) a sampling area of 2 x 6m was delimited and in the central row of this area the transect of collection of forage samples, composed of 6 plants, was collected. The harvested phytomass (leaves, tender and woody stems), was dehydrated in a chapel-type greenhouse, while a cardboard was placed on the counters, to avoid direct contact of the forage

with the cement. During this 72-hour process, samples were flipped 4 times a day (7:00, 10:00, 13:00 and 15:00). Drying was then completed in a Thermo Scientific forced-air oven at 65 °C for 48 h (Toledo 1982). At this temperature, sensitive losses of soluble carbohydrates and the formation of indigestible complexes are avoided (Roza et al. 2011). The dry phytomass was processed in a Thomas Model 4 Wiley® mill with 2 mm mesh and sieved to a particle size of 0.425 mm and stored in plastic containers.

Chemical composition

Dry matter (DM) was terminated at 105 °C in a Thermo Scientific forced air furnace to a constant weight (#7.007, AOAC 2012), and ash by calcination at a temperature of 600°C in a Thermo Scientific muffle (#7.009, AOAC 2012). Neutral detergent fibre (NDF) and acid detergent fibre (FAD) shall be determined by method 12 and 13 of the ANKOM2000 fibre analyser (ANKOM Technology, Macedon, NY, USA), respectively. The crude protein (PC) was determined by elemental analysis (N), for which a LECO CHN 628 (LECO Corporation) was used.

Degradación ruminal in situ

Bulls of approximately two years of age weighing 450 (± 21.2) kg, with a fistula (Diamond Bar, Parma, Idaho, USA) in the rumen were analyzed. The animals were fed a diet based on *Medicago sativa* L. and *Lolium perenne L*. The nylon bag technique was applied to the rumen described by (Ørskov et al. 1980). ANKOM Technology R510 bags (5 cm x 10 cm, porosity 50 ± 10 µ) were placed in each fistulate bull and 5 g of dry matter (DM) from the fodder bushes were incubated for 0, 4, 12, 24, 48, 72 and 96 hours. At the end of the incubation periods, the bags were removed, washed under running water and dried at 60°C. The bags used to measure the loss on washing (0 h) were not incubated in the rumen and were only washed under running water. The waste was stored in polythene bags at -4°C. Nutrient disappearance was calculated as a proportion of incubated and residual material. Data were fitted to equation (1) and effective degradation was fitted by equation (2) considering a step rate of 2, 5 and 8% (Ørskov and McDonald 1979):

$$Y = a + b\,(1 - e^{-ct}) \quad (1)$$

$$ED = a + [(b * c) / (c + k)] \quad (2)$$

Where:

Y = Percentage of degradation accumulated over time, (t%).
SD = Effective degradation, (%/hour).
a = Interception of the degradation curve when t = 0, (initial degradability%).
b = Fraction potentially degraded in the rumen, (%).
c = Degradation rate, (%/hour).
t = Incubation time in the rumen, (hours).
e = Base of natural logarithms.
k = Step rate, (%).

Apparent digestibility and in vitro gas production

For this, the rumen content (liquid and solid fractions) was obtained separately from each fistulate bull. The rumen contents (1000 ml) were collected before feeding in the morning and kept at 39°C in a sealed plastic container during transport to the laboratory. The analysis of digestibility in the laboratory was performed at the time of collection in a nitrogen-rich medium according to Menke and Stengass (1988). Gas production was determined according to the methodology described by (Theodorou et al. 1994). In short, 0.5 g DM of each treatment was placed in glass bottles of 100 ml of nominal capacity, 60 ml of rumen inoculum (70:30 mean/rumen inoculum) were added under a constant flow of$_{CO2}$. The bottles were closed and incubated at 39-40°C. The pressure and gas volume were measured manually at 3, 6, 9, 12, 18, 24, 36, 48, 72 and 96 hours after incubation with a DELTA OHM pressure transducer model DO 9704 (Delta OHM, Padua, Italy) and with plastic syringes the gas volume was measured, followed by the concentration of$_{CO2}$ and CH_4 with a portable gas detector model GX-6000 (RKI Analytical Instruments, Hamburg, Germany). For each treatment, five bottles (replicas) were used for each incubation time and five additional bottles as controls. Total gas production was estimated per 0.5 g of fermentable dry matter (MFS), the results were adjusted according to the monobasic equation (3) described by Groot et al. (1996):

mL de gas = GV/(1 + (B/t) C) (3)

Where:
mL gas = In vitro gas production (mL/0.5 g SPS) at incubation time t
GV = Asymptote or in vitro gas production (mL/0.5 g MSF) when t is infinite
B = Incubation time (h) at which half of the asymptotic gas production is reached
C = Constant that determines the shape of the curve and, therefore, the position of the inflection point, given by: t_I = B ((C-1) / (C + 1)) 1/C
t = Incubation time in the rumen, (h).

In vitro digestibility was estimated at 48 hours of incubation, filtering the residues and correcting them with the residual DM of the vials used as targets, and then dried in an oven at 60 ° C to subsequently interpret the results.

Rumen protozoa

Five flasks per incubation time (6, 12 and 24 h) were used to determine the population of protozoa in the rumen. At the end of each incubation period, 1 ml was removed from each bottle. The protozoa were stored with a drop of formaldehyde and stored at 4 °C until quantification, using an optical microscope (40x) and a Fucsh-Rosenthal camera. The protozoa were stained with a solution of methyl green formamide according to the methodology described by Ogimoto and Imai (1981).

Experiment design and statistical analysis

A completely randomized design (DCA) was used, with five repetitions (fistulated bulls). The treatments were forage plants (Table 1) harvested at 60 days. The results of all variables were subjected to an analysis of variance using the SAS PROC GLM (SAS, 2002). Averages were assessed using Tukey's 0.05% test. The rumen degradation of DM and gas production were analyzed with the program Graphpad Prism 4, Software, Inc. San Diego, CA, USA.

Table 1. Description of forage shrubs evaluated in tropical humid forest conditions, Joya de las Sachas, Orellana, Ecuador.

Tratamiento	Scientific name+	Familytake
T1	*Gliricidia sepium* (Jacq.) Kunth ex Walp.	Fabaceae
T2	*Leucaena leucocephala* (Lam.) de Wit.	Fabaceae
T3	*Moringa oleifera* Lam.	Moringaceae
T4	*Morus alba* L.	Moraceae
T5	*Tithonia diversifolia* (Hemsl.) A. Gray	Asteraceae

The Plant List (2013)

Results

Chemical composition

The high levels of PC found in forage shrubs are attributed to the low content of FDN (<500 g/kg MS), FTO D (<400 g/kg DM) and organic matter (>800 g/kg DM) of the forages at 60 days of regrowth (Table 2).

Table 2. Nutritional quality of tree forages in tropical rainforest conditions.

Chemical composition	Tratamientos				
	T1	T2	T3	T4	T5
MS (g/kg)	919.1	937.8	927.6	905.9	887.5
OM (g/kg)	930.0	936.0	905.0	919.0	845.0
PC (g/kg)	326.1	310.1	298.0	280.0	293.4
FAD (g/kg)	472.9	445.5	418.9	346.3	454.3
FDN (g/kg)	335.4	315.9	306.5	297.1	283.9
Ash (g/kg)	21.6	14.3	19.5	29.6	24.5

MS, Dry matter; OM, Organic Matter; PC, crude protein; FAD: Acid detergent fiber; FDN: Neutral detergent fiber

In vitro digestibility of dry matter and organic matter (IVDDM and IVDOM

Significant differences were found between the treatments (Table 3), the T4 higher record IVIDDM (688.57 g/kg) and IVDOM (671.21 g/kg), the digestion efficiency found in *Morus alba* is attributed to the low levels of FDN (346.3 g/kg) and FAD (297.1 g/kg).

Table 3. In vitro digestibility of nutrients: dry matter (DM), organic matter (OM) of forage shrubs evaluated in tropical rainforest conditions

Treatments	IVDDM (g/kg)	IVDOM (g/kg)
T1	655.88(±51.21)[a]	654.53(±30.48)[from]
T2	550.57(±28.80)[b]	523.05(±31.70)[c]
T3	668.44(±40.86)[a]	639.78(±45.07)[from]
T4	688.57(±31.78)[a]	671.21(±32.87)[a]
T5	642.47(±36.96)[a]	587.39(±41.02)[bc]
WITHOUT	17.32	5.96
R^2	0.66	0.73
P-vrange	0.0002	<0.0001

[abc] Means within columns with different superscripts differ significantly (P<0.05); SEM, standard error of the mean; R^2: Coefficient of determination; P-value: probability

In situ degradation parameters of dry matter (DM)

The parameters of kinetics of rumen degradation showed differences (P<0.0001) between treatments, the T4 and T5 obtained the highest degradation for the soluble fraction (a), degradation potential (a + b), and effective degradation (ED) with the different rate constants (k). However, in the rate of degradation (c) no significant differences (P>0.05) were found between the treatments (Table 4).

Table 4. In situ degradation parameters of DM (g/kg) of forage shrubs evaluated under tropical rainforest conditions.

Degradation	Tratamiento					WITHOUT	R^2	P Value
	T1	T2	T3	T4	T5			
t0	1268	1243	1239	1245	1288			
a[1]	701.9[b]	653.2[c]	644.0[c]	917.4th	892.3[a]	6.32	0.99	<0.0001
b	44.9[b]	44.5[b]	100.3[a]	13.3[c]	55.1[b]	6.27	0.83	<0.0001
c	0.125[a]	0.074[a]	0.056[a]	0.061[a]	0.033[a]	0.03	0.18	0.3858
a+b	746.8[c]	697.6[d]	744.4[c]	930.7[b]	947.4[a]	3.34	1.00	<0.0001
ED 0.02% k	739.3[b]	684.3[d]	716.0[c]	926.7[a]	922.1[a]	3.13	1.00	<0.0001
ED 0.05% k	732.8[b]	676.2[d]	695.6[c]	924.4[a]	910.7[a]	3.76	1.00	<0.0001
ED 0.08% k	728.7[c]	672.0[d]	684.5[d]	923.2[a]	905.7[b]	3.87	1.00	<0.0001

a[1]: soluble fraction (in percent); b: insoluble but potentially degradable fraction (in percent); c: degradation rate (in percentage per hour); a+b: potential degradation; k: step rate (in percent); [abc] Means within rows with different superscripts differ significantly (P<0.05). ED, effective degradation; SEM: standard error of the mean. MS: dry matter.

Production of enteric greenhouse gases

The T1, T3 and T5 presented lower (P = 0.0009) gas production [GV (mL gas)] with 249.6, 232.1 and 257.2 mL gas / 0.5 g of fermented DM respectively and for CH 4 [GV (mLCH 4)] the T4 resulted with the lowest (P = 0.0049) production with 52.4 mL CH 4/0.5 g of fermented DM (Table 4), however, the production of CO2 [GV (mL_{CO2})] showed no statistical differences between treatments (P = 0.1851).

Table 5. Gas production parameters, CH_4 and CO2 in vitro (mL/0.5g of fermented DM) of forage shrubs evaluated under tropical humid forest conditions.

	Tratamientos					WITHOUT	R^2	P Value
	T1	T2	T3	T4	T5			
GV (mL gas)	249.6^{b}	319.6^{a}	232.1^{b}	278.1^{from}	257.2^{b}	12.53	0.59	0.0009
B	20.11^{from}	25.34^{a}	13.91^{b}	20.46^{from}	28.91^{a}	2.56	0.50	0.0061
C	1.09^{abc}	0.88^{c}	1.37^{a}	1.24^{from}	0.99^{bc}	0.08	0.55	0.0024
GV (mL CO_2)	337.2^{a}	269.2^{a}	335.6^{a}	298.6^{a}	274.7^{a}	24.70	0.26	0.1851
B	21.66^{b}	44.3^{a}	14.24^{b}	20.42^{b}	24.56^{b}	4.19	0.60	0.0008
C	1.18^{from}	0.88^{b}	1.42^{from}	1.64^{a}	0.83^{b}	0.05	0.54	0.0029
GV (mL CH_4)	53.9^{from}	65.9^{a}	57.6^{from}	52.4^{b}	53.6^{from}	3.17	0.38	0.0409
B	22.18^{a}	22.65^{a}	19.14^{a}	21.56^{a}	24.52^{a}	1.30	0.31	0.1004
C	0.95^{from}	0.90^{b}	1.00^{a}	1.01^{a}	0.96^{from}	0.02	0.42	0.0211

^{abc} Means within rows with different superscripts differ significantly (P<0.05); GV (mL/0.5g fermented DM), B (gas production asymptote) and C (gas production rate (%/h) are the parameters of the equation: mL gas = GV (1 + (B/t) C) (Groot et al. 1996); SEM: standard error of the mean.

Population of Holotric and Entodinomorphic protozoa of the rumen

The results showed that at 6 h of in vitro incubation of MS, the population of holotri cos protozoa was lower in T3 (P = 0.0206), and in Entodinomorphic protozoa was lower in T3 and T4 (P = 0.0082), at 12 and 24 h no statistical differences were observed (P>0.05) in the count of holometric and entodinomorphic protozoa between treatments.

Table 6. Protozoan populations in the rumen in vitro (log10) of forage shrubs evaluated under tropical rainforest conditions.

Protozoos	Tratamiento					WITHOUT	R^2	P Value
	T1	T2	T3	T4	T5			
6 a.m.								
H	2.53^{a}	1.86^{from}	0.42^{b}	0.90^{from}	2.00^{from}	0.45	0.43	0.0206

And	3.75[a]	3.50[from]	3.35[b]	3.21[b]	3.51[from]	0.09	0.48	0.0082
12:00 p.m.								
H	0.96[a]	1.53[a]	2.60[a]	1.99[a]	1.74[a]	0.49	0.23	0.2360
And	3.67[a]	3.67[a]	3.69[a]	3.58[a]	3.69[a]	0.05	0.16	0.4709
24 h								
H	0.84[a]	0.96[a]	2.19[a]	2.31[a]	1.26[a]	0.49	0.29	0.1308
And	3.56[a]	3.52[a]	3.61[a]	3.53[a]	3.53[a]	0.06	0.07	0.8299

H: Holotric; E: Enthonomorphs

[ab] Means within rows with different superscripts differ significantly ($P<0.05$).

Discussion

The nutritional quality of the forage shrubs studied indicate the potential they have to be incorporated as protein supplements in the diet of ruminants Rodríguez et al. (2014), in this sense the leguminous forage shrubs show a greater potential as a degradable protein supplement in rumen, on the other hand Gallego et al. (2014) mentions that non-legume fodder shrubs such as *Tithonia diversifolia* can be used as food supplements for their protein and soluble carbohydrate content. Sosa et al. (2020) refer to the digestibility of DM in in *vitro* experiments as in *situ* is linked to the contents of cellulose and hemicellulose, negatively affecting the digestion of forages when these contents increase, results that coincide with those found in this research to be *Morus alba* The forage that obtained better digestion. For Widiawati et al. (2018) a better intake of digestible organic matter translates into greater utilization of amino acids present in forage. The best results of IVOMD were for Morus alba (T4), *Gliricidia sepium* (T1) and *Moringa oleifera* (T3), the results in *Morus alba* agree with those obtained by Tesfay et al. (2018) (669 vs 671.2 g / kg), mulberry leaves have an appreciable potential as a source of protein in feed and supplementation for ruminants (Kandylis et al. 2009; Omar et al. 1999), due to its high IVOMD, other fractions of the plant can be used as alternative feed resources for low quality forages (Eshetu et al. 2018), according to Edwards et al. (2012) *Gliricidia sepium* at 8 weeks reports IVOMD values with 600 g / kg being lower than the results found in this study, but similar to those reported by La O et al. (2018) (639.8 vs 642.5 g/kg), the IVOMD results for *Moringa oleifera* in this study were lower than those reported by Melesse et al. (2013) (639.8 vs 750 g/kg). With regard to the results of the degradation parameters of the MS non-legume tree forages *Morus alba* (T4) and *Tithonia diversifolia* (T5) presented a better efficiency (Figure a 2).

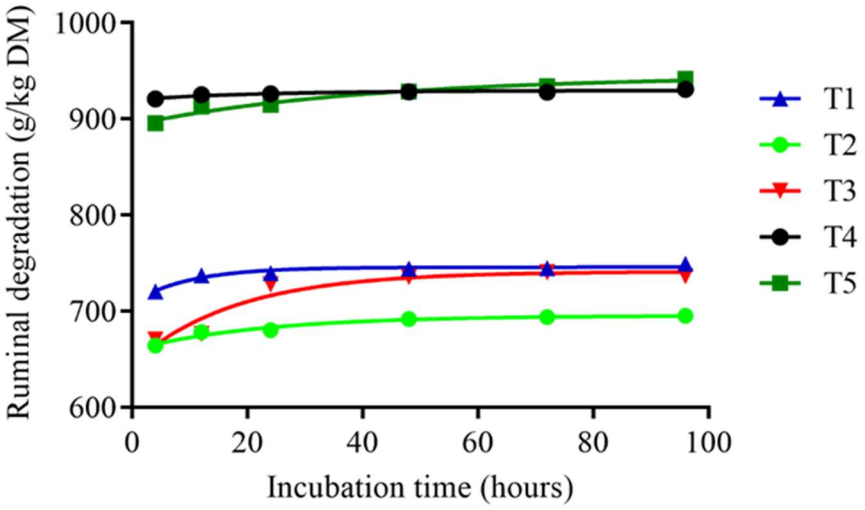

Figure 1. Kinetics of in *situ* degradation of dry matter in forage shrubs evaluated under tropical rainforest conditions.

Torres et al. (2019) in their study shows that *Morus alba* presents the best kinetic parameters of DM at 30, 45 and 60 days of regrowth, on the other hand Boschini (2001) reports values higher than 85% of potential degradability of DM in leaves at ages of 56 and 84 days, However, these results are lower than those obtained 60 days after regrowth in the present investigation; for Martín et al. (2007) the *Morus alba* is a species of great acceptance, used by farmers, especially to feed ruminants and minor species. The results found in Tithonia diversifolia agree with the research carried out by Valenciaga et al. (2018), where they report a potential degradation (a + b) of DM of 94.2% in plant materials of *Tithonia diversifolia* harvested at 60 days. Panadero and Montaña (2019) also indicate that this species contributes to the production of good quality food through strategic supplementation programs for critical times.

Mitigation strategies can be aimed at decreasing GHG production, or increasing the capture mechanisms (sink sources) of critical compounds that promote GHG formation (Alayón-Gamboa 2018); for Jiménez et al. (2019) forage trees incorporated with local energy sources contributes to lower enteric greenhouse gas emissions, in this context *Tithonia diversifolia* (Gas, CO2 and CH_4), resulted with the lowest accumulated production at 96 h of incubation (Figure 3), results that coincide with those reported by Rodríguez et al. (2019) where he evaluated several ecotypes of Tithonia diversifolia at 60 days of regrowth, in this same line of research Cardona et al. (2017b) is their study showed that methaneproduction decreases when including in the base diet for traditional non-legume forage cattle such as *Tithonia diversifolia*, this mitigation property is based on the efficiency of rumen degradation of the DM and the presence of phytochemical compounds such as tannins, flavonoids, essential oils and saponins (Rivera et al. 2018).

Inthis context, theuse of fodder shrubs in ruminant nutrition exerts depressive effects on rumen methanogen and protozoan populations, which benefits rumen microbial ecology, modifies populations of cellulolytic bacteria and fungi (Galindo et al. 2012: 2019; Rodríguez et al. 2019).

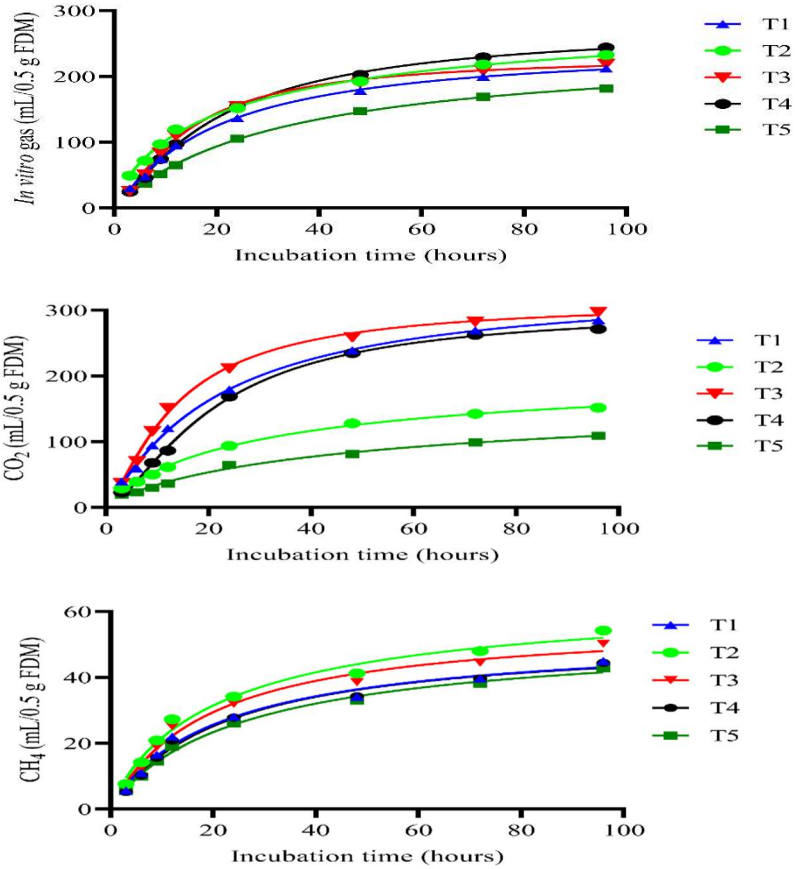

Figure 2. Production of gas (a),$_{CO2}$ (b) and CH_4 (c) mL/0.5 g FDM in tree forages

Galindo et al. (2014) report that forage shrubs can be used to reduce rumen protozoa, because they contain phytochemical compounds such as tannins, flavonoids, saponins, triterpenes, steroids, anthocyanidins, reducers and alkaloids. The role that protozoa play in rumen ecology, are transcendental in the lipid metabolism of ruminants (Salas et al. 2012) and the defaunation of rumen protozoa, is an alternative that is used to reduce rumen methanogens, which contributes to the energy efficiency of the animal (Barros-Rodríguez et al. 2017). in this research *Moringa oleifera* and Morus alba did not affect the count of the Holotricos at 24h if compared with the other forage shrubs studied, as reported by González et al. (2016) the levels of inclusion of *Morus alba* do not affect the main microbial populations that degrade rumen fiber, The mechanism by which this plant is able to reduce rumen methanogenesis is not through the direct effect on methanogens.

Conclusions

The efficiency of *Morus alba* (T4) and Tithonia diversifolia (T5) in the digestion and degradability of dry matter, were influenced by the low levels of FDN and FAD found in these non-legume forage shrubs, in addition *Tithonia diversifolia* showed better efficiency in the emission of gas, methane and enteric carbon dioxide, thereby reducing energy expenditure, which would result in better productive performance of ruminants.

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