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Tree Legumes of Temperate Climate: Effect on the Mitigation of Enteric Methane and Ruminal Fermentation In Vitro

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Tree legumes of temperate climate: effect on the mitigation of enteric methane and ruminal fermentation *in vitro*

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ABSTRACT: The effect of legumes adapted to a temperate climate on gas production kinetics, enteric methane and rumen fermentation *in vitro was evaluated.* The species studied were *Acacia dealbatha, Acacia melanoxylon, Albizzia lopantha, Lupinus pubescens, Inga insignes, Senna multiglandulosa* and *Tecoma stans.* The effective degradation of the dry matter was estimated, the *in vitro gas production* was measured up to 120 hours of incubation, the production of enteric methane, VFAs and ammonia were measured at 24 hours. Dry matter, organic matter, ash, crude protein, neutral detergent fiber, acid detergent fiber and acid detergent lignin were evaluated. The content of total phenols and condensed tannins was quantified. To determine digestibility, the samples were incubated for up to 120 hours. *I. insignes* presented a higher dry matter content and a high crude protein content. *L. pubescens* presented low dry matter digestibility. The neutral detergent fiber content was higher in *I. insignes* and with a higher value in dry matter digestibility and lower content of total phenols and condensed tannins. While *S. multiglandulosa* showed low detergent acid fiber content. The acid detergent lignin value was higher in *I. insignes* and lower in *L. pubescens. In vitro* gas and enteric methane production was higher in *L. pubescens.* propionic acid and ammonia they were higher in *S. multiglandulosa.* It is concluded that the amount of secondary compounds affects methane production, compromises digestibility and rumen kinetics of the legumes studied.

Keywords: Acacia dealbatha; Acacia melanoxylon; Albizzia lopantha; Lupinus pubescens; Inga insignes; Senna multiglandulosa

INTRODUCTION

Greenhouse gas (GHG) emissions from ruminants contribute to global warming and climate change [1], and represent between 7 and 18 percent of total GHG emissions. worldwide of anthropogenic origin [2] [3]. Among the main GHGs identified by international organizations [4] are methane (CH_4), carbon dioxide (CO_2), nitrous oxide (N_2O), Hydrofluorocarbons (HFC), Perfluorocarbons (PFC), and Sulfur Hexafluoride (SF_6).

Methane (CH_4) is one of the main gases that is released as the end product of rumen methane genesis of species linked to animal production. Daily methane production values in *B. taurus* x *B. indicus cross heifers* of 88.08 g/heifer/day (18.9 min –150.1 max) [5]. In sheep, methane production was 13.7 g/sheep/day [6].

In this sense, several works have been carried out in search of reducing greenhouse gas emissions, mainly enteric methane [7] [8] [9], however it should be considered that in ruminants there are several factors that affect enteric methane production, such as the type of microbial population in the rumen, rumen pH, acetate: propionate ratio, animal breed, dry matter intake, diet composition, management practices, stress environmental, among others [6].

Therefore, strategies such as population modification of ruminant animals, biotechnological processes to modify methanogenic microorganisms, and nutritional manipulation have been used to mitigate enteric methane production and alter the different causes related to enteric fermentation [1].

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With regard to nutritional manipulation, work carried out with plant species rich in secondary metabolites such as *Acacia. most anguished, Sesbania. sesban, Leucaena leucocephala, Pennisetum.purpureum* and *Acacia. mearnsii* have been used for their potential to reduce the production of enteric methane [10] [11] [12], which due to its tannin and saponin content have demonstrated their effect on the methanogenesis, the defaunation of methanogenic microorganisms and the non-inhibition of dietary protein synthesis.

The availability of tree legumes in each country and their use associated with traditional grazing [13], would prove to be a model to follow as an alternative system in ruminant feeding to contribute to the reduction of enteric methane emissions into the environment. atmosphere.

These findings support the objective of studying the rumen modulation capacity by tree legumes, considering their species, climate, content of secondary metabolites and their effect on rumen function.

MATERIALS AND METHODS

Animals

Four rambouillet sheep were used (66.6± 0.75 kg body weight; 4 years of age) with permanent rumen cannula were used as donors of rumen contents for *in vitro incubations.* The animals were cared for and handled by trained personnel in accordance with the Spanish guidelines for animal protection in accordance with European regulations, and the experimental procedures were approved by the Animal Experimentation Ethics Committee of the Autonomous Community of Madrid (Authorization number PROEX 035/17). The animals were fed a mixed diet consisting of 600 g of oat hay and 400 g of a commercial concentrate per kg (fresh matter basis). The concentrate consisted (g/kg, fresh matter basis) of 329 g barley grain, 328 g corn grain, 244 g wheat, 62 g soybean meal, 28 g CO_3Ca, 6 g salt and 2 g of mineral-vitamin mixture. The mixed diet contained 112, 350, and 166 g crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) per kg DM, respectively, and was offered at 50 g DM/kg metabolic weight. ($PV^{0.75}$) in two equal portions at 09:00 and 17:00. The consumption of water was at will.

Collection of samples.

Seven tree legumes from the temperate climate of Ecuador located between 2500 - 2800 m.a.s.l were used. The samples collected were from *Acacia dealbatha, Acacia melanoxylon, Albizzia lopantha, Lupinus pubescens, Inga insignes, Senna multiglandulosa* and *Tecoma stans.* Each collected sample corresponded to the green leaves of plant, approximately 5 kg of green leaves were collected, the harvest was carried out during the months of February and March corresponding to the rainy season. The relative humidity of the sector is 55% - 60% and the average annual precipitation is approximately 1390 mm. Subsequently, the leaves were dried in an oven at 60 °C for 72 h. The particle size was reduced using a mill with a 1 mm sieve. Finally, the samples were stored for their subsequent chemical analysis and *in vitro tests.*

Chemical analysis

The content of dry matter (ID 934.01), ash (ID 942.05) and nitrogen (ID 990.03) were analyzed according to [14] AOAC (1999). Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) were measured sequentially using the same sample in filter bags and expressed without including residual ash according to [15] Van Soest et al. (1991), using an ANKOM220 fiber analyzer (ANKOM Technology Corporation, Fairport, NY, USA). The NDF was determined using a thermostable amylase. Acid detergent lignin (ADL) was determined by solubilization of cellulose with sulfuric acid (72%) [15]. Total phenols (FT) were determined with the Folin-Ciocalteau method [16], and tannic acid as standard and condensed tannins (TC) were measured by the hydrochloric acid vanillin method [17], using catechin as standard, using a spectrophotometer Hitachi U2000 UV VIS

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N-NH3 concentrations were determined using an Epoch microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) by the phenol-hypochlorite method [18]. The production of volatile fatty acids (VFA) was determined by gas chromatography [19]. The methane analysis was carried out using a gas chromatograph [20], (Shimadzu GC 14B; Shimadzu Europa GmbH, Duisburg, Germany) equipped with a flame ionization detector and a column loaded with Carboxen 1000 (Supelco, Madrid, Spain).

In vitro incubations

in vitro incubations was obtained from four ewes immediately before morning feeding. The incubations were carried out using the inoculum from each sheep separately, to obtain four replicates per treatment. Glass vials (60 mL) were used in which 200 mg of dry matter from each of the samples were weighed. The rumen contents extracted from each animal were filtered through four layers of gauze and immediately transferred to the laboratory. The ruminal fluid was mixed with a culture medium [21] in a 1:4 (vol/vol) ratio at 39°C under continuous gassing with CO_2, dosing 20 mL of the mixture in each vial by a peristaltic pump (Watson-Marlow 520UIP31). The vials were sealed and incubated at 39°C for 120 hours. Additionally, vials without substrate (two per inoculum) were included to correct the gas production values of endogenous substrates. Gas production was measured in each vial at 3, 6, 9, 12, 14, 22, 26, 30, 34, 48, 58, 72, 96, and 120 hours using a pressure transducer (Delta Ohm DTP704-2BGI; Herter Instruments SL, Barcelona, Spain) the produced gas was released after each measurement. The gas production data measured in each vial at each sampling time were adjusted to the model: mL gas= PG(1+(B/t) c)-1 [22], where PG: total gas production; B: gas asymptote and c: gas production rate.

On the other hand, in the vials incubated at 24 h, the gas produced was measured and a sample (10 mL) was taken in a vacuum tube (Terumo Europe NV, Leuven, Belgium) for methane analysis. The bottles were uncapped and the pH was immediately measured with a pH meter (Crison Basic 20; Crison Instruments, Barcelona, Spain). For AGV analysis, three milliliters of the contents of the vials were taken at the same time and 3 mL of deproteinizing solution (20 g of metaphosphoric acid and 0.6 g of crotonic acid per liter) was added and to determine $N\text{-}NH_3$ used 2 mL of content plus 2 mL of HCl 0.5 mol L^{-1}. The apparent *in vitro digestibility* of dry matter (IVDMS) was measured using a DAYSI II equipment (ANKOM Technology Corporation, Fairport, NY, USA) with samples that were incubated for up to 120 hours.

Statistical calculations and analysis

To estimate the kinetic parameters of fermentation (unpublished data except effective dry matter degradation) the gas production data were fitted to the exponential model: gas = A {1-exp [- c (t - lag)]}, where A is the gas production asymptote, c is the fractional rate of gas production, lag is the initial delay in the start of gas production, and t is the gas measurement time. Parameters A , c and lag were estimated by an iterative least squares procedure using a SAS NLIN process (version 9.2, SAS Institute, Cary, NC, USA) [23]. The mean gas production time ($T_{1/2}$) was the time (h) when half the volume of asymptotic gas (A; mL) was produced and was calculated as $T_{1/2} = [(\ln 2 / c) + lag]$. The average gas production rate (AGPR; mL gas h^{-1}) was defined as the average gas production rate between the start of incubation and $T_{1/2}$, and was calculated as AGPR = Ac/[2 (ln2 + c × lag)]. Finally, the effective degradability of dry matter (DEMS) was estimated assuming a passage rate (Kp) of 0.04 per h according to the equation: $DEMS = [(IVDMS_{120} \times c) / (c + Kp)] e^{(-c \times lag)}$.

The results obtained were analyzed under a completely randomized design (DCA) using the SAS statistical package (version 9.2, SAS Institute, Cary, NC, USA). *In vitro* gas production curves were obtained using Prism 4 software, Graphpad Software, Inc. of San Diego, CA, USA. The comparison of means was carried out using Tukey's test at 5%. The relationship between the content of secondary compounds and the gas and methane production parameters were evaluated by means of a simple correlation analysis using the PROC CORR procedure of the SAS statistical package (version 9.2, SAS Institute, Cary, NC, USA).

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RESULTS

Chemical composition

Table 1 reports the chemical composition of the legumes analyzed, where it is observed that *I. insignis* presented the highest dry matter content (497.00 g/kg $^{-1)}$ with respect to the other species. The range of crude protein content in all species was variable and was between 134.66 - 238.45 g/kg $^{-1}$ DM, however, in *T. stans* the 53.28% of its crude protein was bound to the insoluble cell wall (detergent acid fiber). The neutral detergent fiber content was much higher in *I. insignes* with 647.65 g/kg $^{-1}$ DM, while *S. multiglandulosa* and *L. pubescens* had a lower content of acid detergent fiber (198.25 g/kg $^{-1}$ DM) and lignin. detergent acid (72.29 g/kg $^{-1}$ DM) respectively.

On the other hand, the lowest content of total phenols was found in *T. stans* (1.19%), finally *L. pubescens* presented 0.42% of condensed tannins, a lower value compared to the other species.

Table 1. Chemical composition of different tree legumes from temperate climates expressed as g/kg $^{-1}$ of dry matter (except where otherwise indicated).

Item	*Acacia dealbatha*	*Acacia melanoxylon*	*Albizzia lophanta*	*Lupinus pubescens*	*Inga insignis*	*Senna multi glandular*	*Tecoma stans*
MS [1]	484.00	484.74	457.23	245.64	497.00	336.64	346.66
MO	933.12	934.92	934.94	910.56	908.00	846.75	926.63
ASHES	66.93	65.18	65.13	89.58	92.00	153.38	73.46
CP	162.32	134.66	141.87	238.45	176.85	162.95	165.37
PC-FAD(%)	34.86	32.79	21.98	4.13	40.43	4.64	53.28
NDF	519.88	573.45	329	402.94	647.65	275.92	492.68
FAD	408.64	406.90	242.33	221.15	518.86	198.25	368.00
LAD	285.00	252.25	145.54	72.29	311.29	117.12	229.47
LAD/FAD(%)	69.74	61.99	60.05	32.77	59.99	59.19	62.43
TF[2] (%)	2.32	1.95	2.94	1.73	3.76	1.69	1.19
CT [3] (%)	1.24	1.48	2.18	0.42	3.45	0.35	1.15

DM: dry matter; [1] g/kg of fresh matter; OM: organic matter; PC: crude protein; PC-FAD: crude protein bound to detergent acid fiber.; FND: ash-free neutral detergent fiber; FAD: ash-free acid detergent fiber; LAD: lignin acid free detergent ash; LAD/FAD: lignin in relation to acid detergent fiber; [2] TF: total phenols using tannic acid as standard; [3] CT: condensed tannins using catechin as standard.

In vitro gas production and enteric methane

I. insignes presented a statistically different lower gas production (p=0.0001) when compared with the other species subjected to *in vitro fermentation* (table 2). The gas production asymptote and the fermentation rate were the same or higher in all species evaluated. In fig. 1 distinguishes differentiated groups with higher and lower gas production.

While the species that presented the greatest potential in the reduction of enteric methane produced were *I. insignes* and *A. melanoxylon* (1.09 and 1.35 mL CH4/g DM $^{-1}$ respectively). Additionally, a significant variation (p=0.0001) was evidenced in the pH of the inocula at 24 hours of fermentation in a range of 6.82 - 7.25

Table 2. Gas production parameters and enteric methane of temperate climate tree legumes.

Species	Gas production				Methane production	
	GP	B.	c	pH	mL CH_4/g DM 24h	% CH_4 total 24h
A .dealbatha	84.3 [d]	19.5 [ab]	1.220 [b]	7.23 [a]	1.76 [bc]	21.06
A. melanoxylon	70.4 [ed]	17.3[b]	1.390 [b]	7.25 [a]	1.35 [c]	15.56

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A. lopantha	110.4 ^{c}	17.9 ^{ab}	1.540^{ab}	7.07 ^{b}	1.86 ^{bc}	15.03
L. pubescens	201.9 ^{a}	20.3 ^{ab}	1.540^{ab}	6.87 ^{c}	4.54 ^{a}	21.54
I. insignes	50.7 ^{e}	18.0 ^{ab}	1.467^{ab}	7.29 ^{a}	1.09 ^{c}	15.98
S. multiglandulosa	204.4 ^{a}	15.6 ^{b}	1.856 ^{a}	6.82 ^{c}	3.61 ^{ba}	15.61
T. stans	149.3 ^{b}	30.5 ^{a}	1.322 ^{b}	7.02 ^{b}	2.56 ^{abc}	18.37
EEM	5.51	2.79	0.0949	0.0246	0.450	2.178
Value *P*	0.0001	0.0243	0.0039	0.0001	0.0002	0.1982

GP: total gas production (mL/g DM); B: gas asymptote (mL/g DM); and c: gas production rate (%/hour). ^{abc} Values followed by different letter between rows differ significantly (p <0.005).

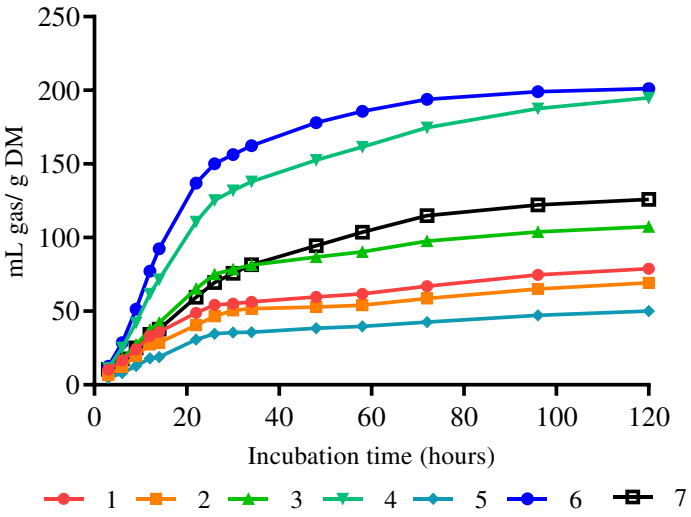

Figure 1. Gas production curves of tree legumes subjected to different incubation times 1: A. dealbatha; 2: A. melanoxylon; 3: A. lopantha; 4: L. pubescens; 5: I. insignes; 6: S. multiglandulosa; 7: T. stans.

Volatile fatty acid profile and rumen function

Table 3 showed the molar proportion of volatile fatty acids (%), where *S. multiglandulosa* presented 61.69, 29.51 and 4.72 for acetate, propionate and butyrate respectively, percentages that differed significantly (p=0.0001) compared to the other species. Similarly, the highest concentration of N-NH_3 was for *S. multiglandulosa.* However, the highest DEMS and DIVMS was for *I. insignes* with 381.28 and 791.46 g/kg respectively.

Table 3. Volatile fatty acids (mol/100mol), ammoniacal nitrogen (N-NH3 mg/L), effective degradation of dry matter (EDDM g/kg $^{-1)}$ and *in vitro digestibility* of dry matter (IVDDM g/kg $^{-1)}$ of temperate climate tree legumes.

	A. dealbatha	***A. melanoxylon***	***A. lopantha***	***L. pubescens***	***I. insignes***	***S. multiglandulosa***	***T. stans***	**EEM**	**Value P**
Acetate	69.77 ^{b}	71.74 ^{b}	70.99 ^{b}	70.20 ^{b}	71.36 ^{b}	61.69 ^{c}	75.65 ^{a}	0.771	0.0001
Propionate	20.63 ^{b}	21.53^{b}	22.77 ^{b}	21.87 ^{b}	17.45 ^{c}	29.51 ^{a}	16.36 ^{c}	0.491	0.0001
butyrate	6.25 ^{ba}	4.31^{c}	4.37 ^{c}	5.37 ^{bc}	7.40 ^{a}	4.72 ^{bc}	5.73 ^{bc}	0.353	0.0001
isobutyrate	0.82 ^{a}	0.64 ^{a}	0.46 ^{a}	0.60 ^{a}	0.93 ^{a}	0.80 ^{a}	0.66 ^{a}	0.116	0.1296
isovalerate	1.32 ^{ba}	0.98 ^{ba}	0.69 ^{b}	1.11 ^{ba}	1.65 ^{a}	1.32 ^{ba}	0.90 ^{b}	0.147	0.0036
valerate	1.18 ^{b}	0.80 ^{cb}	0.70 ^{c}	0.84 ^{cb}	1.19 ^{b}	1.65 ^{a}	0.67 ^{c}	0.098	0.0001
A/P	3.38 ^{b}	3.33 ^{b}	3.11 ^{b}	3.20 ^{b}	4.12 ^{a}	2.11 ^{c}	4.64 ^{a}	0.117	0.0001
N-NH3	133.65	125.12	115.70	128.53	161.15	211.53	109.88	26.199	0.1459
$EDDM_{4\%}$	339.24 ^{ab}	363.61 ^{a}	309.58 ^{ab}	173.18 ^{c}	381.28^{a}	232.16 ^{bc}	272.63^{abc}	2.637	0.0001
IVDDM	748.57 ^{ab}	686.58 ^{ab}	661.44 ^{b}	477.05 ^{c}	791.46^{a}	469.91 ^{c}	698.82 ^{ab}	2.806	0.0001

^{abc} Values followed by a different letter between columns are significantly different (p <0.05).

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The correlation analysis (table 4) indicates a strong associativity between the content of total phenols, condensed tannins and the production of total gas (r= -0.664, r= -0.813) and enteric methane (r= -0.602, r= -0.796). A direct relationship was observed between the content of secondary compounds and effective degradation of dry matter.

Table 4. Parameters of Pearson correlation between the content of secondary compounds and the production of gas, methane and ruminal function of tree legumes from temperate climates.

	Gas production		methane production		ruminal function					
					pH		N-NH3		EDDM 4%	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
TF	-0.664	0.104	-0.602	0.153	0.051	0.913	0.097	0.836	0.616	0.141
TC	-0.813	0.026	-0.796	0.032	-0.093	0.843	-0.143	0.760	0.781	0.038

FT: total phenols; TC: condensed tannins.

DISCUSSION

The dry matter content is related to the availability of nutrients in the plant [24], in this sense I. *insignis, A. dealbatha, A. melanoxylon and A. lophanta* presented amounts close to 500 g/kg MS $^{-1}$ which would apparently reflect a higher biomass available for the animal compared to the other species studied.

However, when analyzing the nutritional components, it was observed that in species such as *T. stans* and *I. insignes,* despite their acceptable levels of crude protein, 53.28 and 40.43% of that protein, respectively, were bound to detergent acid fiber, thus reducing the availability of protein for its degradation at the ruminal level.

In the other hand, if we consider the fibrous components of plants as predictors of dry matter intake [25], indicates that species with high lignin content (LAD) such as *I. insignes, A. dealbatha and A. melanoxylon* would reduce consumption levels by animals and, on the contrary, it has been shown that there is a relationship between consumption preference in animals and NDF content (r = 0.737; P < 0.0001).

The content of secondary compounds (total phenols and condensed tannins) was lower than that reported in other studies in tropical legumes [26] [27] [28] due to their different metabolic pathways (C_3 and C_4) used for photorespiration [29].

Regarding the amount of phenols found in this research, they would not represent any limitation on fermentation parameters, because at the rumen level non-tannic phenols do not have the affinity to bind with proteins and other nutrients [16]. Meanwhile, tannins have a different mechanism of action, being the most effective hydrolysables in reducing methanogenesis by having a direct effect on rumen microorganisms, inhibiting their growth. In this investigation, the amount of condensed tannins (between 0.42 - 3.45%) was determined. These low toxicity compounds [30], have an indirect effect, but more evident at the rumen level, exerting their action on nutrients, decreasing digestibility, gas and methane production [31]. In addition, those shrubs that have a higher content of condensed tannins and protein would be related to a greater availability for the formation of tannin-protein complexes [25], as was the case of *I. insignes* in this research.

However, it has also been reported [32] [33] the action of condensed tannins on ruminal microorganisms as a selective process, acting especially on the population of ruminal methanogens, cellulitic bacteria and protozoa associated with the methanogenesis, causing a decrease in the availability of hydrogen in the rumen, which may affect hydrolysis, nutrient degradation and microbial protein synthesis. Studies relates these effects based on the concentration and type of tannins in the forage and adaptation of the rumen population, qualifying the use of forages rich in tannins as beneficial [34].

The content of condensed tannins found in the tree legumes was significantly related ($p<0.05$) with the production of gas and enteric methane, expected results as detailed by studies [35], in this context, it was evidenced that as the concentration of condensed tannins increased, gas and methane

production decreased. However, the methane reduction was more marked when it was calculated per unit of nutrient (mL/CH4/g DM) compared to the total percentage of methane produced at 24 hours of incubation, behavior perhaps related to the type of tannins (condensed or hydrolyzable) as mentioned by studies [31], which also indicates that the greater the number of hydroxyl groups in the condensed tannins, the greater their potential to reduce methane production. In this sense, lower gas production may also be related to higher protein utilization and microbial protein synthesis [36].

Fermentation kinetics patterns (asymptote, fermentation rate) were also affected by condensed tannin levels, perhaps due to variability in carbohydrate (FAD) and lignin content present in plants [37]. On the other hand, in others studies [24] it was observed that the higher molar proportion of propionate was not related to a decrease in methane production, perhaps due more to the amount of condensed tannins present in the plants and their antimethanogenic activity rather than decrease fiber digestibility.

The effective degradation of dry matter was higher in those forages with higher content of condensed tannins, behavior similar to that reported in similar studies [38]. On the other hand, the condensed tannins decreased (not significantly) the concentration of N-NH_3, a desirable characteristic up to a certain point according to others authors [39], in this sense, one investigation [38] mention that N-NH_3 is part of the nutrient supply for proteolytic and cellulolytic bacteria. Furthermore, [39] indicates that the presence of tannins could decrease the ammonia concentration, exhibiting the efficient use of volatile fatty acids (VFA) for the synthesis of microbial proteins.

The digestibility values were low in those legumes with a lower content of condensed tannins (*S. multiglandulosa)* due to a greater extent to the lignin content that could be related to low rates of particle size reduction and slower outflow rates affecting at the end to consumption [25].

CONCLUSION

The effects caused by secondary metabolites present in the legumes studied on enteric methane production and ruminal kinetics were different, these results being conditioned to the type and amount of secondary compounds present. On the other hand, it seems that the detrimental effects on digestibility would be related to the content of indigestible fibers rather than the content of condensed tannins.

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