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In vitro production of gases with mixtures of *Hyparrhenia rufa* (Nees) and *Leucaena leucocephala* (Lam) de Wit

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ABSTRACT

Objective: To evaluate total *in vitro* gas and methane (CH_4) production in different mixtures of *Hyparrhenia ruffa* (Hr) and *Leucaena leucocephala* (Ll).

Design/methodology/approach: In airtight biodigesters with 200 mL of culture medium, 20 g of the following treatments were incubated by triplicate: T1: 100% Hr, T2: 80% Hr + 20% Ll, T3: 60% Hr + 40% Ll, T4: 40% Hr + 60% Ll; these were inoculated with 20 mL of fresh rumen fluid and incubated at 38±0.5 °C for 24, 48, 72 and 96 h. The total gas and CH_4 production were assessed; the data were analyzed in a completely randomized design.

Results: The addition of 20%, 40% and 60% Ll in mixture with Hr decreased the neutral detergent fiber (NDF), acid detergent fiber (ADF), total gas and CH_4 production, while the crude protein content increased.

Study limitations/implications: *In vivo* studies are required / by including amounts higher than 20% Ll may improve energy utilization efficiency.

Findings/conclusions: Adding more than 20% *L. leucocephala* in a mixture with *H. ruffa* decreases total gas and CH_4 production.

Keywords: methane, ruminants, tropical grasses, forage shrubs.

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INTRODUCTION

The digestive process of the food consumed by ruminants involves physical, microbiological and chemical processes. The fermentation of food in the rumen is carried out by microorganisms, including bacteria, protozoa and fungi; the products of fermentation are: energy in the form of adenine triphosphate (ATP), which is used by microorganisms to grow and reproduce, the process involves the synthesis of microbial mass that will be later digested in the abomasum and used by the ruminant as a true protein source; volatile fatty acids such as acetic, propionic and butyric are also produced, which will be used by the ruminant as a principal energy source. As secondary products of fermentation,

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carbon dioxide (CO_2), hydrogen (H_2) and CH_4 are produced, these are synthesized from the fermentation of structural carbohydrates products by rumen methanogenic archaea such as *Methanobrevibacter ruminantium*, *Methanobacterium formicicum*, *Methanomicrobium mobile* (Cobos *et al.*, 2018).

The CH4 production is necessary for the oxidation of nicotin adenine dinucleotide (NAD), it is a step in the process for obtaining the energy contained in the neutral detergent fiber (NDF) and other nutrients in the diet, due that this process reduces the accumulation of H_2, it regulates pH and reduces ruminal pressure (Sharp *et al.*, 1998; Ley de Coss *et al.*, 2018) to maintain ruminal stability (Galindo *et al.*, 2010). In a second process, with the manure fermentation, nitrous oxide (N_2O) is produced, a gas with 310 times the potential of heat retaining than CO_2 (Ellis *et al.*, 2012).

Methane emissions from bovine enteric fermentation account for about 39% of the greenhouse gases (GHG) produced by the livestock sector (Arbre *et al.*, 2016), it is necessary to reverse the negative trends of extensive livestock farming, where the increase of CO_2 was of 70% and 40% for CH_4 during the 1970 to 2004 period (IPCC, 2016). To achieve this, it is required to design and evaluate methodologies to accurately calculate the energy flow and its relationship with GHG emissions in livestock activity (De-Vries and de-Boer, 2010).

In the southern and southeastern regions of Mexico, the ruminants' diet is based on forage grasses and shrubs; the former has a higher NDF, acid detergent fiber (ADF) and lower protein content, which favors higher CO_2, H_2 and CH_4 production. Methane production in tropical regions is of 20 L kg^{-1} of fermented dry matter (Piñeiro *et al.*, 2017), with energy losses in the form of CH_4 of up to 18%, so that, by using feeding strategies that allow greater efficiency in energy utilization, emissions of this gas could be reduced and cattle productivity improved (Vanlierde *et al.*, 2016).

The rate of CH_4 emissions from ruminal fermentation is directly related to the intake, nutrient content, physical and chemical characteristics and diet digestibility. Therefore, a strategy to reduce enteric methane production is through feeding management that allows changes in the ruminal fermentation pattern (Piñeiro *et al.*, 2017). The inclusion of forage trees and shrubs in the feeding of ruminants has improved NDF degradation, due to the higher crude protein contribution. When 20% of *L. leucocephala* was included in the diet, CH_4 production was reduced by 26% (Piñeiro *et al.*, 2015). Reduction in CH_4 emission was also observed with the shrubs as *L. leucocephala*, *Sapindus saponaria*, *Calliandra calothyrsus*, *Pithecellobium dulce*, *Heliocarpus velutinus* and *Guazuma ulmifolia*, when supplied with low quality pasture or with *Megathyrsus maximus* (Gaviria *et al.*, 2015; Lopez *et al.*, 2016). Based on the above, the objective was to assess the total gases and CH_4 production, when combined with *Hyparrhenia ruffa* grass and *Leucaena leucocephala* in *in vitro* incubation.

MATERIALS AND METHODS

Location of the study. The research took place from May 1 to June 30, 2019, in the Jaulas Metabólicas area and the Laboratorio de Sanidad Agropecuaria at the Facultad de Ciencias Agronómicas, Campus V of the Universidad Autónoma de Chiapas located at the Centro Universitario de Transferencia de Tecnología (University Center for Technology Transfer, CUTT) "San Ramón", Carretera al ejido 16 de septiembre km 2.5 in Villaflores,

Chiapas, Mexico (16° 27' 59" N and 93° 28' 43" W). From September 2 to 16, 2019, the analyses were performed at the Laboratorio de Nutrición Animal of the Programa en Ganadería from the Colegio de Postgraduados, Campus Montecillos, México (19° 27' 39.24" N and 98° 54' 29.19" W).

Chemical analysis of samples. The mixtures of *H. ruffa* and *L. leucocephala* were analyzed for total dry matter (TDM), crude protein (CP) following the Kjeldahl method, ethereal extract (EE) via the Soxhlet method, crude fiber (CF) by the Weende method, ash (C) and organic matter (OM) by difference, all using the techniques described by the AOAC (2012). The fiber fractions (NDF and FDA) determination was performed with alpha-amylase without ash correction as specified by Van Soest *et al.* (1991).

Growth medium and treatments. Rumen liquid (RL) from a 525 kg live weight male Brown Swiss bovine was used, the RL was extracted with an esophageal tube with a vacuum pump. The bovine received a diet with 85% *Hyparrhenia rufa* and 15% of a concentrated feed, containing 2.7 Mcal of ME and 14% crude protein (CP), the culture medium used to determine total gas (TG) production, CO_2 and CH_4 ratios and DM degradation (Table 1), was prepared in sterile conditions and low CO_2 flow.

The evaluated treatments were: T1: 100% *Hyparrhenia rufa* (Hr), T2: 80% Hr + 20% *L. leucocephala* (Ll), T3: 60% Hr + 40% Ll, T4: 40% Hr + 60% Ll.

Table 1. Culture medium for *in vitro* fermentation of dry matter of *Hyparrhenia rufa* and *Leucaena leucocephala* mixtures.

Compound	Quantity (mL) for 100 mL of medium
Destilled water	52.9
Clarified rumen fluid (1)	30.0
Mineral solution I (2)	5.0
Mineral solution II (3)	5.0
Sodium carbonate (Na_2CO_3), 8% (4)	5.0
Sulfide-cysteine solution (5)	2.0
Resazurin solution, 0.1 % (6)	0.1

(1) Clarified rumen fluid filtrated and centrifugated at 17,664 g for 15 min and sterilized 20 min at 21 °C a 15 psi. (2) Contains (in 1000 mL) 6 g K_2HPO_4. (3) Contains (in 1000 mL H_2O), 6 g KH_2PO_4, 6 g $(NH_4)_2SO_4$, 12 g NaCl, 2.45 g $MgSO_4$ y 1.6 g $CaCl_2 \cdot H_2O$. (4) 8 g Na_2CO_3 in 100 mL H_2O destilled. (5) 2.5 g L-cysteine (in 15 mL 2N NaOH) + 2.5 g Na_2S-$9H_2O$ (in 100 mL H_2O). (6) 0.1 mL resazurin in 100 mL.

Production of total gases and CH_4. The *in vitro* production of TG and CH_4 was determined by triplicate with repetition throughout time using 2.0 L capacity biodigesters with a hermetic look, to which the following mixture was added in aseptic conditions and CO_2 flow: 20 g of DM of each treatment plus 200 mL of culture medium (Table 1), each treatment was inoculated with 20 mL of fresh RL, which was filtered in cotton gauze and kept in a water bath at 38±0.5 °C and CO_2 flow. The initial total bacteria concentration was counted with the most probable number (MPN) technique with 1.30×10^8 CFU mL^{-1}

at pH 6.72. The inoculated treatments were incubated for 24, 48, 72 and 96 h in a water bath at 38±0.5 °C. At the end of the incubation period, the TG pressure was measured with a manometer adapted to the equipment. Afterward, the amount of produced TG in the system was assessed using the liquid displacement technique through a trap with Mariotte flasks.

The displaced water was collected in a graduated cylinder and the amount of gas produced per gram of fermented DM (mL of gas g^{-1} of MSf) was determined. In a second run and under the same culture conditions and temperature, a 2N NaOH solution (20 g L^{-1}) pH 13.57 was added to the Mariotte flask traps; the NaOH solution upon reacting with CO_2 formed Na_2CO_3 and the released gases were a mixture of CH_4, H_2, N_2 and H_2S according to the technique described by Ley de Coss *et al.* (2018); the CO_2 trap was coupled to the biodigester using a Taygon hose (5 mm internal 35 cm length) to which a 31.8 mm 20 G hypodermic needle was attached. In all TG production evaluations, the result was corrected by difference to the gas production of the blank samples (200 mL of culture medium plus 20 mL of RL), as well as with the CH_4 concentration measurements using the gas trap technique with saline solution.

Statistical analysis. The experimental design was in a completely randomized design, total gas and CH_4 production data were analyzed with the PROC GLM procedure of the Statistical Analysis System (SAS) (SAS, 2011) and the means of the treatments were compared with Tukey's test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Chemical composition of the diets

Table 2 shows the chemical composition of the *H. ruffa* and *L. leucocephala* evaluated mixtures. By increasing the *L. leucocephala* proportion from 0.0% to 60% of the mixture, the NDF content decreased from 55.7% to 38.2%; FDA from 28.7% to 18.4%; while the CP content increased from 8.3% to 18.1% and the ethereal extract from 0.8% to 2.4%. The increase in CP content and reduction of NDF and FDA could have an impact on the production of TG and CH_4, during *in vitro* fermentation (Piñeiro *et al.*, 2017). One of the factors that modify the fermentation pattern and products is the amount of fermentable NDF; in this study, the 100% *H. ruffa* fermentation, showed a higher production of total gases (Table 3). Grasses from tropical regions such as *H. ruffa* have a higher NDF amount than shrubs, which generates fermentation patterns with higher CH_4 production (Archimède *et al.*, 2011).

Total gas production

Table 3 shows that increasing the *L. leucocephala* content in the mixture from 0.0% to 20% decreased the TG production at 24 and 96 h of incubation ($P<0.05$), at 48 and 72 h there was no difference between both treatments ($P>0.05$). When the *L. leucocephala* proportion increased to 40%, TG production was lower at all incubation hours ($P<0.05$) compared to the treatment without *L. leucocephala*, and there was also a difference ($P<0.05$) compared to the treatment where 20% *L. leucocephala* was included at all incubation hours.

Table 2. Chemical composition of *Hyparrhenia ruffa* and *Leucaena leucocephala* mixtures (g per 100 g of dry matter).

	Proportion of *H. ruffa: L. leucocephala*			
Nutrient (%)	100	80:20	60:40	40:60
Dry material	93.4	92.8	92.1	91.5
Ashes	9.0	8.9	8.8	8.7
Raw Fiber	28.8	26.7	24.7	22.6
NDF	55.7	49.9	44.0	38.2
ADF	28.7	25.3	21.8	18.4
Raw protein (N×6.25)	8.3	11.5	14.8	18.1
Ethereal extract	0.8	1.3	1.9	2.4
NFE	53.3	52.0	50.7	49.4

NDF, Neutral detergent fiber; ADF, Acid detergent fiber; N, Nitrógen; NFE, Nitrogen free extract.

Table 3. Cumulative *in vitro* total gas production (mL of gas per 20 g of fermented DM).

Treatments ***Hyparrhenia ruffa*: *Leucaena leucocephala***	**Time (h)**			
	24	**48**	**72**	**96**
100	150.03^{a}	248.82^{a}	357.75^{a}	605.47^{a}
80:20	99.60^{b}	245.69^{a}	353.37^{a}	480.89^{b}
60:40	91.77^{c}	143.91^{b}	263.26^{b}	348.04^{c}
40:60	89.93^{c}	123.78^{b}	234.72^{b}	300.75^{c}
SEM	2.84	9.17	26.48	22.85
DMS	7.43	23.99	69.24	42.08

SEM: Standart error of the mean; MSD: Minimum significant difference. $^{a, b, c}$ Means with different letters within the same column differ from each otther, according to Tukey test ($P<0.05$).

When the *L. leucocephala* proportion of the mixture increased to 60%, there was no difference in total gas production ($P>0.05$) compared to the treatment with 40% of *L. leucocephala*, indicating that with higher than 40% levels in the mixture, TG production did not increase. In all assessed treatments, the highest TG production was recorded between the 48th and 72nd hours of incubation.

When analyzing the proportion increase in the TG at 48 h, when the proportion of *L. leucocephala* increased from 0 to 20%, the production decreased by 1.25%, while when the proportion increased from 20% to 40%, the decrease was 41.42%, and when the proportion of *L. leucocephala* increased from 40% to 60%, the total gas production decreased by 13.98%.

In this research, the result indicates that the greatest effect in the TG decrease production is obtained when 40% of *L. leucocephala* is included. This effect could be related to the NDF and CP content of the different mixtures (Table 2), since, as the proportion of *L. leucocephala* in the mixture increases, the NDF content decreases and the CP content increases, due to a lower proportion of cell walls. With that, the fermentation pattern changes, the amount of potentially fermentable cell walls decreases, and consequently the amount and proportion of volatile fatty acids decreases. This decreases the proportion of acetate, H_2, CO_2 and increases the proportion of propionate. It is reported that in diets

with *L. leucocephala* containing 14% of CP, methane production is reduced (Lovett *et al.*, 2004), and when NDF and FDA levels are lower, there is lower total gases production (Waghorn and Hegarty, 2011).

CH_4 production

A similar trend was observed in the CH_4 production to that obtained in TG production. The highest amount of CH_4 produced was in the treatment with 100% *H. ruffa* at 24 and 96 h of incubation ($P<0.05$). At 24 h of incubation, when the proportion of *L. leucocephala* increased from 0.0% to 20%, CH_4 production decreased ($P<0.05$), and the same response was observed when the *L. leucocephala* content was of 40 and 60%. At 48 and 72 h, there was a difference when 40 and 60% of *L. leucocephala* were included. The results obtained in this research show that increasing *L. leucocephala* content in the mixture decreases CH_4 production. Higher NDF and FDA content has been related to higher CH_4 production (Lopez *et al.*, 2016; Vélez *et al.*, 2018). When there is lower availability of digestible nutrients and higher cell wall content (NDF and FDA), CH_4 production is favored, while high levels of fast-fermenting carbohydrates and CP reduce CH_4 production (Owens *et al.*, 1998).

Table 4. Cumulative CH_4 production in *in vitro* incubations (mL of gas per 20 g of fermented DM).

Treatments *Hyparrhenia ruffa*: *Leucaena leucocephala*	Time (h)			
	24	48	72	96
100	111.97[a]	176.19[a]	272.45[a]	451.81[a]
80:20	66.43[b]	173.09[a]	253.46[a]	354.99[b]
60:40	47.89[b]	74.68[b]	125.21[b]	167.47[c]
40:60	40.59[b]	57.58[b]	114.22[b]	148.15[c]
SEM	10.41	16.25	14.34	21.43
MSD	27.22	42.47	37.49	40.32

SEM: Standart error of the mean; MSD: Minimum significant difference. [a, b, c] Means with different letters within the same column differ from each otther, according to Tukey test ($P<0.05$).

CONCLUSIONS

By including higher than 20% levels of *L. leucocephala* in mixtures with *H. ruffa* grass in *in vitro* incubations, total gas production and CH_4 production decrease, due to the change in the fermentation pattern caused by decreasing the NDF and FDA content of the mixture, and at the same time increasing the crude protein content.

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