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Originales

In vitro production of gas methane by tropical grasses

Produccion *in vitro* de gas metano por gramineas forrajeras tropicales

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Abstract:

Objetive. Estimate the production of methane (CH_4) by tropical grasses fermented *in vitro*. **Materials and methods**. A sample of 20 g dry matter of *Cynodon nlemfuensis, Hyparrhenia rufa, Megathyrsus maximus* and *Digitaria swazilandensis* plus 200 ml of culture medium were plated in triplicate flasks sterile stainless steel with CO_2 flux, inoculated with 20 ml of ruminal fluid bovine, incubated at 38 °C for 24, 48, 72 and 96 h. Total production of gas, CH_4, volatile fatty acids, and pH were evaluated in a completely randomized design with three replicates per treatment and comparison of means with Tukey; the concentration of total and cellulolytic bacteria were analyzed with the Kruskal-Wallis, and the GLM procedure independent data Wilcoxon rank. **Results.** *H. rufa* and *D. swazilandensis* both had the lowest total gas production ($p<0.05$), while D. swazilandensis had lower production of CH_4, increased production of propionic acid ($p<0.05$) and lower pH 96 hours of incubation ($p<0.05$). *D. swazilandensis* showed greater efficiency in energy production due to reduced production of CH_4 and increased propionate production. The concentration of total bacteria was similar between treatments ($p>0.05$), while the concentration of cellulolytic bacteria was lower in *C. nlemfuensis y D. swazilandensis* when 96 of incubation ($p<0.05$). **Conclusions**. The *Digitaria swazilandensis*, showed favorable conditions to have lower total methane and total gas production.

Keywords: Grasses, *in vitro* digestibility, methane.

Resumen:

Objetivo. Estimar la producción de metano (CH_4) por gramíneas tropicales fermentadas *in vitro*. **Materiales y métodos.** Una muestra de 20 g de materia seca de *Cynodon nlemfuensis, Hyparrhenia rufa, Megathyrsus maximus y Digitaria swazilandensis* más 200 ml de medio de cultivo se depositaron por triplicado en frascos de acero inoxidable estériles con flujo de CO_2, se inocularon con 20 ml de líquido ruminal de bovino e incubaron a 38 °C por 24, 48, 72 y 96 h. Se evaluó producción total de gas, CH_4, ácidos

grasos volátiles, y pH en un diseño completamente al azar con tres repeticiones por tratamiento y la comparación de medias con Tukey; la concentración de bacterias totales y celulolíticas, se analizaron con la prueba de Kruskal-Wallis, y el procedimiento GLM con datos de rangos independientes de Wilcoxon. **Resultados.** *H. rufa y D. swazilandensis* tuvieron la menor producción total de gases ($p<0.05$), mientras que *D. swazilandensis* tuvo menor producción de CH_4, mayor producción de ácido propónico ($p<0.05$) y menor pH a las 96 horas de incubación ($p<0.05$). *D. swazilandensis* mostró mayor eficiencia en la producción de energía por la menor producción de CH_4 y mayor producción de propionato. La concentración de bacterias totales fue similar entre tratamientos ($p>0.05$), mientras que la concentración de bacterias celulolíticas fue menor en *C. nlemfuensis y D. swazilandensis* a la hora 96 de incubación ($p<0.05$). **Conclusiones**. La *Digitaria swazilandensis*, mostró condiciones favorables para tener menor producción total de metano y gases totales.

Palabras clave: Digestibilidad *in vitro* , gramíneas, metano .

INTRODUCTION

Ruminants emit between 18 and 25% of the greenhouse gases (GHG), depending on the feeding strategy that has been established, CH_4 is the second largest contributor to this effect (1,2,3,4). Ruminant feed in tropical and subtropical regions is mainly based on the use of forage grasses whose cellulose and hemicellulose content is higher than in temperate climate grasses (5), this higher cell wall content being potentially fermented by cellulolytic bacteria species such as *Ruminococcus flavefaciens, Ruminococcus albus* and *Fibrobacter succinogenes*, which transform glucose into acetate and butyrate, whose metabolic pathway produces hydrogen (H_2) and carbon dioxide (CO_2), which are the main substrates for methanogenic archaea such as *Methanobacterium formicicum, Methanobrevibacter ruminantium, Methanomicrobium mobile, Methanosarcina bacteri* and *Methanosarcina majei* (6), where the highest production of CH_4 is produced by this metabolic pathway (6,7).

The production of CO_2 and CH_4 is a necessary process in ruminal biochemistry to obtain energy, this process reduces the accumulation of H_2 and pH reduction, to maintain ruminal ecology under favorable conditions (8). However, this process reduces the efficiency of energy use by the animal by 6.3% for sheep and 6.5% for cattle (9).

The use of tropical forage grasses with higher cell content and lower potentially fermentable cell wall content in ruminant feed could allow for greater energy efficiency that contributes to reducing GHG emissions for the purpose of mitigating climate change, Zheng et al (3) y Iñamaga et al (4), reported that feed strategies influenced GHG emissions, also indicate that CO_2 emissions based on the production of fat-corrected milk were higher for high forage feeding strategies. Therefore, the objective of this research was to evaluate the production of total gases (GT) and CH_4 emitted by tropical fodder grasses on *in vitro* incubation (3,4).

MATERIALS AND METHODS

Area of study. The study was developed in the Laboratory of Animal Science of the Faculty of Agricultural Sciences, Campus IV of the University Autonomous University of Chiapas located in Huehuetán, Chiapas, Mexico and the Ruminal Microbiology and Microbial Genetics Laboratory of the Postgraduate School Livestock Program, Montecillos Campus, Texcoco, Mexico.

Treatments and chemical analysis of grasses. The treatments (pastures) evaluated were T1: *Cynodon nlemfuensis*; T2: Hyparrhenia rufa; T3: *Megathyrsus maximus* and T4: *Digitaria swazilandensis*; with an age of 75 days, during the month of May 2015 (average temperature of 24.81°C, relative humedad of 72.86% and 277 mm of accumulated monthly precipitation at the time of sampling, and 1438.9 mm of precipitation during the year), was obtained from a cattle ranch (El Carmen 9, in Mazatán, Chiapas) located at 14°54´23.93" N and 92°25´37.81" O; 35 meters above sea level. With soil of the Phaeozem (Feosem)

type, characterized by a high accumulation of organic matter and by being saturated at the top, the soil is mainly prairie soil, with a móllic epipedion (a relatively thick, dark, humus-rich surface horizon) and without calcium carbonate in the first meter of depth; no fertilization was carried out on the areas of the sampled forage.

The samples were dried in a drying oven at 60°C for 24 hours and ground in an ED-5 electric mill equipped with a 1 mm screen. For each of the samples, crude protein (CP) was determined by the Kjeldahl method, as well as ethereal extract (EE) and ash content after incineration of the sample in a muffle at 550° C per 4 h according to AOAC (10). Determination of the neutral detergent fiber (NDF) and acid detergent fiber (ADF) fractions according to the technique described by Van Soet et al (11).

The culture medium (Table 1) used to determine the production of total gases (GT) and methane (CH_4), in addition to the degradation of MS, was prepared under sterile conditions and CO_2 flow. The inoculum was fresh rumen fluid (FRF) extracted at 2 h *pos-prandium* from a 500 kg BW bovine (F1, zebu x swiss) with rumen cannula, which received at *libitum* (received the first ration at 6:00 am and second at 4:00 pm) a diet based on 85% *C. nlemfuensis* and 15% of a concentrated feed containing 2.7 Mcal of ME and 14% crude protein.

TABLE 1

Table 1. Culture medium for measuring total gas production, methane and *in vitro* degradation of dry matter.

Compound	Quantity (ml) for 100 ml of medium
Distilled water	52.9
Clarified rumen liquid (1)	30.0
Mineral solution I (2)	5.0
Mineral solution II (3)	5.0
Sodium carbonate (Na2CO3), 8% (4)	5.0
Sulphide-cysteine solution (5)	2.0
Resazurin solution 0.1% (6)	0.1

(1)Clarified filtered rumen liquid filtered centrifuged at 17664 g for 15 min and sterilized 20 min at 21°C at 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 $(NH_4)2SO4$, 12 g NaCl, 2.45 g $MgSO_4$ and 1.6 g $CaCl_2$-H_2O. (4) 8 g Na_2CO_3 in 100 ml H_2O distilled. (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 resazurine in a volume of 100 ml.

Production of CH4. The *in vitro* production of GT and CH_4 was determined in triplicate with repetition over time of each treatment (grasses) using bottles (biodigesters) with a capacity of 2.0 L with hermetic seal, where the following mixture was added under aseptic and CO_2 flow conditions: 20 g of MS from each grass (1 g of MS for each 10 mL of medium) according to the Williams technique (12) plus 200 ml of culture medium (Table 1) each treatment was inoculated with 20 ml of FRF filtered in cotton gauze, incubated at 38±0.5°C under CO_2 flow for 24, 48, 72 and 96 h in a thermoregulation bath. The initial total bacterial concentration was 1.35 x10^8 CFU ml^{-1} based on the most probable number technique (MPN, 13) at pH 6.74. At the end of the incubation period, the production of total gases (GT) in the system was measured by moving liquids through a trap with Mariotte flasks. The displaced water was collected in a 500 ml graduated cylinder and thus the amount of GT per 20 g of fermented MS was determined.

To determine the amount of CH_4 produced in each treatment, in a second test and under the same culture conditions, times and repetitions, in the Mariotte flask traps was added a solution of NaOH (2N) with pH of 13.67 according to the technique described by Stolaroff (14); the NaOH solution reacts with CO_2 to form sodium carbonate (Na_2CO_3) and the remaining gases released are a mixture of CH_4, H_2, N_2 and hydrogen sulphide (15). The CO_2 trap was coupled to the biodigesters using a Tygon hose (internal Φ 5 mm and a length of 35 cm) that was fitted with a hypodermic needle (31.8 mm) and 10 cm long). In all GT production evaluations, the results of each treatment and its respective repetition were corrected for difference with the gas production of the blank samples (200 ml of culture medium plus 20 ml of FRF).

Production of volatile fatty acids (VFA) and microbiological variables. At the end of each incubation period 5 ml of culture medium were obtained and centrifuged at 18000 G for 10 min; 2.0 ml of the supernatant was mixed 4:1 with 25% metaphosphoric acid, the vials were shaken in a Vortex and re-centrifuged at 35000 G for two minutes, the concentration of VFA was measured using a Claurus 500 gas chromatograph, using the technique and conditions described by Ley de Coss et al (16). In addition, per incubation period, 0.5 ml of culture medium was obtained from each treatment to estimate the concentration of total bacteria (BT) and cellulolytic bacteria (BC) using the MPN technique and culture media similar to those reported by Ley de Coss et al (17), which consisted to BT: 0.06 g D-(+)-glucose + 0.06 g D-cellobiose + 0.06 g starch, 30 ml clarified FR, 5.0 ml mineral solution I [6 g K_2HPO_4 in 1000 ml H_2O], 5.0 ml mineral solution II[6 g KH_2PO_4 + 6 g KH_2PO_4 + 6 g $(NH_4)2SO_4$ + 12 g NaCl + 2.45 g $MgSO_4$ + 1.6 g $CaCl_2.H_2O$ in 1000 ml H_2O], 2.0 ml 8% Na_2CO_3 solution, 2 ml sulphide-cysteine solution (2.5 g L-cysteine in 15 ml NaOH (2N) + 2.5 g Na2S-9H_2O dissolved in 100 ml H_2O), 0.2 g peptone tripticase and 0.1 ml of 0.1% resazurine solution; and for BC a similar medium was used, and only the energy source (glucose+cellobiose+starch) was replaced by a strip of Whatman paper as a cellulose source (18).

Design and statistical analysis. The experimental design was completely randomized with three repetitions per treatment for each incubation period. Data on total gas production, CH4, AGV concentration and pH of the culture medium were analyzed with the SAS GLM procedure (19), while data on BT and BC concentration were analyzed with the Kruskal-Wallis test, with the GLM procedure with data from independent ranges (Wilcoxon) and averages were compared with the Tukey test ($p<0.05$) with SAS.

RESULTS

The lowest total gas production was in *H. rufa* and *D. swazilandensis*, in the latter species it had lower CH_4 production, indicating higher energy production efficiency due to higher propionic acid synthesis. There was no change in BT concentration; however, in pastures with lower CH_4 synthesis there was lower BC concentration. Table 2 shows the results of the chemical composition of the grasses, showing that the crude protein content of *H. rufa* was less than 7%, while in *C. nlemfuensis, M. maximus* and *D. swazilandensis* it was greater than 9%. The NDF content, the lowest value was *H. rufa* (63.25%), while *D. swazilandensis* had the highest content of this compound (71.40%), with an 8.15% difference between the two species, when related to the ADF content that was similar among the four species (42.25 to 43.40%), it can be attributed that the highest content of NDF in *D. swazilandensis* could be due to the higher content of hemicellulose.

TABLE 2.
Table 2. Chemical composition (%) of tropical grasses *C. nlenfuensis, H. rufa, M. maximus* and *D. swazilandensis* at the age of 75 days.

per	C. nlemfuensis	H. rufa	M. maximus	D. swazilandensis
	%			
CP	9.56	6.36	9.54	10.35
EE	1.85	1.25	1.92	2.35
NDF	67.24	63.25	67.25	71.40
ADF	42.56	42.25	42.25	43.40
Hemicellulose	24.68	21.00	25.00	28.00
Ashes	6.72	8.78	8.25	9.25

Total production of gases and CH_4. In all the fermented pastures, the highest proportion of gases (Table 3) was obtained in the period from 48 to 72 h, which indicates that in this period the highest activity of the bacteria to degrade the substrate was obtained. When considering the total accumulated gas production per g^{-1} of dry matter fermented (DMf), it was lower for *H. rufa* and *D. swazilandensis* ($p<0.05$).

TABLE 3.
Table 3. Total gas production of tropical grasses *C. nlemfuensis, H. rufa, M. maximus* and *D. swazilandensis* on *in vitro* incubation.

Time	C. nlemfuensis	H. rufa	M. maximus	D. swazilandensis	SEM[1]
	ml g DMf^{-1}				
96	156^{b}	239^{a}	212^{a}	129^{b}	17.6
72	525^{a}	356c	521^{ab}	442^{a}	21.4
48	255^{a}	242^{a}	252^{a}	254^{b}	42.3
24	170^{a}	148^{ab}	128^{b}	122^{b}	27.7
Total	1106^{a}	985.0^{b}	1113^{a}	947^{b}	58.7

$^{a, b, c}$ Means with different letters in the same row are different ($p<0.05$) [1] Standard error of mean.

In the same way as GT production, the largest proportion in the production of CH_4 (Table 4) occurred in the period from 48 to 72 h, but the total accumulated production of CH_4 was similar between *C. nlemfuensis, H. rufa* and D. swazilandensis ($p>0.05$) as well as between C. nlemfuensis, H. rufa and M. maximus ($p>0.05$), while there was a difference between *M. maximus* and *D. swazilandensis* with lower production ($p<0.05$).

TABLE 4.

Table 4. CH_4 production by period and total accumulated CH_4 of tropical grasses *C. nlemfuensis, H. rufa. M. maximus* and *D. swazilandensis* on *in vitro* incubation.

Time	C. nlemfuensis	H. rufa	M. maximus	D. swazilandensis	SEM[1]
	ml g DMf^{-1}				
96	118.5^{b}	183.3^{a}	162.6^{a}	98.9^{b}	21.2
72	373^{a}	273.8^{b}	398.8^{a}	338.8^{a}	32.1
48	195.1^{a}	185.1^{a}	192.7^{a}	192.0^{a}	16.3
24	130.1^{a}	113.4^{ab}	95.9^{b}	93.3^{b}	13.5
Total	816.7^{ab}	755.8^{ab}	852.2^{a}	723.9^{b}	101.9

$^{a, b, c}$ Means with different letters in the same row are different ($p<0.05$) [1] Standard error of mean.

In relation to the percentage of CH_4 of total gas production, for the grasses *H. rufa, M. maximus* and *D. swazilandensis* represented 76.5%, while for *C. nlemfuensis* it was 73.9%, which indicates that the highest proportion of gas produced during fermentation corresponds to this GHG.

The total production of VFA and acetic acid production was similar in the grasses evaluated ($p>0.05$), while *D. swazilandensis* had higher production of propionic ($p<0.05$) and butyric acids ($p<0.05$). The acetic: propionic ratio showed that during the fermentation of *D. swazilandensis* the energy loss was lower and was related to the lower production of CH_4 obtained (Table 5).

TABLE 5

Table 5. Production of volatile fatty acids from tropical grasses *C. nlemfuensis, H. rufa, M. maximus* and *D. swazilandensis in vitro* incubation.

	C. nlemfuensis	H. rufa	M. maximus	D. swazilandensis	SEM[1]
	mmol L^{-1}				
Acetic	74.28a	73.81^{a}	73.81^{a}	64.17^{a}	14.6
Propionic	18.80^{b}	16.20^{b}	16.20^{b}	38.23^{a}	9.3
Butyric	9.43a^{b}	4.86^{b}	4.86^{b}	12.26a	4.3
Total	102.43^{a}	94.87^{a}	94.87^{a}	114.60a	33.25
A:P	3.95^{b}	3.50^{b}	3.5^{b}	1.67a	0.34

$^{a, b, c}$ Means with different letters in the same row are different ($p<0.05$) [1] Standard error of mean.

Table 6, shows the pH of the medium during 96 h of fermentation. *D. swazilandensis* and *M. maximus* had the lowest pH at 24, 72 and 96 h of incubation, even less than 6 at 96 h .

TABLE 6

Table 6. pH of the culture medium in which the tropical grasses *C. nlemfuensis, H. rufa, M. maximus* and *D. swazilandensis* were fermented *in vitro*.

Hours	C. nlemfuensis	H. rufa	M. maximus	D. swazilandensis	SEM[1]
96	6.11^{ab}	6.53^{a}	5.95^{b}	5.88^{b}	0.12
72	6.74^{a}	6.64^{a}	6.09^{b}	6.24^{ab}	0.26
48	6.65^{a}	7.02^{a}	6.84^{a}	6.34^{a}	0.30
24	7.14a	6.95^{a}	6.95a	6.30^{b}	0.31

$^{a, b, c}$ Means with different letters in the same row are different ($p<0.05$) [1] Standard error of mean.

There was no difference in BT concentration among treatments ($p>0.05$) during the entire incubation period and the maximum concentration, in all treatments, was 109 cells ml^{-1} of culture medium. Regarding the concentration of cellulolytic bacteria, at 24 h of incubation, the highest concentration was observed in *C. nlemfuensis* ($p<0.05$), at 48 and 72 hours there was no difference among treatments ($p>0.05$); while at 96 hours it was lower ($p<0.05$) in *C. nlemfuensis* and *D. swazilandensis* (Table 7).

TABLE 7

Table 7. Concentration of total and cellulolytic bacteria in the culture medium in *in vitro* incubation.

Hours	C. nlemfuensis	H. rufa	M. maximus	D. swazilandensis	SEM[1]
	Total bacteria 1×10^{9}				
96	11.6	6.09	2.13	3.53	3.14
72	7.77	4.03	1.26	2.23	3.09
48	5.95	3.08	0.94	1.71	3.08
24	5.14	2.66	0.77	1.35	3.09
	Cellulolytic bacteria 1x10^{7}				
96	6.74^{b}	19.80^{a}	31.20^{a}	6.50^{b}	2.70
72	4.31^{a}	17.60^{a}	19.90^{a}	4.20^{a}	2.74
48	2.60^{a}	14.50^{a}	12.00^{b}	2.50^{a}	2.24
24	11.10^{a}	1.11^{b}	1.08^{b}	1.08^{b}	2.20

$^{a, b, c}$ Means with different letters in the same row are different ($p<0.05$) [1] Standard error of mean.

DISCUSSION

Generally, grasses have a low crude protein content, with a lower nitrogen content that limits microbial activity in the rumen (20), Avellaneda et al (21), report values of 6.37 and 71.96% crude protein and NDF, respectively in Guinea grass (*Panicum maximum var Mombasa*), harvested at 90 days of age, similar results to those found in this study. Maximum methane production was obtained at pH 7.0 to 7.2, and may even occur in the range of 6.6 to 7.6 (3), in this study *D. swazilandensis* showed a lower concentration of cellulolytic bacteria, a pH below 6.5 and therefore a lower concentration of CH_4, due to the reduction of the activity of bacteria that degrade fiber by the pyruvate-lase pathway such as *Ruminococcus flavefaciens, Ruminococcus albus, Butyrivibrio fibrisolvens* and *Fibrobacter succinogenes* (22,23) and consequently the substrates (CO_2

and H_2) necessary in the formation of CH_4; However, species such as Streptococcus bovis, Ruminobacter amylophilus, Succinomonas amylolytica and Selenomonas ruminantium proliferate, fermenting soluble carbohydrates and cellulose fragments to produce propionate via succinate (24), which generates a different profile in the production of VFA, producing a higher proportion of propionic acid and therefore less CH_4. On the other hand, the ruminal fermentation of forages with a higher content of cell wall does not cause a significant decrease in pH, because the greater amount of glucose released is fermented by acetate, in this case, the released H_2 can be used as a substrate by methanogenic archaea, which is associated with higher production of CH_4 (3), as in the case of *H. rufa* and *C. nlemfuensis*, while with forages that cause low rumen pH, methanogenesis is decreased as in the case of *M. maximus* whose pH was less than 6.5 since 72 hours of incubation and *D. swazilandensis* since 24 hours.

One of the important factors affecting the production of CH_4 is the ratio of produced VFA, specifically the acetic: propionic ratio, which regulates the production and availability of H_2 and subsequent production of CH_4; this ratio can vary from 0.9 to 4 and energy utilization is more efficient if the ratio is close to 1.0 (25). The production of CH_4 has been used as an indicator of the fermentative activity of bacteria in anaerobic fermentation processes (26) in which different groups of bacteria are involved: such as hydrolytic bacteria that fractionate polysaccharides to sugars, VFA formers and methanogenic archaea that synthesize CH_4 from H_2 and CO_2 (27,28). Acetate and butyrate originate the production of CH_4, due to the increased availability of CO_2 and H_2 for methanogenic archaea, while for propionate formation in the rumen it is considered a competitive form of H_2 uptake that causes a lower synthesis of CH_4 (29). Rumen protozoa produce H_2 as the main end-product of their metabolism and is closely associated as a substrate for methane formation by methanogenic archea. These methanogenic bacteria associated with rumen protozoa are apparently responsible for 9 to 25% of methanogenesis, but this can be reduced by around 13% when the protozoa are killed; however, this reduction occurs when the animal consumes starchy diets, which is when the protozoa generate more H_2, which is not the case when the diets are high in forage resulting in less methane formation (30). Conversely, a high proportion of acetate: propionate is related to low energy efficiency, which involves higher CH_4 production as was the case with *C. nlemfuensis, H. rufa* and *M. maximus.*

In conclusion, the tropical grasses analyzed show a high cell wall concentration, which limits their digestibility and reduces their quality as fodder; however, Digitaria swazilandensis showed a lower total production of methane and total gases, possibly due to a higher concentration of propionic acid, lower concentration of cellulolytic bacteria, a pH and a lower acetic:propionic ratio, being the most efficient in energy use.

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