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In vitro biogas production and degradations of sheep diets containing *Crotalaria* or Chipile at two different regrowth ages

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ABSTRACT

Objective: To determine the *in vitro* biogas production and fermentative characteristics of diets for fattening lambs containing 20% chipile or crotalaria at 30 or 40 d of regrowth.

Methodology: The treatments were whole diets containing 20% crotalaria with 30 d (T1) or 40 d of regrowth (T2), as well as 20% chipile with 30 d (T3) or 40 d of regrowth (T4). *In vitro* gas production was measured at 2, 4, 6, 8, 10, 12, 24, 48, and 72 h and the following elements were determined: kinetic estimators (A, b, k), dry matter (DMD), organic matter (OMD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD) degradation, metabolizable energy (ME), and short-chain fatty acids (SCFA). The experimental design was completely randomized.

Results: Regarding the accumulated biogas production, T4 presented higher production from 2 to 24 h, T3 and T4 higher at 48 h, and T1 higher at 72 h. Kinetic estimators showed that T1 was higher in A and k and T4 was higher in b. T2 presented the lowest DMD, OMD, NDFD, ADFD, ME, and SCFA.

Study Limitations: Scale production of chipile, aimed to obtain a greater biomass volume, is limited since it has not been domesticated yet.

Conclusions: Sheep diets containing 20% chipile or crotalaria with 30 d of regrowth have proven to be an alternative for the manufacturing of whole diets for the intensive fattening of lambs in the tropics.

Keywords: Biogas, degradation, metabolizable energy, alternative feed.

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INTRODUCTION

Stock breeding, supplier of food and raw materials of animal origin, constitutes one of the main economic activities of the country's primary sector. In Mexico, the inventory of sheep production is 8,725,882 heads, and the State of Mexico (15.8%) and Hidalgo (12.9%) have the largest inventory, while the state of Guerrero only has a 2% stake (SIAP, 2021). Sheep production in Mexico is carried out under traditional grazing systems, with little technology and low productivity. Meat production is the most widespread productive

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activity in rural areas, since it is carried out even under adverse weather conditions that hinder other productive activities. However, there have been significant changes in recent years because it is no longer a backyard activity, a forced savings activity, or an activity that makes marginal use of summer pastures (Hernández-Marín, 2018).

In the animal production systems of the tropical zones of Mexico, feeding is based on grazing. However, the nutritional quality is not enough to cover the animals' nutritional requirements; therefore, the use of legumes is an alternative in animal feed (Gámez *et al.*, 2019). There are between 16,000 and 19,000 species of legumes distributed all over the world. They are divided into 750 genera and characterized by their high protein content (20 to 45%) (Benites, 2020). The Crotalaria genus comprises about 600 species that are distributed throughout the tropical and subtropical regions of the world. Chipile (*Crotalaria longirostrata*), native to Mesoamerica, is a legume of the Fabaceae family. It is a wild plant that grows in Chiapas, Mexico, and is traditionally used for human consumption and as forage for animals (Miranda-Granados *et al.*, 2018; Córdova-Ballona *et al.*, 2022). For its part, crotalaria (*Crotalaria juncea*) is a legume with high biomass production; it is native to India and is used as forage, green manure, and biological agent (Avendaño, 2011); due to its phenolic content, part of the seed has a significant antibacterial activity (Chouhan and Singh, 2010). Crotalaria foliage can be used as a high-quality and palatable forage source during the vegetative phase. Approximately six to eight weeks after planting or cutting, it has a high concentration of nitrogen and protein (18-22%), phosphorus (0.29%), and calcium (1.4%), as well as 60% digestibility (Gámez *et al.*, 2019). The hypothesis was that the use of chipile with 30 and 40 d of regrowth will achieve better biogas production values and in vitro fermentative characteristics in diets for fattening lambs than crotalaria with the same number of regrowth days. Therefore, the objective was to determine the *in vitro* biogas production and fermentative characteristics of diets for fattening lambs containing 20% chipile (*Crotalaria longirostrata* Hook. & Arn) or crotalaria (*Crotalaria juncea* L.) with 30 or 40 d of regrowth.

MATERIALS AND METHODS

Study site

This work was carried out in the Laboratorio de Nutrición Animal of the Facultad de Medicina Veterinaria y Zootecnia No. 2 of the Universidad Autónoma de Guerrero, located at km 197 of the Acapulco-Pinotepa Nacional highway, Cuajinicuilapa, Guerrero, Mexico. It is located at 16° 28' 28" N and 98° 25' 11.27" W, at 46 m.a.s.l.

Chemical analysis

Chipile and crotalaria samples (Table 1) were dehydrated at 60 °C for 72 h in an oven (Riossa HCF-41, Mexico). Subsequently, they were ground using a 1mm screen in a Thomas-Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). Dry matter (DM), crude protein (CP), and ash (As) were determined in the samples according to the AOAC methods (2005) no. 930.15, 920.105, and 942.05, respectively. Organic matter (OM) was estimated by subtracting the ash value from 100. In addition, neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the ANKOM Technology Method according

Table 1. Chemical composition of crotalaria and chipile with 30 and 40 d of regrowth.

	Crotalaria		Chipile	
	30 d	**40 d**	**30 d**	**40 d**
Dry matter (%)	18.23	21.86	16.29	12.33
Crude protein (%)	15.86	10.37	25.82	24.71
Neutral detergent fiber (%)	58.85	68.84	55.16	48.79
Acid detergent fiber (%)	39.54	52.89	34.47	32.23
Hemicellulose (%)	19.31	15.95	20.69	16.57
Organic matter (%)	90.91	94.63	91.75	92.56
Ash (%)	9.09	5.37	8.25	7.44

to Van Soest *et al.* (1991). Hemicellulose was calculated by the difference between NDF and ADF.

Treatments

The treatments were whole diets for fattening lambs (Table 2) containing chipile (*Crotalaria longirostrata*) or crotalaria (*Crotalaria juncea*) with 30 or 40 d of regrowth (Table 2).

In vitro test

The medium components contained 30 mL of clarified rumen fluid [fresh bovine rumen fluid centrifuged for 10 min at 12,857 *x g* and sterilized (All American® 1941X,

Table 2. Composition of the treatments that include 20% chipile or crotalaria with 30 or 40 days of regrowth.

	T1	**T2**	**T3**	**T4**
Dry basis composition (%)				
Ground corn	63.0	61.0	65.3	64.7
Soybean paste	11.0	14.0	5.7	6.3
Crotalaria 30 d	20.0	-	-	-
Crotalaria 40 d	-	20.0	-	-
Chipile 30 d	-	-	20.0	-
Chipile 40 d	-	-	-	20.0
Star grass	3.0	2.0	6.0	6.0
Urea	1.0	1.0	1.0	1.0
Mineral mix*	2.0	2.0	2.0	2.0
Chemical composition (calculated)				
ME (Mcal kg^{-1} DM)	2.81	2.81	2.83	2.83
Crude protein (%)	17.0	17.0	17.0	17.0
Neutral detergent fiber (%)	23.6	23.8	21.2	21.3

*17.58% calcium, 2.40% phosphorus, 36.50% sodium chloride, 11.70% sulfur, 0.71% zinc, 0.14% copper, 0.0007% iodine, 0.0016% cobalt, 0.0029% selenium, 0.024% fluorine, and 5.0% moisture.

USA) for 15 min at 121 °C and 15 psi], 5 mL of mineral solution I [6 g K_2HPO_4 (J. T. Baker®) in 1,000 mL of distilled water], 5 mL of mineral solution II [6 g KH_2PO_4 (J. T. Baker®)+$(NH_4)_2SO_4$ (J. T. Baker®)+12 g NaCl (Meyer®)+2.45 g $MgSO_4$ (Meyer®)+1.6 g CaCl-$2H_2O$ (Meyer®) in 1,000 mL of distilled water], 0.1 mL of 0.1% resazurin (Sigma-Aldrich®), 0.2 g of soy peptone (MCDLab®), 0.1 g yeast extract (BD Bioxon®), 4 mL cysteine-sulfide solution [3.125 g L-cysteine (Sigma-Aldrich®) in 15 mL 2N NaOH (Meyer®)+3.125 g Na_2S-$9H_2O$ (Meyer®) with a graduate mark of 250 mL of distilled water], 5 mL of 8% Na_2CO_3 solution (J. T. Baker®), and 50.6 mL of distilled water. The medium was sterilized for 15 min in an autoclave at 121 °C and 15 psi, according to the modifications proposed by Sánchez-Santillan *et al.* (2016) to the methodology of Cobos and Yokoyama (1995).

A 120-mL glass sample vial with 0.5 g of DM from a treatment and 45 mL of culture medium was considered a biodigester and experimental unit. The vials were kept under anaerobic conditions with CO_2, hermetically sealed with a 20-mm Ø neoprene stopper and an aluminum ring. The biodigesters were sterilized for 15 min at 121 °C and 15 psi. The biodigesters were inoculated with 5 mL of total ruminal bacteria obtained from the rumen fluid of a Suiz-bu cow and incubated at 39 °C for 72 h in a hot water bath. The cow grazed in pangola grass grasslands before the sample of the rumen fluid was taken. The rumen fluid was centrifuged at 1,157 *x g* for 3 min to precipitate protozoa and fiber particles (Torres-Salado *et al.*, 2019).

Biogas production was measured by displacing the plunger of a 50-mL glass syringe (BD Yale®, Brazil). Biogas was measured at 2, 4, 6, 8, 10, 12, 24, 48, and 72 h and the accumulated production was reported. The values of the accumulated biogas production were used to estimate the biogas production kinetics, using the Gompertz model:

$$Y = A * \left\{ \exp\left[-b * \exp(-k * t) \right] \right\}$$

Where: Y=volume of biogas at time t (mL g^{-1} of DM), A=total biogas production potential when $t=\infty$ (mL g^{-1} of DM), b=constant rate of biogas production of the potentially degradable material (mL h^{-1}), k=lag time (h), microbial efficiency constant factor, defined as the intercept of the time axis of the tangent line at the inflection point, t=incubation time (Lavrenčič *et al.*, 1997).

At 72 h of incubation, the pH was measured with a potentiometer (Hanna® HI2211, Italy; calibration: pH 7 and 4). The residual sample of the biodigester was filtered in ANKOM® F57 bags with constant weight. The bags with the residue were dried for 24 h at 60 °C in a drying oven. ANKOM® bags were heat sealed and NDF and ADF content was determined (Van Soest *et al.*, 1991). The degradation of dry matter (DMD), of NDF (NDFD), and of ADF (ADFD) were estimated by difference (Hernández-Morales *et al.*, 2018). The models described by Menke *et al.* (1979) were used to estimate organic matter digestibility (OMD) and metabolizable energy (ME). Additionally, the model developed by Getachew *et al.* (2004) was used for short-chain fatty acids (SCFA).

Statistical analysis

The experimental design was completely randomized with 10 replications per treatment. Data on biogas production, biogas production kinetics, and *in vitro* fermentative characteristics were analyzed with the GLM procedure (SAS Institute Inc., 2011). The mean values were compared with Tukey's test ($p \leq 0.05$).

RESULTS AND DISCUSSION

The chipile treatment with 40 d of regrowth (T4) recorded the highest production of accumulated biogas, 2 to 24 h after incubation ($p \leq 0.05$). Meanwhile, 40-d crotalaria (T2) and 30-d chipile (T3) did not show differences 2, 4, 6, 8, and 10 h after incubation (Table 3). Therefore, T4 contained a greater amount of non-structural carbohydrates available for fermentation, since this type of carbohydrate (Texta *et al.*, 2019) and the protein fraction (Rodríguez *et al.*, 2010) are fermented in the first 24 h.

At 48 h, chipile treatments (T3 and T4) recorded an average of 152.35 mL g^{-1} DM: 12.3% more biogas than the average of crotalaria treatments. However, at 72 h, the 30-d crotalaria treatment (T1) produced the highest volume of accumulated biogas. This represented 12.3% more biogas than the average of the chipile treatments (T3 and T4). It can be deduced that structural carbohydrates of crotalaria with 30 d of regrowth are more susceptible to fermentation by cellulolytic bacteria than those contained in chipile, since such carbohydrates are fermented after 24 h (Texta *et al.*, 2019). Espinoza-Sánchez *et al.* (2020) reported higher *in vitro* biogas production values (242.3 mL g^{-1} DM) than those recorded in this study for sheep diets containing 40% corn grain, 1% urea, 25% *Samanea saman* pod, 6% molasses, 26% pangola grass, and 2% mineral mix. Those values can be attributed to the availability and nature of the carbohydrates included in the diets evaluated by both authors, since biogas production is the result of their fermentation (Amanzougarene and Fondevila, 2020).

The estimators of the fermentation kinetics show that the treatment that contained crotalaria with 30 d of regrowth (T1) produced the highest value of A ($p \leq 0.05$) (Table 4).

Table 3. Biogas production (mL g^{-1} DM) of diets that include 20% crotalaria or chipile with 30 or 40 d of regrowth.

Incubation time (h)	T1	T2	T3	T4	SEM
2	7.5 c	16.1 b	15.3 b	20.9 a	0.86
4	20.5 c	28.4 b	28.8 b	40.6 a	1.25
6	33.2 c	42.0 b	41.6 b	59.2 a	1.61
8	50.7 c	56.5 b	58.9 b	79.7 a	1.86
10	67.3 b	65.9 b	70.5 b	90.9 a	1.74
12	80.3 b	72.5 c	81.3 b	98.9 a	1.69
24	115.1 b	99.7 c	117.9 b	124.5 a	1.61
48	141.0 b	130.4 c	150.0 a	154.7 a	1.66
72	185.3 a	145.6 c	163.5 b	166.5 b	2.39

[a,b,c] Means with different letters within a row are different ($p \leq 0.05$). SEM=standard error of the mean. T1=crotalaria with 30 d of regrowth; T2=crotalaria with 40 d of regrowth; T3=chipile with 30 days of regrowth; T4=chipile with 40 days of regrowth.

This result indicates greater biogas production, which matches the direct measurement of biogas (Table 3). Therefore, the treatment containing crotalaria with 30 d of regrowth (T1) showed the greatest availability of carbohydrates for in vitro fermentation (Amanzougarene and Fondevila, 2020). However, estimator b showed that the 40-d chipile (T4) treatment had a better biogas production rate, although it did not produce the greatest volume of the said gas. This indicates that its passage through the rumen would be faster than the rest of the treatments, as a result of its fermentation rate (b) (Table 4) and biogas production at 24 h (Table 3).

The *k* estimator refers to the time it takes for the microorganisms to adhere to the substrate; consequently, 40-d crotalaria (T2) had the lowest *k* (Table 4). Overall, none of the treatments in this study had the best kinetic estimators. The qualities of each treatment depend on the ingredients it contains. Crotalaria and chipile, as well as their age at regrowth, influence these estimators.

Crosby *et al.* (2017) reported higher estimator values (A, *b*, and *k*) than the values recorded in this study (Table 4) for diets containing 40% corn stover, 30% winter squash dry residue, 18% ground corn, 10% soybean paste, 1% urea, and 1% mineral mix. For their part, Rodríguez *et al.* (2017) recorded similar values to those of the present study: similar for A, higher for *k*, and lower for *b*; their study diet contained 67.5% grain sorghum, 5% alfalfa hay, 11% soybean paste, 4% molasses, 10% corn stover, 1.5% urea, and 1% mineral mix. The difference between the values of the estimators reported in this study and the values recorded by the abovementioned authors may be the result of two factors: a) nutritional composition of the treatments, and b) methodology used to establish the *in vitro* gas production.

Table 4. Fermentation kinetics and in vitro fermentative characteristics of diets that include 20% crotalaria or chipile with 30 or 40 d of regrowth.

Variables	T1	T2	T3	T4	SEM
A (mL g^{-1} DM)	163.9 a	135.9 c	154.8 b	153.7 b	1.77
b (mL h^{-1})	0.101 c	0.107 bc	0.112 b	0.151 a	0.031
k (h)	2.83 a	2.37 d	2.63 b	2.50 c	0.003
pH	6.9 a	6.7 b	6.8 a	6.8 ab	0.01
DMD (%)	69.0 a	50.4 b	67.9 a	67.2 a	1.86
NDFD (%)	56.7 a	35.7 c	58.7 a	48.6 b	2.28
ADFD (%)	65.9 a	10.2 c	49.6 b	43.4 b	4.82
ME (Mcal kg^{-1} DM)	1.49 b	1.31 c	1.66 a	1.65 a	0.03
OMD (%)	43.0 b	37.6 c	48.3 a	48.0 a	1.07
SCFA (mmol g^{-1} DM)	2.50 a	2.15 b	2.65 a	2.65 a	0.05

[a,b,c] Means with different letters within a row are different ($p \leq 0.05$). SEM=standard error of the mean. T1=crotalaria with 30 d of regrowth; T2=crotalaria with 40 d of regrowth; T3=chipile with 30 days of regrowth; T4=chipile with 40 days of regrowth; A=total biogas production potential; *k*=lag time; *b*=constant rate of biogas production of the potentially degradable material; pH=potential of hydrogen; DMD=dry matter degradation; NDFD=Neutral Detergent Fiber Degradation; ADFD=acid detergent fiber degradation; ME=metabolizable energy; OMD=organic matter degradation; SCFA=short chain fatty acids.

After 72 h of incubation, the pH value of the culture medium of the treatments ranged from 6.7 to 6.9 (Table 4). Those values are within the range required for the growth of ruminal microorganisms (Nagaraja, 2016). Therefore, the crotalaria or chipile fermentation products did not take the pH values beyond the standards for ruminal bacteria. *In vitro* degradation determinations are useful because they establish a correlation with *in vivo* digestibility (Gosselink *et al.*, 2004). The treatment that contained crotalaria with 40 d of regrowth (T2) showed the lowest dry matter degradation (DMD) and organic matter degradation (OMD) ($p \leq 0.05$), while the rest of the treatments did not record DMD differences ($p > 0.05$) (Table 4). Meanwhile, chipile treatments (T3 and T4) recorded higher OMD ($p \leq 0.05$) (Table 4).

The 40-d crotalaria treatment (T2) had the least degradation of detergent fibers (NDFD and ADFD) (Table 4). In addition, treatments with 30 d of regrowth (T1 and T3) showed the highest NDFD ($p \leq 0.05$), with no difference between them ($p > 0.05$). Likewise, the crotalaria treatment with 30 d of regrowth (T1) showed the highest ADFD ($p \leq 0.05$). The changes in fiber degradation are assumed to be caused by the state of maturity of the regrowth, since it recorded greater physiological changes at 40 d, due to the lignification process that hindered the adherence of ruminal bacteria (Hoffman *et al.*, 2007). Espinoza-Sanchez *et al.* (2020) reported higher DMD values (74.9%) than all the treatments of the present study and lower NDFD values (54.4%) than the 30-d chipile (T3) and crotalaria (T1) treatments for sheep diets that include 50 % ground ripe mango, 1% urea, 25% *Samanea saman* pod, 6% sugar cane molasses, 16% pangola grass, and 2% mineral mix.

Chipile treatments (T3 and T4) showed higher ($p \leq 0.05$) content of metabolizable energy (ME) and short-chain fatty acids (SCFA) (Table 4). This indicates that chipile treatments showed a higher energy contribution to the microbial fermentation and a better production of SCFA, as fermentation products under the *in vitro* conditions of the present experiment. SCFA and biogas production showed the same trend, since biogas production is the result of the fermentation of carbohydrates and their transformation into volatile fatty acids (Amanzougarene and Fondevila, 2020). Espinoza-Sánchez *et al.* (2020) recorded higher ME values than those reported in this study for sheep diets using ripe mango as an energy source and *Samanea saman* pod as a protein source.

CONCLUSIONS

Given their biogas production and *in vitro* fermentative characteristics, sheep diets containing 20% chipile or crotalaria with 30 d of regrowth proved to be an alternative in the elaboration of whole food diets for the intensive fattening of lambs in the tropics. However, further studies are required to prove the feasibility of producing these legumes as sheep feed.

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