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Gas production and environmental impact indicators from *in vitro* fermentation of diets with nopal silage (*Opuntia ficus-indica* L.)

Castañeda-Trujano, Francisco J.[1]; Miranda-Romero, Luis A.[1*]; Tirado-González, Deli N.[2]; Tirado-Estrada, G.[2]; Achiquen-Millán, Jimena[3], Améndola-Massiotti, Ricardo D.[1]; Martínez-Hernández, Pedro A.[1]

[1] Universidad Autónoma Chapingo, Posgrado en Producción Animal, Departamento de Zootecnia, Estado de México, México. C.P. 5623.
[2] Tecnológico Nacional de México/Instituto Tecnológico El Llano Aguascalientes, Aguascalientes Departamento de Ingenierías, México. C.P. 20256.
[3] Universidad Autónoma de Baja California Instituto de Ciencias Agrícolas. Ejido Nuevo León, Baja California, México. C.P. 21705
* Correspondence: microbiologia.pecuaria08@gmail.com

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ABSTRACT

Objective: To evaluate the global warming potential index (GWPI) and in vitro gas production (GP) of fattening diets in lambs fed with silage of agricultural by-products of nopal cladodes and prickly pear (*Opuntia ficus-indica* L.) - hibiscus grain (*Hibiscus sabdariffa* L.) - oats straw (*Avena sativa* L.).

Design/methodology/approach: The GP technique was used to obtain the GWPI of isoproteic (crude protein (CP)) and isoenergetic diets (15% CP and 2.8 Mcal ME (metabolizable energy)) without silage (DWS; control), with corn silage (CSD) and with 10 or 20% of nopal-prickly pear-hibiscus grain-oat straw silage (DEN10, DEN20), fed for 60 days to 24 Creole fattening sheep.

Results: *In vitro* dry matter digestibility at 72 h ($DIVMS_{72}$) was better in CSD, but similar for DEN10, DEN20, and CSD. DEN10 and DEN20 had the lowest CH_4 production, GWPI, and environmental impact index (EII). The low fermentable fraction (LF; GP=24-72 h) was related to $DIVMS_{72}$.

Findings/conclusions: The cactus pear-hibiscus grain silage inclusion (DEN10, DEN20) in conventional diets had no effect on $DIVMS_{72}$, but decreased CH_4 emissions and the GWPI.

Keywords: Greenhouse effect gases, environmental impact, *Opuntia-Hibiscus*-straw by-products, *in vitro* gas production technique.

INTRODUCTION

Ruminant production systems must have diverse ingredients that are a good source of nutrients, and as far as possible, reduce greenhouse gas emissions, [1], [2]. Given the scarcity and food prices increase, the utilization of agricultural by-products is an alternative to partially replace conventional sources of energy and protein used in diet formulations [3]–[5]. However, the energy and protein of these agricultural by-products do not always cover the requirements of the animals and create an imbalance in the rumen

flora, generating higher carbon dioxide (CO_2) and methane (CH_4) production [1], [6], [7]. Incomplete fermentation of structural carbohydrates represents up to 10% of energy loss from foo, [8] having an environmental impact, since CH_4 is considerably more polluting than CO_2 [9].

The CH_4 and CO_2 emission estimation was carried out with *in vivo* techniques such as sulfur hexafluoride (SF6), and relatively expensive breathing chambers [10]. At the same time, ruminal fermentation CH_4 emission can be derived from an *in vitro* gas production technique, which is low-cost and less polluting [11], given that ruminant productive behaviour depends on the digestibility, fermentation, and nutritional contribution of their food, such as the energy: protein ratio [12], [13]. Recent studies have tested this technique to determine the potential production of greenhouse gases [14], [15].

The objective of this research was to evaluate the global warming potential index (GGWPI) and the in vitro gas production (PG) of fattening diets for sheep fed with nopal cladode-prickly pear-hibiscus grain-oatmeal straw silage.

MATERIALS AND METHODS

Location. The research was conducted at the sheep module of the Experimental Farm and the Livestock Microbiology Laboratory of the Universidad Autónoma Chapingo.

Silage. Except for the hibiscus grains, all ingredients were chopped ($<$2.5 cm). For the silage, a 64% nopal cladode, 11% prickly pear, 10% hibiscus grain, and 15% oat stubble mixture was compacted in 200-liter plastic drums to a 650 kg m^{-3} density. Subsequently, the drums were covered, and hermetically sealed. The silage was stored 60 days before use.

Treatments. Correspond to four isoproteic (15% CP) and isoenergetic (2.8 Mcal of ME kg^{-1}) diets: diet without silage (DWS, control), diet with corn silage (CSD), and diets with 10 or 20% of nopal cladode-prickly pear-hibiscus grain-oatmeal straw silage (DEN10 and DEN20) (Table 1). These diets were fed for 60 days to 24 creole male sheep (26.9±3 kg BW MS), housed in individual pens, and randomly assigned to one of the four diets (n=6). Between 30-45 and 46-60 d, three samples of the supplied sheep Diets were dried (DS), grounded, and used for *in vitro* fermentation.

Gas production kinetics and *in vitro* digestibility at incubation 72 h. The diet samples were fermented, and their produced gas was assessed via the GP technique [16], [17] following a modified and described procedure [15]. The maximum volume (Mv; mL g^{-1}), rate (S; h^{-1}), and the Lag phase (L; h) of the GP were estimated using a one-phase function [18] optimized with the SAS statistical software [19]; also, the dry matter degradability ($DIVMS_{72}$), calculated from the initial DS and the residual DS.

Also, the fast, medium, slow, and total fermentation fractions (FFF, MFF, SFF, TFF; g kg^{-1}) of the food were also obtained, transforming the accumulated gas volumes in the 0 to 8, 8 to 24 h, and 24 to 72 h of incubation intervals and the linear regression models described by [15].

Environmental impact indicators and *in vitro* digestibility after 24 hours of incubation. A modification to the GP methodology described was followed to determine the degradability ($DIVMS_{24}$), total gas volume (VOLT; mL g^{-1}), and methane

Table 1. Diets ingredients and nutritional composition.

Ingredient %	**Diet**			
	DSE	**DEM**	**DEN10**	**DEN20**
Oat straw	16	10	9.5	3
Ground corn	57.3	56.6	53	39
Rolled corn	-	-	-	10
Soybean paste	13.6	13.8	13	12.2
Corn Gluten	7.6	4.1	9	10.3
Mineral mix[†]	2	2	2	2
Calcium	2	2	2	2
Urea	0.5	0.5	0.5	0.5
Salt	1	1	1	1
Corn silage	-	10	-	-
Cactus-Prickly pear-ibiscus silage	-	-	10	20
Aporte nutrimental				
DM%[§]	89.7	74.2	74.7	73.3
ME (Mcal/kg^{-1})	2.8	2.8	2.8	2.8
CP%	15	15	15	15
NDF%	23.5	23.9	20.7	21.3

[†] Vitasal ovino plus: calcium, phosphorus, magnesium, sodium, chlorine, potassium, sulfur, y antioxidants (24, 3, 2, 8, 12, 0.5, 0.5, y 0.5%, respectively); lasolacide, chromium, manganese, iron, zinc, iodine, selenium, and cobalt (2000, 5, 4000, 2000, 5000, 100, 30, y 60 ppm respectively); A, D, and E vitamins (500,000, 150,000, y 1000 UI, respectively). [§] DM, dry mater; ME, metabolizable energy; CP, crude protein; NDF, neutral detergent fiber.

production plus minor gases (CH_{4+GM}). The latter was adjusted to theoretical methane (% CH_4) with a 0.77 factor [14, 20]. The CO_2 and CH_4 volumes were used to calculate the Global Warming Potential Indicator (GWPI) with the equation:

$$GWPI\left(\text{mL CO}_2\text{ eq g}^{-1}\text{DS}\right)=\text{CO}_2\left(\text{mL g}^{-1}\right)+\left[\text{CH}_4\left(\text{mL g}^{-1}\right)\times 2.3\right].\ [21]$$

GWPI and total gas volume (VOLT) were used to calculate the environmental impact index $\left(EII\left(\text{CO}_2\text{ Eq}\right)=GWPI\,/\,VOLT\right)$.

Both fermentations for 24 and 72 h of incubation were repeated over time. Rumen inoculum from Dorpper male sheep with rumen cannula was used, adapted for 20 d to a diet without silage (DWS).

Statistical analysis. Analyzes of variance (ANOVA) were performed using diets as a fixed effect and repetitions (Rep) within the experiment run time (Time) as random effects (Model 1). The probability (P) of the fixed effects, the coefficients of determination (R^2), and variation (CV; %) were done with the Proc GLM [19], and the adjusted probabilities of Rep (Time) and standard errors (SE) se with Proc Mixed [19].

Model (1)

$$Y = \mu + Rep\left(Time\right)_{ij} + Diet_k + \varepsilon_{ijk}$$

Where: Y=Mv, S, L, $DIVMS_{72}$, FFF, MFF, SFF, TFF, CH_4, GWPI, EII, $DIVMS_{24}$, VOLT; μ=general average; $Rep\left(Time\right)_{ij}$ =effect of the i^{th}-repetition within the j^{th} execution time of the experiment; $Diet_k$=k^{th} diet effect; and ε_{ijk}=random error.

The LsMeans mean comparison test [19] was performed and the difference between means was analyzed using DMS, considering the SE, P=0.05 significance value, and the degrees of freedom (DF; 95) of the model error.

Simple linear correlations were obtained between pairs of variables (r) (Proc Corr; [19]). The correlation validity was obtained through the P values; as well as the multiple linear regression models by Forward) of Stepwise (Proc Reg; [19]), considering the variables inclusion with $P<0.15$. The models' validity was analyzed considering the regressor variables (βi) contribution to R^2 and its Mallow PC value.

RESULTS AND DISCUSSION

Table 2 shows that the Mv fluctuated between 303.45 and 321.38 mL g^{-1} at 72 h of incubation. These data are like those obtained by Martínez-Loperena *et al.* [22] for conventional diets, and by Vázquez-Mendoza [23] for corn or nopal silage diets; however, lower than those reported by Lazalde-Cruz [24] when 5 to 25% hibiscus seed (410.17 ml g^{-1}) was included. The $DIVMS_{72}$ varied from 79.16 to 81.77 %, higher than that reported by Miranda-Romero *et al.* [15] (52.3 to 54.8%) and by Muciño-Castillo [25] (61.44 to 69.55%), which suggests that the here evaluated diets show high degradation potential.

There were differences between Mv and $DIVMS_{72}$ diets ($P<0.0001$). DWS had higher Mv (321.38 ml g^{-1}) than DEN10 and DEN20 ($P<0.05$). The $DIVMS_{72}$ of the CSD was higher compared to DEN10 ($P<0.05$), but equal to DWS and DEN20 ($P>0.05$). DEN10 and DEN20 had the same degradation potential, but lower fermentative potential (Mv) than DWS.

The S was not different between diets, but DWS and CSD had higher L than DEN10 and DEN20 ($P<0.05$). Furthermore, the L of DEN20 was the lowest ($P<0.05$), indicating that increasing this silage proportion in the diet reduces the lag time. Higher L values were reported by Jiménez-Santiago *et al.* [27], Muciño-Castillo [26] values of 5.15 to 8.35 h when evaluating diets with nopal flour, while Lazalde-Cruz [24] reported L=3.91 h, in diets with hibiscus grain. Muciño-Castillo [26] and Lazalde-Cruz [24] reported 0.04 and 0.036 h^{-1} S values, like this research, which had no differences between diets.

Table 2 also shows FFF, MFF, SFF, and TFF values. The FFF was different between diets ($P<0.002$). Although previously studies reported similar values [15], [28], it increased as cactus silage in the diet increased ($P<0.05$), suggesting higher nonstructural carbohydrate content (NSC) previously related to the FFF [29].

The DWS, DEN10, and DEN20 had lower MFF than CSD ($P<0.05$). MFF is related to starch content [29]. CSD may have had a higher content of fermentable starch (a result of silage) than DEN10 and DEN20, which have fewer starch sources; including cactus silage tends to decrease MFF [15]. However, the MFF values of the present research were similar to previous ones when evaluating diets with cassava [28] and hibiscus [24]. The SFF was higher in DWS and CSD compared to DEN10 and DEN20 ($P<0.05$). The SFF is associated with cellulose content [29]. DEN10 and DEN20 could have lower NDF than DWS and CSD due to their oat straw and ME contents (28-37%; [23] *vs.* ≥59% [30], [31]).

DEN10 and DEN20 had lower TFF in DWS ($P<0.05$), which is again attributed to the 10% hibiscus grain inclusion in the EN, as a non-fermentable energy source in the rumen [32]. The TFF values are similar to those reported in diets with corn and nopal silage by Miranda-Romero *et al.* [15].

Table 3 shows the values for the environmental impact variables (CH_4, GWPI, EII), $DIVMS_{24}$, and VOLT were different among the diets ($P<0.0001$). The CH_4% values were similar to those of previous research in which whole grain high-concentrate diets were evaluated [14], [24], [33]. The CH_4%, GWPI, and EII were lower in the DWS than in the silage diets ($P<0.05$) and decreased when the proportion of EN increased. The IPGC and EII include the CH_4 volume (mL g^{-1} DS) in their calculation, which allows us to better understand the impact that diets can have on global warming (IPGC; mL CO_2 eq g^{-1} DS) in relation to the VOLT, which was similar in DWS, CSD, and DEN10 ($P>0.05$).

Studies mention that the CH_4% increase with the increasing low digestibility fiber in the diet [34], in this research DWS had better $DIVMS_{24}$, but due to its lower content of oat straw, diets with silage decreased the EII (*i.e.* DEN20 decreased 1.02 times the EII). When comparing Mv, $DIVMS_{24}$ and $DIVMS_{72}$, it was observed that during the first 24 hours, 86.5% of the potentially digestible dry matter is digested, and 64.5% of the total gas is produced (72 h).

Table 2. Gas production kinetics, *in vitro* dry matter (DM) degradability, and fermentable fractions of sheep diets with corn silage and nopal silage.

Tratamiento	Mv mL g^{-1}	S h^{-1}	L h	$IVDMD_{72}$ %	FFF	MFF	SFF	TFF
					ml g^{-1} DM			
DSE[†]	321.38 ^{a}	0.0408 ^{a}	3.57 ^{a}	80.10 ^{ab}	179.85 ^{b}	263.63 ^{ab}	289.63 ^{a}	733.12 ^{a}
DEM	316.20^{ab}	0.0412 ^{a}	3.85 ^{a}	81.77 ^{a}	172.94 ^{b}	272.84 ^{a}	280.69 ^{a}	726.47 ^{ab}
DEN (10%)	303.45 ^{b}	0.0400 ^{a}	3.24 ^{b}	79.16 ^{b}	180.64 ^{b}	253.75 ^{b}	260.27 ^{b}	694.67 ^{bc}
DEN (20%)	303.77 ^{b}	0.0404 ^{a}	2.71 ^{c}	80.36 ^{ab}	200.13 ^{a}	249.57 ^{b}	242.59 ^{b}	692.30 ^{c}
Valor de P	<0.0001	<0.13	<0.0001	<0.0001	<0.002	<0.0001	<0.0001	<0.0001
R^2	0.55	0.35	0.53	0.66	0.48	0.64	0.70	0.60
VC (%)	9.11	7.43	19.38	5.18	13.24	7.69	14.45	0.34
SE	8.19	0.0009	0.19	1.20	7.01	5.77	11.19	19.19
DMS 0.05=	13.68	0.0015	0.31	2.01	11.71	9.64	18.70	32.07

[†] Vm, maximum volume; S, rate of gas production; L, time Lag; $IVDMD_{24}$, *in vitro* dry mater digestibility 24 h; FFF, MFF, SFF, y TFF, fast, medium, slow and total fermentable fraction, respectively; DSE, diet without silage; DEM, diet with corn silage; DEN, diet with cactus silage; R^2 y VC determination and variation coefficient ; SE, standard error; ^{a,b,c} averages in the same column with different literals are different.

Table 3. *In vitro* atmospheric impact variables and dry matter digestibility (DM).

Tratamiento	CH_4 %	GWPI $mLCO_2$ eq g^{-1} MS	EII CO_2 eq	$DIVMS_{24}$ %	VOLT mL g^{-1} MS
DSE[†]	22.16^{a}	1601.64^{a}	5.81^{a}	71.30^{a}	205.33^{a}
DEM	20.54^{b}	1450.41^{b}	5.45^{ab}	69.70^{ab}	203.69^{a}
DEN (10%)	19.12^{c}	1243.18^{c}	5.15^{bc}	68.53^{b}	203.06^{a}
DEN (20%)	17.48^{d}	1187.92^{c}	4.79^{c}	68.46^{b}	190.52^{b}
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
R2	0.6297	0.5782	0.6299	0.554	0.6222
VC (%)	20.12	21.52	16.32	5.36	6.58
EE	1.15	85.16	0.24	1.07	3.81
DMS 0.05=	1.9216	142.3023	0.4010	1.7879	6.3665

[†] CH_4, % theorical methane; GWPI, global warming potential index; EII, Environmental impact indicator: VOLT, total volume of CH_4; DSE, diet without silage; DEM, diet with corn silage; DEN, diet with cactus silage; R^2, determination coefficient; VC, variation coefficient; SE, standard error; ^{a,b,c} averages in the same column with different literals are different.

Pearson correlations. Table 4 shows the simple correlations between the variables evaluated in this research. The highest FFF value relates to a decrease in L ($r=-0.75$; $P<0.0001$). Higher FFF and Mv relate to better $DIVMS_{24}$ ($r=0.26$ to 0.37; $P<0.01$) and VOLT with $DIVMS_{72}$ ($r=0.48$; $P<0.0001$). Also, degradability may have similar trends after 24 and 72 h of incubation ($r=0.46$ $DIVMS_{24}$ with $DIVMS_{72}$; $P<0.0001$). VOLT negatively correlated with the CH_4 % ($r=-0.28$; $P<0.01$), since the highest VOLT comes from the soluble carbohydrates and starch fermentation (0-24 h), expecting less CH_4 production [34].

Table 4. Correlations between the potential global warming index variable calculation via the *in vitro* gas production technique.

	GWPI	VOLT	CH_4	IVD_{24}	MV	S	L	FFF	MFF	SFF	TFF	IVD_{72}
EII	0.83^{***}	-0.28^{**}	0.99^{***}	-0.13_{NS}	-0.12_{NS}	-0.13_{NS}	0.07_{NS}	-0.28^{**}	-0.29^{**}	0.08_{NS}	-0.13_{NS}	-0.14_{NS}
GWPI		-0.01_{NS}	0.83^{***}	-0.26^{**}	-0.08_{NS}	-0.07_{NS}	0.21^{*}	-0.37^{**}	-0.22^{*}	0.16_{NS}	-0.08_{NS}	-0.11_{NS}
VOLT			-0.28^{**}	0.18_{NS}	-0.05_{NS}	0.31^{**}	0	0.08_{NS}	0.06_{NS}	-0.19_{NS}	-0.08_{NS}	0.48^{***}
CH_4				-0.13_{NS}	-0.12_{NS}	-0.13_{NS}	0.07_{NS}	-0.28^{**}	-0.29^{**}	0.08_{NS}	-0.13_{NS}	-0.14_{NS}
$IVDI_{24}$					-0.24^{**}	0.182_{NS}	-0.35^{**}	0.26^{**}	-0.15_{NS}	-0.47^{***}	-0.29^{**}	0.46^{***}
MV						-0.43^{***}	0.22^{**}	0.37^{**}	0.84^{***}	0.85^{***}	0.96^{***}	-0.25_{NS}
S							0.06_{NS}	-0.19_{NS}	-0.23^{*}	-0.54^{***}	-0.50^{***}	0.36^{**}
L								-0.75^{***}	0.41^{***}	0.43^{***}	0.18_{NS}	-0.10_{NS}
FFF									0.26^{**}	0.01_{NS}	0.41^{***}	0.10_{NS}
MFF										0.67^{***}	0.85^{***}	-0.05_{NS}
SFF											0.89^{*}	-0.51_{NS}
TFF												-0.32^{**}

EIIA, Enviromental impacto indicator; GWPI, global warming potential index; VOLT, total volume; CH_4, % theorical methane; $IVD_{24, 72}$, *in vitro* dry mater degradation 24 and 72 hours; MV, MAXIMUN volume; s, rate of gas production; L, lag time; FFF, fast fermetable faction; MFF, medium fermentable fraction; SFF, slow fermentable fraction; TFF, total fermentable fraction. ***$P<0.0001$; **$P<0.01$; *$P<0.05$; NS, $P>0.05$.

Better FFF and MFF could also relate to greater degradability at 24 and 72 h and lower GWPI ($r=-0.22$ to -0.37 GWPI with FFF, MFF, and $DIVMS_{24}$; $r=-0.25$ to $r=-0.26$ CH_4 with FFF and MFF; $P<0.05$) which corroborates that reported by Rasmussen and Harrison [34]. Although SFF positively correlated with Mv ($r=0.85$; $P<0.0001$), it negatively correlated with $DIVMS_{24}$ ($r=-0.47$; $P<0.0001$), likewise, $DIVMS_{72}$ also negatively correlated with TFF ($r=-0.32$; $P<0.01$).

Multiple linear regression models. Consistently, the SFF variable would allow obtaining the $DIVMS_{72}$ (Y) ($P<0.0001$). When including all diets: $Y=93.92-0.05\ SFF+\varepsilon_{ij}$ ($R^2=0.26$); DWS: $Y=94.7-0.05\ SFF+\varepsilon_{ij}$ ($R^2=0.34$); and CSD $Y=105.53-0.085\ SFF+\varepsilon_{ij}$ ($R^2=0.77$). In DEN10 and DEN20 the VOLT contributed 0.16 and 0.20 to the R^2 ($P<0.0001$) [$Y=11.6+0.63\ DIVMS_{24}+0.13\ VOLT+\varepsilon_{ij}$ ($R^2=0.42$); $Y=47.67+0.16\ VOLT+\varepsilon_{ij}$ ($R^2=0.20$)]. The amount of fiber contained in diets relates to gas production [15].

CONCLUSIONS

The diets containing the silage composed of nopal cladode-prickly pear-hibiscus grain-oatmeal straw showed the same degradability potential as the control diet and were similar to the diet with corn silage. The accumulated volume of gas at 24 h negatively correlated with the methane percentage, therefore, diets high in concentrate and low in fiber have lower methane emissions. The diet with silage mainly composed of nopal cladode and prickly pear provides a greater amount of non-structural carbohydrates and more degradable fiber, which reduced the average fermentable fraction, as well as the environmental impact index 1.01 times. The slow fermentation fraction could predict the degradability at 72 h. According to these results, including this compound silage would not negatively affect the productive behavior of fattening lambs.

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