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In vitro gas and methane production and dry matter degradation of pumpkin (*Cucurbita argyrosperma*) silages with pangola grass (*Digitaria decumbens*) hay

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ABSTRACT

Objective: Determine the *in vitro* production of gas and methane and dry matter degradation of pumpkin shell and pulp silages (PSP; *Cucurbita argyrosperma*) with pangola grass hay (*Digitaria decumbens*) and, as additives, urea and two inclusion percentages of molasses fermented for 14 and 21 days.

Methodology: Silages (2 kg) were: S1 = 72.5% of PSP, 22.5% of pangola grass, 3% of molasses, and 2% of urea; S2 = 72.5% of PSP, 19.5% of pangola grass, 6% of molasses, and 2% of urea. Both silages were fermented for 14 and 21 days. We evaluated gas and methane (CH_4) production, total bacteria count, and dry matter degradation (DMD). The experiment followed a completely randomized design with a 2 × 2 factorial arrangement, with types of silage and fermentation time as factors.

Results: The S1 treatment, at 21 d, showed the lowest production of gas at 72 h (46.96 mL g^{-1} DM) and the lowest DMD (35.78%; $p \leq 0.05$). Methane production and total bacteria count were not significantly different ($p > 0.05$) between the types of silage or fermentation time.

Study limitations: The inclusion of 3% of molasses with a fermentation time of 21 d showed the lowest gas production and dry matter degradation of silages with pumpkin shells and pulp with pangola grass hay.

Conclusions: The silages made of pumpkin shells and pulp are viable alternatives to preserve and produce ruminant feed during drought periods. Moreover, these silages represent an alternative use for potentially polluting materials, such as pumpkin shells and pulp.

Keywords: *Digitaria decumbens*, *Cucurbita argyrosperma*, gas production, silage, *in vitro*.

INTRODUCTION

Pumpkin is a vegetable from the Cucurbitaceae family that originated from the American continent (Martínez-Valdiviezo *et al*., 2015); of the 825 documented species. Mexico cultivates 13 species of nutritional importance for humans and 128 wild species. One of the most cultivated native species is *Cucurbita argyrosperma*; this species has medicinal properties and is also consumed by humans (Lira *et al*., 2002)

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in tropical and subtropical regions (Ireta-Paredes *et al*., 2018). In Mexico, the states with the greatest diversity of cultivated species are Oaxaca, Chiapas, Jalisco, Michoacán, Veracruz, and Guerrero (Lira *et al*., 2002). While the main producers of *Cucurbita argyrosperma* are Guerrero (in the Costa, Centro, la Montaña, Tierra Caliente, and Norte regions; Ayvar *et al*., 2004), Tabasco, Zacatecas, and Campeche, whose main objective is to obtain seeds, with a production of 0.5 t ha^{-1} (Ireta-Paredes *et al*., 2018). The rest of the vegetable (shell and pulp) is not consumed by humans, and thus, it is discarded in fields or plots, causing an environmental problem. Therefore, the production of silages using the agricultural by-products available in the region represents a viable alternative in the tropics, which favors obtaining food, particularly during the dry season, reducing feed costs, and taking advantage of potentially polluting agricultural residues (Guzmán *et al*., 2012).

The pumpkin shell and pulp (PSP) contains: 13.71% of dry matter (DM), 15.40% of raw protein (RP), 48.01% of neutral detergent fiber (NDF), 24.21% of acid detergent fiber (ADF), 23.80% of hemicellulose, 13.78% of ashes, and 86.22% of organic matter (OM; Lorenzo-Hernández *et al*., 2019). Moreover, PSP has an approximate yield of 1 t ha^{-1} of DM (Dorantes-Jiménez *et al*., 2016). The silage production process allows the fermentation of soluble carbohydrates into lactic acid to preserve the nutrients of the processed material (Yitbarek and Tamir, 2014). Moreover, using additives like urea and sugarcane molasses improves the lactic acid bacteria fermentation and increases the nutritional value of the silage (Araiza-Rosales *et al*., 2015).

The gas production technique simulates the ruminal environment under laboratory-controlled conditions (temperature, pH, anaerobiosis, and minerals) and evaluates the fermentation of different substrates (Storm *et al*., 2012); this technique is also used to evaluate the degradation of unconventional ruminant feeds (Sánchez-Santillán *et al*., 2018). This study aimed to determine the *in vitro* production of gas and methane and the dry matter degradation of silages made of pumpkin shells and pulp, pangola grass hay, urea, and two percentages of sugarcane molasses fermented for 14 and 21 days.

MATERIALS AND METHODS

Study localization

The research was performed in the Animal Nutrition laboratory of the School of Veterinary Medicine and Zootechnics No. 2 of the Universidad Autónoma de Guerrero, located in Cuajinicuilapa, Guerrero, México (16° 28' 18'' N, 98° 24' 55'' W, 50 masl).

Silages

Silages were produced using pumpkin shells with pulp without seeds (*Cucurbita argyrosperma*), pangola grass hay (*Digitaria decumbens*), and, as additives, sugarcane molasses and urea. The pumpkin shells with pulp and the pangola grass hay were ground in a multifunction mill (M.A.GRO® TR-3500, Mexico) with a 2.54 cm diameter sieve. Two molasses inclusion percentages (3 and 6%) were evaluated, silages were: S1 = 72.5% of *C. argyrosperma*, 22.5% of *D. decumbens*, 3% of molasses, and 2% of urea. S2 = 72.5% of *C. argyrosperma*, 19.5% of *D. decumbens*, 6% of molasses, and 2% of urea. Silages (2 kg) were produced in polypropylene bags (40 × 40 cm), the air was extracted with a vacuum (Koblenz®, Spain), and bags were closed with raffia to maintain the anaerobic conditions required to start the 14 and 21 day fermentation process.

Culture medium

The culture medium contained: 30 mL of clarified rumen fluid [fresh bovine rumen fluid centrifuged at 12,857 g for 10 min and sterilized 15 min at 121 °C and 15 psi], 5 mL of mineral solution I [6 g of K_2HPO_4 (Sigma-Aldrich®) in 1 L of distilled water], 5 mL of mineral solution II [6 g of KH_2PO_4 (Sigma-Aldrich®) + 6 g of $(NH_4)_2SO_4$ (Merck®) + 12 g of NaCl (Sigma-Aldrich®) + 2.45 g of $MgSO_4$ (Sigma-Aldrich®) + 1.6 g of CaCl-$2H_2O$ (Sigma-Aldrich®) in 1 L of distilled water], 0.1 mL of resazurin at 0.1% (Sigma-Aldrich®), 0.2 g of peptone from soybean (Merck®), 0.1 g of yeast extract (Sigma-Aldrich®), 2 mL of cysteine-sulfide solution [2.5 g of L-cysteine (Sigma-Aldrich®) in 15 mL of 2N NaOH (Meyer®) + 2.5 g of Na_2S-$9H_2O$ (Merck®) made up to 100 mL with distilled water], 5 mL of a Na_2CO_3 solution at 8% (Merck®), and 50.6 mL of distilled water, according to Torres-Salado *et al*. (2019). The medium was sterilized in an autoclave at 121 °C and 15 psi for 15 min based on the Cobos and Yokoyama (1995) methodology, modified by Sánchez-Santillán *et al*. (2016).

Solutions

Saturated saline solution: In 1 L of distilled water, we dissolved 370 g of NaCl and added 5 mL of 0.1% methyl orange. The pH was adjusted to 2. This solution was poured into serum vials (120 mL), at full capacity, to

obtain saline solution gas traps. NaOH (2 N) solution: In 1 L of distilled water, we dissolved 80 g of NaOH. This solution was poured into serum vials (60 mL), at full capacity, to obtain NaOH (2 N) traps.

Biodigesters

We added 0.5 g of the sample at a constant weight and 45 mL of culture medium to serum vials (120 mL). Vials were kept under a continuous CO_2 flow to maintain anaerobic conditions. Each vial was hermetically sealed with a neoprene septum (Ø 20 mm) and an aluminum crimp seal. Biodigesters were sterilized and subsequently inoculated with 5 mL of the total ruminal bacteria obtained from the rumen fluid of a Suiz-bu cow. Biodigesters were incubated in a water bath at 39 °C for 72 h. The fresh rumen fluid was obtained from a cannulated cow [fed in pangola grass (*Digitaria decumbes*) pastures] and filtrated through a double gauze layer to eliminate organic matter macroparticles. Bovines were handled following the internal bioethics and well-being regulation of the Universidad Autónoma de Guerrero, which is based on the Official Mexican Standard NOM-062-ZOO-1999.

In vitro gas production

The biodigesters' gas production was determined at 24, 48, and 72 h with a Tygon® hose (internal Ø: 2.38 mm, length: 45 cm) with hypodermic needles (20 G × 32 mm) at the ends. Needles were used to couple a biodigester with the gas trap vial (saturated saline solution). The trap vial was placed upside down in a modified graduated cylinder, which collects the saline solution displaced by the gas produced during the incubation through a hypodermic needle positioned as an outlet valve (Torres-Salado *et al.*, 2019).

Methane (CH_4) production

To measure methane (CH_4) production, we followed the same procedure described to measure gas production, but tramp vials were filled with a NaOH (2 N) solution, modified from the methodology described by Stolaroff *et al.* (2008). CH_4 production was measured as milliliters displaced from the NaOH (2 N) solution at 24, 48, and 72 h. CO_2 reacts with NaOH and forms Na_2CO_3 (Prada-Matiz and Cortés-Castillo, 2011).

pH and total bacteria count

At the end of the incubation period (72 h), we measured the pH with a pH meter (Hanna® HI2211, Italy; calibration: pH 7 and 4). We used a micropipette (Corning®, USA) to extract 1 mL of the medium contained in each biodigester and placed it in a test tube (Pirex®) with 0.25 mL of 10% formaldehyde (Sigma-Aldrich®). The total bacteria count was calculated by direct counting in a Petroff-Hausser cell-counting chamber (Hausser #39000, Electron Microscopy Sciences, USA), with an area of 0.0025 mm^2 and a depth of 0.02 mm. Cell-counting was performed using a microscope (BX31, Olympus, USA) at a magnification of 1000 (Sánchez-Santillán *et al.*, 2016). Total bacteria count was calculated with the equation: Total bacteria count = (average) (dilution factor, 2×10^7) (Sánchez-Santillán and Cobos-Peralta, 2016).

Dry matter degradation

After the incubation period, the residual content in the biodigester was filtered through ANKOM® bags (ANKOM® Technology) to constant weight. Filter bags were then sealed. The bags were dried at 60 °C in a forced-air oven (RIOSSA® HCF-41, Mexico) for 24 h. Dry matter degradation (DMD) was calculated with the equation DMD % = (initial sample - final sample / initial sample) * 100 (Sánchez-Santillán *et al.*, 2016).

Statistical analysis

Results (four replicates per interaction) were analyzed in a completely randomized design with a 2 × 2 factorial arrangement (SAS, 2011). Factors were type of silage (S1 or S2) and fermentation time (14 and 21 d). Averages were adjusted by least squares and compared using the Tukey test.

RESULTS AND DISCUSSION

The *in vitro* gas production technique has been used to evaluate the effect of different forages, feeds, diets, and ruminal fermentation additives (Crosby-Galván and Ramírez-Mella, 2018). This technique simulates the digestive processes of the microbial fermentation of carbohydrates to determine the nutrient quality and availability for rumen microorganisms (Antolín *et al.*, 2009). The gas production analysis was performed separately for the ingredients used to prepare silages (PSP and pangola grass hay) and the silages. In silages, we observed interactions between factors in all the variables analyzed. The gas production of PSP was higher (32.47, 50.09, and 37.76 mL of gas g^{-1} DM at 24, 48, and 72 h, respectively) than that of pangola grass hay (Table 1); this is associated with the higher content of non-structural carbohydrates in the PSP. At 24 h, the gas production of S1 with 21 d of fermentation was lower ($p\leq0.05$) than the rest of the silages. However, at 48 h,

the highest gas production was observed in S1 with 14 d of fermentation (p≤0.05). At 72 h of incubation, S1 with 21 d had a lower gas production (p≤0.05), representing 73.88% of the total gas production averaged by the rest of the evaluated silages (63.56 mL of gas g^{-1} DM; Table 1). The levels of gas production were obtained from the anaerobic fermentation of soluble or structural carbohydrates, but silages originate from anaerobic fermentation (Sánchez-Santillán *et al*., 2015). Antolín *et al*. (2009) reported higher values than those observed in this study, with a production of 217.5 mL of gas g^{-1} DM at 96 h of incubation in hybrid corn silages. Navarro-Villa *et al*. (2012) evaluated the *in vitro* gas production of silages made of *Lollium perenne* grass; their results show that gas production was 2.4 times higher than what is reported in this study. Moreover, compared to this study, Rojas-García *et al*. (2020) reported higher values at 24 h (112 mL of gas g^{-1} DM) and similar values at 48 h (47.6 mL of gas g^{-1} DM); however, at 72 h, the production is lower (33.9 mL of gas g^{-1} DM) in the complements prepared with PSP flour (70%) and parota pod flour (30%).

Methane (CH_4) production differ (p≤0.05) at 24 h, but there were no differences at 48 and 72 h (p>0.05) between S1 and S2 (Table 2). The difference at 24 h was observed in the fermentation times of S1. The fermentation time of 21 d produced 3.46 mL of CH_4 g^{-1} DM more than the 14-d fermentation (p≤0.05). Silages averaged 13.43 and 17.21 mL of CH_4 g^{-1} DM at 48 and 72 h of incubation (Table 2).

These data are lower than the 22.9 mL of CH_4 g^{-1} DM reported by Navarro-Villa *et al*. (2012) in *Lollium perenne* grass silages. However, the CH_4 production reported by Navarro-Villa *et al*. (2012) represented 15.3% of the total gas produced during the gas production assay; this value is lower than what we observed in this study; CH_4 production represented between 24.75 and 46.72% of the total gas produced during rumen microbial fermentation of the silages.

Table 1. *In vitro* gas production (mL g^{-1} of DM) in silages with pumpkin shells and pulp, pangola grass hay, urea, and two levels of molasses fermented for 14 and 21 days.

Silage	Fermentation (d)	Incubation time		
		24	48	72
S1	14	25.43[a]	53.96[a]	69.77[a]
S1	21	21.04[b]	34.36[b]	46.96[b]
S2	14	28.03[a]	40.22[b]	59.52[a]
S2	21	26.92[a]	41.20[b]	61.38[a]
Cucurbita argyrosperma		95.53	140.40	154.12
Digitaria decumbens		63.06	90.31	116.36
SEM		4.404	6.14	6.08

[a,b] Means with different letters in the same column indicate statistical difference (p≤0.05).
S1 = 72.5% of *C. argyrosperma*, 22.5% of *D. decumbens* hay, 3% of molasses, and 2% of urea; S2 = 72.5% of *C. argyrosperma*, 19.5% of *D. decumbens* hay, 6% of molasses, and 2% of urea; SEM = standard error of the mean.

Table 2. CH_4 production (mL g^{-1} of DM) in silages with pumpkin shells and pulp, pangola grass hay, urea, and two levels of molasses fermented for 14 and 21 days.

Silage	Fermentation (d)	Incubation time (h)		
		24	48	72
S1	14	6.37[a]	14.40[a]	17.27[a]
S1	21	9.83[b]	13.94[a]	18.84[a]
S2	14	7.32[a]	12.68[a]	16.19[a]
S2	21	8.03[a]	12.70[a]	16.56[a]
Cucurbita argyrosperma		21.48	31.60	35.25
Digitaria decumbens		10.32	18.42	24.90
SEM		0.67	0.91	0.96

[a,b] Means with different letters in the same column indicate statistical difference (p≤0.05).
CH_4 = Methane gas; S1 = 72.5% of *C. argyrosperma*, 22.5% of *D. decumbens* hay, 3% of molasses, and 2% of urea; S2 = 72.5% of *C. argyrosperma*, 19.5% of *D. decumbens* hay, 6% of molasses, and 2% of urea; SEM = standard error of the mean.

Methane production was higher in PSP than in pangola grass hay at the three evaluated incubation times (Table 2). The CH_4 production of PSP was 2.5, 1.7, and 2.0 times higher than that of the silages at 24, 48, and 72 h of fermentation. The CH_4 production of hay was 1.2, 1.1, and 1.5 times higher than S1 and S2. This is probably due to the lactic acid concentration of silages (Lorenzo-Hernández *et al*., 2019), which favors less methanogenic *in vitro* fermentations (Counotte *et al*., 1981; Navarro-Villa *et al*., 2012) compared to the unprocessed ingredients. The differences in CH_4 production can be associated with the structural and non-structural carbohydrates available during fermentation (Ferro *et al*., 2017). The acetate and hydrogen produced by the fermenting microorganisms (Zhang *et al*., 2015; Sánchez-Santillán and Cobos-Peralta, 2016) favor a syntropic relationship between methanogenic microorganisms, which use hydrogen (H_2) and carbon dioxide (CO_2) as substrates to produce CH_4 as a metabolic pathway for energy production (Torres-Salado *et al*., 2019).

The pH of the biodigester's culture medium was not significantly different (p>0.05) between silages or

between the unprocessed ingredients (Table 3), with an average pH of 6.78. These values remain between the normal range (6.0 to 7.0) of the ruminal microbiota (Kolver and de Veth, 2002), particularly of cellulolytic bacteria, since their activity is not inhibited (Rojas-García *et al*., 2020). However, pH values lower than 6.0 inhibit their activity (Ley de Coss *et al*., 2016). In this study, the pH values are similar to those reported by Navarro-Villa *et al*. (2012). They reported a pH of 6.69 in the culture media after the *in vitro* ruminal anaerobic fermentation of silages made of *Lollium perenne* grass. Furthermore, the pH in this study is similar to that reported in complements prepared with 70% PSP flour and 30% of parota pod flour (Rojas-García *et al*., 2020). After 72 h of *in vitro* fermentation, the total bacteria count was not significantly different between the ingredients used to prepare the silages. The molasses inclusion percentages and the silage fermentation time did not differ in the total bacteria count at 72 h of incubation ($p>0.05$). The average bacteria count in the silages was 8.2×10^8 bacteria mL^{-1} (Table 3), which classifies them as fibrous feed since they have 50.76 and 56.99% of NDF (Lorenzo-Hernández *et al*., 2019). The total bacteria count in this study was lower than that reported by Rojas-García *et al*. (2020). They determined the total bacteria count after the fermentation period in the culture media elaborated with pumpkin shell and pulp, reporting 16.7×10^8 bacteria mL^{-1}.

Dry matter degradation (DMD) was lower ($p\leq0.05$) in S1 with 21 d of fermentation. There were no differences in the remaining silages ($p>0.05$; Table 3). The DMD of silages differed from the degradation observed with PSP. The silages with no observed differences ($p>0.05$) had a DMD average of 49.5%, which represents 69.3% of the DMD of PSP. Higher values (74.6% of DMD) were reported in complements that include 70% of PSP (Rojas-García *et al*., 2020). To find alternative ruminant feeds, different potential products that can cause environmental contamination problems have been evaluated *in vitro*. However, DMD results are variables and depend on the type of products to process. De Haro *et al*. (2001) reported a DMD of 55% in pepper silages; Martínez-Teruel *et al*. (2007) reported a DMD of 63.6% in artichoke silages; Araiza-Rosales *et al*. (2015) reported a DMD ranging from 65 to 70% in silages with increasing apple and molasses concentrations; Gusha *et al*. (2015) reported a DMD ranging from 61 to 75% in cacti silages with tropical legumes; Caicedo *et al*. (2015) reported a DMD between 63.83 and 74.75% in silages with increasing taro concentrations; and Espinoza-Guerra *et al*. (2016) reported DMD between 52.9 and 59.1% in passion fruit silages. This variation and the use of various products impede the comparison with the DMD observed in this study (Table 3). Degradation is affected by the bromatological composition of the products used to prepare the silages (Posada and Noguera, 2005), specifically the amount of soluble sugars available (Araiza-Rosales *et al*., 2015) and the cell wall composition of the products (Ramírez *et al*., 2002).

Table 3. pH, total bacteria count, and dry matter degradation in silages with pumpkin shells and pulp, pangola grass hay, urea, and two levels of molasses fermented for 14 and 21 days.

Silage	Fermentation (d)	pH	[B] (10^9 cells mL^{-1})	DMD (%)
S1	14	6.77	0.81	50.47[a]
S1	21	6.84	0.80	35.78[b]
S2	14	6.86	0.72	50.35[a]
S2	21	6.78	0.93	47.57[a]
Cucurbita argyrosperma		6.71	1.20	71.47
Digitaria decumbens		6.69	1.14	43.91
SEM		0.018	0.0040	1.700

[a,b] Means with different letters in the same column indicate statistical difference ($p\leq0.05$).
[B] = total bacteria count; DMD = dry matter degradation; S1 = 72.5% of *C. argyrosperma*, 22.5% of *D. decumbens* hay, 3% of molasses, and 2% of urea; S2 = 72.5% of *C. argyrosperma*, 19.5% of *D. decumbens* hay, 6% of molasses, and 2% of urea; SEM = standard error of the mean.

CONCLUSIONS

The silages made of pumpkin shells and pulp are viable alternatives to preserve and produce ruminant feed during drought periods. Moreover, these silages represent an alternative use for potentially polluting materials. The inclusion of 3% of molasses with a fermentation time of 21 d showed at 72 h the lowest gas production and dry matter degradation of silages with pumpkin shells and pulp with pangola grass hay.

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