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Huizache Leaves and Agave Bagasse Incorporated into Granulated and Pelletized Concentrates and Their Effects on Methane Production and *in vitro* Fermentation Patterns in Ruminant Diets

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ABSTRACT

Background: The use of non-conventional food sources is a key element in facing the current problems derived from climate change and food shortages that demand the establishment of new sustainable feeding systems for ruminants. Moreover, densification methods like pelleting constitute a way to improve the quality and acceptability as well as to facilitate the handling of supplements. The objective of this study was to evaluate Huizache leaves and agave bagasse incorporated into granulated and pelletized concentrates and their effects on methane production and *in vitro* fermentation patterns in ruminant diets.

Methods: A protein concentrate granulated and pelletized containing Huizache leaves and agave bagasse were mixed with a high-quality forage (alfalfa hay) using a 2×3 factorial arrangement with a total of six treatments. In each treatment were determined crude protein, carbohydrates, *in vitro* digestibility, *in vitro* gas, methane, carbon dioxide, volatile fatty acids, ammoniacal nitrogen and microbial nitrogen. The variables were submitted to an analysis of variance using the procedure MIXED SAS and statistical differences were declared at P<0.05.

Result: There was an interaction (P<0.01) between concentrate source (granulated vs pelleted) and relation (alfalfa hay + concentrate source) on maximum gas production "A" (P<0.05). Also, there was an interaction between concentrate source (granulated vs pelleted) and relation (alfalfa hay: concentrated) on microbial nitrogen production (P<0.05). The microbial nitrogen production increased in 55 % with pelleted concentrate in relation to granulated concentrate (P<0.05).

Key words: Agave bagasse, Alfalfa hay, Concentrate, Huizache leaves, Pellet.

INTRODUCTION

The world is currently experiencing unprecedented urban growth that requires significant adaptations in food systems to ensure sufficiency, resilience and sustainability (FAO, 2019). The livestock sector must face the gradual increase in demand for beef and milk, as a result of population increase, urbanization and improvement in the economic level of some sectors of society (Ku-Vera *et al.*, 2020; Henchion *et al.*, 2021; FAO, 2021).

However, an increase in livestock production comes with the generation of greenhouse gases (GHG) and products of enteric rumen fermentation as methane which represents 18% of total GHG emissions of anthropogenic origin. Numerous studies have focused on the mitigation of this gas since its global warming potential is 28 times greater than that of CO_2 (INECC, 2018).

One factor that limits livestock production in rangeland is the low consumption of dry matter and energy, due to the low availability of quality forage (Stockdale, 2000). In arid and semi-arid areas, the inclusion of shrub foliage in the livestock diet is a relevant option to implement, since they do not compete with human food and can also provide good quality nutrients throughout the year (Murillo-Ortiz *et al.*, 2019; Araiza-Ponce *et al.*, 2020; Herrera-Torres *et al.*, 2021). An additional benefit of shrubs is the contribution of secondary metabolites that can modify rumen fermentation,

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improve protein utilization and reduce methane emissions (Canul-Solis *et al.*, 2020).

Some plants, including various species of Acacia, are considered an important source of protein for animals. Their characterization has allowed us to observe that they have secondary metabolites such as condensed tannins with the potential to reduce methane synthesis during rumen digestion and favor the passage of protein beyond the rumen by acting as protective agents, improving nitrogen absorption (Rodriguez *et al.*, 2014; Ruacho-González *et al.*, 2017; Ku-Vera *et al.*, 2020; Araiza-Rosales *et al.*, 2022).

Another potential source of non-conventional food is agave bagasse, a waste that is generated as residual fiber in large quantities from the production of tequila and mezcal in various regions of Mexico. Its fiber and micromineral content indicate that it can be used as food, especially in the dry season; although it does not meet the nutritional requirements of animals, it has been proposed as an alternative to being enriched with a nitrogen source, in addition to the fact that its aroma could favor the acceptance of the food in a similar way to other byproducts of alcoholic fermentations (Delgadillo- Ruiz *et al.*, 2015).

It must be highlighted that the use of fresh foliage and byproducts in rangeland conditions entails a series of drawbacks since they are very unstable when exposed to the outside conditions and, if not handled properly, they lose their nutritional quality, in addition to being prone to contamination, molding and rotting due to high moisture content (Perez-Ruchel *et al.*, 2017). One way to address this problem is the inclusion of supplementary feeds, in the form of concentrates that include non-conventional forage sources rich in secondary metabolites (Gutiérrez León *et al.*, 2019).

The use of densification processes such as extrusion and pelleting allows the inclusion in the diet of diverse ingredients that translate into nutritional improvements, transportation, handling and storage are also facilitated, costs are reduced and acceptability by animals is improved. Its use for the manufacture of supplements has resulted in improvements in fermentation and reduction of methane production *in vitro* (Reyes-Jáquez *et al.*, 2011; Kang *et al.*, 2016; Nguyen *et al.*, 2020).

The objective of this study was to evaluate the inclusion of Huizache leaves and agave bagasse into granulated and pelletized formulated concentrates and their effects on methane production and *in vitro* fermentation patterns in ruminant diets.

MATERIALS AND METHODS

Location of study area

The experiment was carried out in the Animal Nutrition Laboratory of the Faculty of Veterinary Medicine and Animal Science of the Juarez University in Durango (Mexico) and in the Laboratory of Postgraduate and Research Unit of the Technological Institute of Durango (Mexico) from October of 2022 to June of 2023. Surgical procedures and management of rumen fistulated steers that were used to obtain rumen fluid were performed in accordance with the Official Mexican Standard (NOM-062-ZOO-1999) and were approved and certified by the Animal Protection Committee of the State of Durango (Mexico).

Collection and sampling

Samples of *Acacia farnesiana* and *Acacia schaffneri* were collected in autumn 2022, in two areas located in the vicinity of the city of Durango, Dgo. Mexico with abundant Huizache vegetation. Branches of approximately 1m were cut from 5 randomly selected shrubs. The leaves were separated manually and mixed in a pool by species. The agave bagasse was donated by a local mezcal plant. The samples were dried in a forced air oven at 55°C (Calisa Alley Mod. 550R) until constant weight and were processed in a mill (Arthur H. Thomas Willey, Philadelphia, PA, USA) and sieved using a size mesh of a 1mm.

Pelleting and granulating

The processing of the pelleting was carried out in a pilot plant scale pelletizer Mill (Mod. ZSLP-R300) with a 6mm cylindrical inlet diameter and an inlet temperature of 50 to 55°C. The pellets were allowed to cool and stored at room temperature until use, whereas in the granulating process, the ingredients of concentrates were ground in a mill (Arthur H. Thomas Willey, Philadelphia, PA, USA) (Bear Cat #1A-S, Westerns Land and Roller Co., Hastings, NE, USA), equipped with a 1 mm screen.

Ingredients of the concentrates

Two concentrates were evaluated, one was subjeted to a granulation process and the other to a pelleting process. Two varieties of Huizache (*A. farnesiana* leaves and *A. schaffneri* leaves) and agave bagasse were included in the formulation of the concentrates. The ingredients used to prepare the concentrates are shown in Table 1.

Chemical composition of concentrates

The alfalfa hay and both concentrates (granulated and pelleted) were analyzed to determine dry matter (DM), ether extract (EE), ashes (Ash) and crude protein (CP) according to standardized procedures by the AOAC (2010). The fractions of neutral detergent fiber (NDF), acid detergent fiber (ADF), hemicellulose (HEM), cellulose (CEL) and lignin

Table 1: Proportion of ingredients in concentrates.

Ingredient	% DM
Distillers dried grains	15
Agave bagasse	6
Ground corn	9
Soybean paste	18
Cottonseed	15
Wheat bran	17
Huizahe (*A. farnesiana leaves*)	15
Huizache (*A. schaffneri leaves*)	5

(LIG) were estimated using an ANKOM fiber analyzer (Fiber Analyzer 200, ANKOM Technology, USA) following the manufacturer's instructions. Non-structural carbohydrates (NSC) were estimated using the following equation: NSC=[100-(PC+EE+Ash+NDF)]. True *in vitro* digestibility of dry matter (IVDDM) and organic matter (IVDOM) were determined at 48h using a Daisy incubator (ANKOM Technology, USA). Condensed tannins (CT) were extracted and estimated according to Heimler *et al.* (2005) using a UV-VIS spectrophotometer (Spectronic Instruments, Genesys 10S, Mod. 336003); CT were expressed in Catechin equivalents. Both concentrates were isoproteic and provided the same amount of neutral detergent fiber. The nutritional composition of the alfalfa hay and concentrates is shown in Table 2.

Experimental treatments

In each experimental treatment, alfalfa hay, were used as forage source. Six treatments were evaluated: (T1) alfalfa hay + without concentrate (100:00, DM); (T2) alfalfa hay + granulated concentrate (75:25, DM); (T3) alfalfa hay + granulated concentrate (50:50, DM); (T4) alfalfa hay + without concentrate (100:00, DM); (T5) alfalfa hay + pelleted concentrate (75:25, DM); (T6) alfalfa hay + pelleted concentrate (50:50, DM). Composition of experimental treatments is shown in Table 3.

***In vitro* gas production and fermentation patterns**

Fermentations for *in vitro* gas determinations were carried out in 100 mL glass syringes (FORTUNA, Germany) according to what was done by Yusuf *et al.* (2020), using 0.200 g of sample and 30 mL of a buffer solution and ruminal fluid in a 2:1 ratio for incubation for 24 h at 39°C. Rumen fluid was collected approximately 3 h after morning feeding from two steers with rumen fistula. Rumen fluid was immediately squeezed through four layers of gauze and transported to the laboratory in a sealed thermos. The reading times for measuring gas production were 0, 3, 6,12, 24, 36, 48, 72 and 96 hours. The accumulated gas production kinetics were adjusted to the model proposed by France *et al.* (2002).

Table 2: Nutritional composition of alfalfa hay and concentrate.

		Concentrate	
	Alfalfa Hay	Granulated	Pelleted
DM %	93.6±0.06b	96.17±0.09[a]	96.33±0.03a
OM %	87.15±0.01b	93.27±0.02[a]	93.26±0.03a
CP %	16.8±0.35b	25.57±0.32[a]	25.47±0.58a
EE %	1.5±0.06b	3.2±0.21[a]	3.5±0.001a
NDF %	55.17±0.20a	31.2±0.70b	31.1±0.17b
ADF %	36.33±0.72a	11.8±0.17b	12.03±0.26b
HEM %	18.9±0.9b	19.37±0.49[a]	19.07±0.43a
CEL %	30.97±0.55a	9.60±0.23b	9.97±0.033b
LIG %	4.3±0.11a	2.0±0.06b	1.87±0.20b
NSC %	13.7±0.17b	33.33±1.09[a]	33.17±0.75a
IVDDM %	75.94±0.78b	80.25±0.21[a]	76.66±0.85b

[1]a,b indicate that values within the same row are significantly different ($p<0.05$).

Table 3: Chemical composition of experimental treatments.

Proportion (%)	Treatments (T)					
	Granulated			Pelleted		
	Relation (R)					
	100:00	75:25	50:50	100:0	75:25	50:50
DM	93.6±0.11	95.1±0.09	95.04±0.12	93.6±0.11	95.3±.15	94.7±0.07
OM	87.2±0.03	88.7±0.08	90.38±0.22	87.1±0.03	88.7±0.13	90.2±0.17
CP	16.8±0.60	17.7±0.47	20.57±0.02	16.8±0.60	19.3±0.07	19.6±0.24
NDF	55.18±34	56.0±2.49	53.3±0.83	55.2±0.34	52.1±0.63	52.1±1.77
ADF	35.8±1.57	36.30±1.75	31.9±2.44	35.8±1.57	32.8±0.062	29.2±2.78
Lignin	4.3±0.20	5.48±0.37	6.4±0.60	4.3±0.204	4.4±0.18	4.3±0.54
IVDDM	75.9±1.35	76.1±0.38	76.1±0.44	75.9±1.35	76.6±0.52	75.5±0.92
IVDOM	75.2±1.16	75.04±0.61	75.7±0.37	75.2±1.16	75.5±0.39	75.6±0.84
NFC	26.5±0.15	24.33±24.33	23.5±0.88	26.5±0.152	26.2±0.54	25.4±1.38
EE	1.48±0.123	1.9±0.10	2.6±0.09	1.5±0.12	2.3±0.05	2.9±0.194
CT[1]	44.0±1.86	53.1±1.86	60.1±2.27	44.0±1.86	60.1±2.27	57.6±1.14

[1]CT expressed in mg of catequine equivalents/mg extract.

$$GP = A \times [1 - e - kd (t-L)]$$

Where;
GP = Volume of gas produced at time t.
A = maximum gas production from the fermentable fraction.
Kd = constant rate of gas production (h^{-1}).
L = delay time before gas production.

At the end of the fermentation, the pH of each sample was measured with a digital potentiometer (Hanna Instruments, Mod. HI83141) and ammoniacal nitrogen ($N\text{-}NH_3$) and volatile fatty acids were quantified (Galyean, 2010). Prior to the quantification of volatile fatty acids, a liquid-liquid extraction was performed using dichloromethane and a 20% NaCl solution following the procedure proposed by Luyt *et al.* (2021) with some modifications.

In vitro methane and carbon dioxide production

According to Fievez *et al.* (2005), to quantify the volume of methane produced, a two-way valve was used to transfer the gas contained in the glass syringe to a plastic syringe with 4 ml of sodium hydroxide (10 M). After stirring, the residual volume was measured and was considered methane. The total amount of methane produced per sample was subtracted from the total gas to obtain the carbon dioxide value.

Microbial nitrogen

Incubation was carried out in glass syringes mentioned above using 0.5 g of sample and 40 mL of buffered rumen fluid in the ratio 1:2 for 24 h at 39°C. Microbial nitrogen was calculated according to methodology proposed by Getachew *et al.* (2000)

$$MN = TN - (NDF\text{-}N + NH_3\text{-}N)$$

Where;
MN = Microbial nitrogen.
TN = Total nitrogen in the syrinx before incubation (Nitrogen in the feed + Nitrogen in buffered ruminal fluid).
NDF-N and NH_3-N = Fiber-bound nitrogen and ammoniacal nitrogen in the supernatant after 24 hours of incubation.

Statistical analysis

A completely randomized design with factorial arrangement 2x3 was used and to detect differences between minimum quadratic means, Tukey's multiple range test ($P<0.05$). The MIXED procedure of SAS (2003) was used in all analyses.

RESULTS AND DISCUSSION

In vitro gas production parameters and methane

Treatments effects on *in vitro* gas production parameters and methane are shown in Table 4. There were no concentrate source and ratio (alfalfa hay: concentrate) interactions on GP_{24}, kd, L, CO_2 , and CO_2:CH_4 ($P>0.05$). There was no treatment nor relation effect on GP_{24} ($P>0.05$). The Kd was not affected by concentrate source ($P>0.05$). However, it was greater for alfalfa hay + granulated vs alfalfa hay + pelleted granulated ($P<0.05$). The CO_2 concentration was not affected by concentrate source (granulated vs pelleted) ($P>0.05$). Nevertheless, there was an interaction ($P<0.01$) between concentrate source (granulated vs pelleted) and relation (forage + concentrated) on maximum gas production "A" ($P<0.05$). However, "A", not was affected by concentrate source ($P>0.05$). Also, there was an interaction ($P<0.01$) between concentrate source (granulated vs pelleted) and relation (forage: concentrated) on the methane production ($P<0.05$). The concentrate source affected methane production ($P<0.05$). The methane production increased with pelleted concentrate while with granulated concentrate decreased.

The use of Huizache leaves and agave bagasse in ruminant diets has been studied partially, but not integrated to concentrates granulated and pelleted. In fact, there are few scientific reports which have evaluated the concentrates granulated and pelleted containing Huizache leaves and agave bagasse which makes it difficult to compare our results with other research. The result of the interaction between concentrate source (granulated vs pelleted) and relation (alfalfa hay:concentrate) on maximum gas production "A" (maximum gas production) indicates that the treatment with

Table 4: Treatment effects on *in vitro* gas production parameters and methane.

	Treatments (T)									
	Granulated concentrate			Pelleted concentrate						
	Relation (R)						P<value			
	100:0	75:25	50:50	100:0	75:25	50:50	S*	R**	TxR	SEM
A, ml/g	129.6	51.1	51.7	50.47	49.5	48.2	0.34	0.97	0.73	1.1
Kd, ml/h	0.56	126.9	130.0	127.3	128.4	124.2	0.57	0.04	0.02	2.2
L, h	5.0	0.27	0.20	0.27	0.33	0.25	0.02	0.58	0.76	0.02
CH_4, ml/g DM	10.7	5.2	4.2	5.1	5.3	5.0	0.04	0.41	0.94	0.81
CO_2, ml/g DM	39.8	5.2	8.1	10.7	7.0	7.6	0.03	0.04	0.01	0.92
CO_2:CH_4 ratio	3.7	44.8	36.3	42.0	42.3	40.6	0.15	0.22	0.92	0.90

Treatment designations: Alfalfa hay + granulated concentrate; Alfalfa hay + pelleted concentrate.
*Source= granulated concentrate, pelleted concentrate.
**Relation= Alfalfa hay:concentrate (100:0, 75:25, 50:50).
SEM= Standard error of the means.

Table 5: Treatment effects on *in vitro* ruminal fermentation patterns.

	Treatments (T)									
	Granulated			Pelleted						
	Relation (R)						P<value			
	100:0	75:25	50:50	100:0	75:25	50:50	S*	R**	TxR	SEM
pH	7.1	7.0	7.0	7.1	7.0	7.1	0.99	0.45	0.15	0.53
TVFA, Mm	483.2	334.3	263.4	439.6	207.3	231.2	0.02	0.05	0.29	0.39
Acetic acid, %	68.8	65.0	65.0	68.9	61.2	58.2	0.09	0.32	0.19	1.30
Propionic acid, %	24.6	35.9	39.2	34.4	25.5	37.5	0.27	0.14	0.27	0.82
Butyric acid, %	6.4	1.7	2.2	4.4	1.7	1.8	0.10	0.01	0.21	0.32
A:P, ratio	2.7	1.7	1.5	2.7	1.4	1.6	0.12	0.35	0.44	0.02
NH_3-N, mg/dl	6.9	5.2	6.1	7.1	7.3	6.0	0.79	0.52	0.93	0.60
Microbial N, mg/gDM	1.5	2.0	3.5	4.0	5.3	6.0	0.05	0.04	0.01	0.67

Treatment designations: Alfalfa hay + granulated concentrated; Alfalfa hay + pelleted concentrated.

*Source= granulated concentrate, pelleted concentrate.

**Relation= Alfalfa hay:concentrate (100:0, 75:25, 50:50).

SEM= Standard error of the means.

the highest gas production is the granulated concentrate in a 50:50 relation (alfalfa hay: concentrate). Regarding the effects of treatments on GP_{24}, their response can be associated with a similar composition of the experimental treatments (Gaviria *et al.*, 2015). The pelleting process seems to have a negative effect on gas production; this could be due to a smaller contact surface that could increase the colonization time by the rumen microorganisms and decrease the transformation efficiency as happens with milling (Nguyen *et al.*, 2020). In this study, the methane production increased with pelleted concentrate while with granulated concentrate decreased. A lower methane production in the presence of the concentrate can be attributed to a faster fermentation, since it has been observed that more digestible diets tend to produce less methane; this effect was greater in the presence of the granulated concentrate (Ku-Vera *et al.*, 2020). Likewise, the observed effect on methane production due to the alfalfa hay: concentrate relation could be related to the inclusion of secondary metabolites naturally present in Huizache leaves (Salami *et al.*, 2019).

In vitro ruminal fermentation patterns

Treatments effects on *in vitro* ruminal fermentation patterns are shown in Table 5. There was no concentrate source by ratio interactions on pH, acetic acid, propionic acid, butyric, acid A:P ratio and NH_3-N (P>0.05). Nevertheless, there was an interaction between concentrate source (granulated vs pelleted) and ratio (alfalfa hay:concentrated source) on TVFA concentration (P<0.05). The TVFA concentration increased in 23% with granulated concentrate in relation to pelleted concentrate (P<0.05). Likewise, there was an interaction between concentrate source (granulated vs pelleted) and ratio (forage: concentrated) on microbial nitrogen production (P<0.05). The microbial nitrogen production increased in 55% with pelleted concentrate in relation to granulated concentrate (P<0.05).

The increase in TVFA production is positively related to gas production (A), Cherdthong *et al.*, (2019) report that pelleting process increases the density of the food and difficult access to rapidly fermentable nutrients, this is reflected in a lower production of TVFA. The effect on the forage:concentrate ratio can be attributed to the presence of secondary metabolites in the concentrate. Sandoval-Pelcastre *et al.* (2020) describe that condensed tannins negatively affect cellulolytic bacteria and, consequently, the fermentation of carbohydrates to VFA is reduced.

The increase in microbial nitrogen production in treatments with pellet concentrate can be attributed to the fact that there is a greater amount of N-NH_3. N-NH_3 is the main substrate used for microbial protein synthesis (Koenig and Beauchemin, 2018). These results coincide with Cherdthong *et al.* (2019), who report that microbial protein synthesis is favored by the inclusion of palletized foods. An effect was observed in the forage:concentrate relation, where microbial nitrogen production increases with increasing the proportion of concentrate since Getachew *et al.* (2000) found that the inclusion of energy sources increases microbial protein synthesis.

CONCLUSION

Pelletizing is a way to avoid the selectivity of livestock and favor the acceptance of sources of condensed tannins; however, according to the results of this work pellets and granules of the proposed formulation represent alternatives with similar positive effects, in consequence, both could be used by livestock producers to improve diet quality mainly by enhancing microbial protein synthesis.

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Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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