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Effect of Alfalfa Hay Substitution by Raw Garlic Leaves on *In vitro* Gas Methane Production and Ruminal Fermentation

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ABSTRACT

Background: The aim of present research was to evaluate under *in vitro* conditions, the effect of alfalfa hay substitution by raw garlic leaves on ruminal fermentation patterns and methane production in diets ruminants.

Methods: Four treatments were evaluated: (T1) alfalfa hay (50%); (T2) alfalfa hay (33%) + raw garlic leaves (17%); (T3) alfalfa hay (17%) + raw garlic leaves (33%) and (T4) raw garlic leaves (50%).

Result: The highest values of fractional rate of gas production (kd), ammonia-nitrogen (NH3-N), propionate and microbial biomass synthesis (MBS) was recorded in T4 and the lowest in T1 ($P<0.05$). In contrast, the highest methane production was recorded in T1 and the lowest in T4 ($P<0.05$). It was concluded that the substitution of alfalfa hay by raw garlic leaves in diet with 50% roughages and 50% concentrate result in an improvement *in vitro* rumen fermentation pattern and decreases the methane production.

Key words: Garlic leaves, *In vitro* ruminal fermentation, Methane production.

INTRODUCTION

In the recent years there is an increasing global demand for garlic consumption which leads to the inescapable production of agricultural waste consisting primarily of husk, straw and leaves (Kallel and Ellouz, 2017). In fact, garlic leaves generated abundantly during the harvesting period, are usually made into waste which is incinerated (Han *et al*., 2013). Nevertheless, agricultural waste generated in the garlic production may be used as forage, since the fiber and protein contents in leaves are higher than the ones contained in common forages sources. Moreover, raw garlic leaves contains several compounds, including sulfur compounds as thiosulfates and allicin, as well as enzymes, free amino acid, sterols, glycosides, flavonoids and phenols (Lawson, 1996). Some of these compounds have shown activity on decreasing methane production on *in vitro* assays (Kamra, *et al*., 2012). Moreover, Busquet *et al*. (2005), using the same *in vitro* system, showed that garlic components reduced the proportions of acetate and branched-chain volatile fatty acids (VFA) and increased the proportion of propionate and butyrate and small peptides. In this study, we hypostatized that under *in vitro* conditions the alfalfa hay substitution by raw garlic leaves can improve the ruminal fermentation and reduce the methane production in ruminants diets. Therefore, the aim of present research was to evaluate under *in vitro* conditions, the effect of alfalfa hay substitution by raw garlic leaves on ruminal fermentation patterns and methane production in diets ruminants.

MATERIALS AND METHODS

Study location

The experiment was conducted at the animal metabolic unit and the laboratory of animal nutrition of the Faculty of Veterinary and Husbandry of Juarez University at the Durango (México).

Raw garlic leaves sampling

The samples (leaves with 25 cm of length) of raw garlic (*Allium sativum*) used in this study, were collected from North region of Mexico. To ensure representative sampling, the samples were collected eight times between June 2019 and January 2020.

Experimental treatments

Four treatments were evaluated: (T1) alfalfa hay (50%, DM)

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+ raw garlic leaves (0%, DM); (T2) alfalfa hay (33%, DM) + raw garlic leaves (17%, DM); T3 alfalfa hay (17%, DM) + raw garlic leaves (33%, DM); T4 alfalfa hay (0%, DM) + raw garlic leaves (50%, DM). The other 50% of the DM was supplied for a concentrate composed of ground corn, cottonseed and minerals. Nutritional composition of experimental treatments is shown in Table 1.

Chemical composition

The samples of each experimental treatment were analyzed in triplicate for dry matter (DM), organic matter (OM) crude protein (CP) and ether extract (EE) AOAC, (2000). Acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent lignin (ADL) analyses were determined using the filter bag technique with a fiber analyzer (ANKOM Technology, Fairport, NY, USA). Total carbohydrates (TCHO) contents were calculated according to the equation proposed by Sniffen *et al.* (1992): %TCH = 100 – (%CP+%EE+% ash); whereas, the nonfibrous carbohydrate (NFC) content was calculated using the difference between %TCH and %NDF.

True degradability of dry matter

The true degradability of dry matter (TDMD) at 48 h was carried out in polyethylene bags (ANKOM®), to which they were weighed 0.5 g of each diet. The bags were introduced into glass bottles of ANKOM gas production system. Immediately, 125 mL of ruminal fluid and a buffer solution were added to each glass bottle. Ruminal fluid was obtained from of two rumen cannulated steers fed with a diet containing fed 60% oat hay and 40% concentrate. The glass bottles were introduced in Daisy incubator (ANKOM Technology, Fairport, NY, USA) with controlled temperature (39°C).

***In vitro* gas parameters and methane production**

In vitro gas production was measured using the ANKOM gas production system. Ruminal fluid was collected approximately 3 h after morning feeding from two rumen fistulated steers. Approximately 1 g of dried and ground samples was weighed and placed into glass bottles. The gas production was recorded after 2, 4, 6, 8, 12, 16, 24, 36, 48, 72 and 96 h of incubation. The cumulative gas production kinetic was fitted to model proposed by France *et al.* (2002) as: GP = A * [1-e-kd(t-L)]. Where: GP is the volume of gas produced at time t, "A" is potential gas production (ml/g DM) from the fermentable fraction of forage, "kd" is the fractional rate of gas production (h^{-1}) and "L" is the discrete lag time prior to gas production. The average GP rate (AGPR) at half of A was calculated according to the equation of García-Martínez *et al*. (2005). For methane production measure once the incubation period is over at 24 h, pressure release valve was opened during 2 secs in every glass module and the released gas in each module was passed through a tube and connected to a portable gas analyzer (GEMTM5000, LANDTEC, USA).

Ruminal fermentation patterns

After termination of the incubation at 24 h, two samples (5 mL) of the glass bottle fluid were collected. The first subsample was acidified with 0.3 mL of 50% H_2SO_4 and the second subsample with 2.5 mL of 25% metaphosporic acid. Both subsamples frozen immediately at -40°C and later analyzed for ammonia nitrogen (NH_3-N) and total volatile fatty acids (TVFA), respectively (Uden *et al*., 1980).

Calculation

The microbial biomass synthesis (MBS) yield was calculated

Table 1: Chemical composition of experimental treatments.

Ingredients	Treatments			
	T1	T2	T3	T4
Alfalfa hay	50	33	17	0
Raw garlic leaves	0	17	33	50
Corn milled	39	39	39	39
Cottonseed	10	10	10	10
Minerals	1	1	1	1
	Chemical composition (% DM)			
DM	93.1	93.5	93.3	93.6
OM	91.1	91.7	92.0	92.4
CP	15.0	14.8	14.7	15.2
EE	2.0	2.0	2.5	2.1
NDF	53.3	49.3	50.9	47.1
ADF	28.3	23.3	21.7	19.2
TCHO	74.5	74.9	76.3	75.1
NFC	21.5	25.6	25.4	28.2
ME Mcal kg^{-1} DM*	5.5	5.2	5.2	6.2

DM= Dry matter; OM= Organic matter; CP= Crude protein; EE= Ether extract; NDF= Neutral detergent fiber; ADF=Acid detergent fiber; TCHO= Total carbohydrate; NFC= Non-fibrous carbohydrate. *Estimated from the equation ME (Mcal kg^{-1} DM) = 2.20 + 0.136 Gas $production_{24h}$ + 0.057 CP + 0.0029 ether $extract^2$/4.184 (Menke and Steingass,1988).

using the TDMD (mg) and gas volume (24 h) and stoichiometric factor as follows: MBS (mg^{-1}g DM) = TDMD (GP_{24} × 2.25) (Blümmel *et al*., 1997). Additionally, the efficiency of microbial biomass synthesis (EMBS) was estimated as the ratio of MBS to TDMD (Blümmel *et al*., 1997). The partitioning factor (PF) was calculated as the ratio between TDMD (mg) and the gas produced at 24 hours of incubation of substrate truly degraded (Blümmel *et al*. 1997).

Statistical analysis

Analysis of variance for completely random design was carried out to compare the *in vitro* gas production, methane and ruminal fermentation patterns using the procedure GLM of SAS (2002).

RESULTS AND DISCUSSION

Chemical composition

In this study, the forage to concentrate ratios were 50%:50%, respectively. As would be expected, the four experimental treatments provided the same amount of protein and energy. Nevertheless, the highest content of NDF was recorded in the treatment containing a higher proportion of alfalfa (T1). Alfalfa provides a high fiber content (NDF, 18.68% DM) (Van Soest, 1994). Cell wall content of forages is related to their dry matter digestibility. In fact, in this study NDF content seems to have reduced the TDMD of T1.

In vitro gas parameters and methane production

The *in vitro* gas parameters and methane production of experimental treatments are summarized in Table 2. The "A" fraction was affected significantly by treatments (P< 0.05). The higher "A" value was found in T4 and the lower in T1 (P<0.05). The higher values fractional rate of gas production "kd" and average gas production rate when half of "A" occurred "AGPR" were found in T4 and the lower in T1 (P<0.05); though "kd" and AGPR values was unaffected by T2 and T3 (P>0.05). The average "A" value in the present study, was of 116.5 mL 200 DM mg^{-1}. These values agree with values reported by Sahli *et al.* (2018) when incubated *in vitro*, garlic powder a doses of 32 mg in a diet composed of 50% ryegrass hay and 50% commercial concentrate. The "kd" value registered with T4 (9.0 mL h^{-1}), was higher to that found by Anassori *et al.* (2012) (3.5 mL h^{-1}), who evaluated the *in vitro* gas production kinetics of raw garlic bulb. Likewise, the AGPR value registered in T4 suggests that the inclusion raw garlic leaves accelerated microbial fermentation of digestible components in the diet. There were differences in the methane production among treatments (P< 0.05). The low methane concentration was obtained in T4 (high in garlic leaves) may be compared with results reported by Kongmun *et al*. (2010) who report a decrease in the methane production when evaluated garlic powder in ruminants diets. These results suggest two possible reduction pathways for methane: a) an inhibitory action in growth and expression of methanogens and, b) the acétate pathway was affected by affecting microorganisms which contribute to produce methane from it. (Murillo *et al*., 2018).

True degradability of dry matter, microbial biomass synthesis and efficiency microbial biomass synthesis

The true dry matter degradability, microbial biomass synthesis and efficiency microbial biomass synthesis are presented in Table 3. The TDDM and GP_{24} were different between treatments (P<0.05). The highest TDDM values and GP_{24} were recorded in T4 and the lowest in T1 (P<0.05). In the same way, the MBS values were affected significantly by treatments (P< 0.05). The highest MBS value was recorded in T4 and the lowest in T1. However, the highest EMBS value was recorded in T3 and the lowest in T2 and significant differences were observed between both treatments (P<0.05). The highest PF was recorded in T1 and was different to the others treatments (P<0.05). The TDDM values obtained in study current, partially agreement with reported by Zafarian and Manafi (2013) who evaluated under *in vitro* conditions garlic powder in wheat straw based diets. The result obtained in T1 for MBS values is not agreement with reported by Arbabi *et al*. (2017) who evaluated under *in vitro* conditions alfalfa hay more concentrate in a range 50:50; though the MBS obtained with T4, could be explained from by CP and NFC contents which promoting a better balance between protein and energy provided by the diet (Van Soest, 1994). The average PF value in all treatments, was of 5.9 mg of TDMD/mL was higher to the suggested range of 2.74 to 4.41 mg TDMD/mL gas produced for stimulate the microbial protein production (Makkar, 2004).

Table 2: *In vitro* gas parameters, methane production and rate of passage of experimental treatments.

	Treatments				Mean	SEM
	T1	T2	T3	T4		
A (ml 0.2 g^{-1} DM)	111.2^{d}	118.4^{b}	115.8^{c}	120.9^{a}	116.5	1.10
Kd (mL h^{-1})	0.06^{c}	0.08^{b}	0.08^{b}	0.09^{a}	0.07	0.025
AGPR (mL h^{-1})	12.4^{c}	18.1^{b}	17.7^{b}	37.4^{a}	21.4	0.83
L (h)	0.04^{a}	0.03^{a}	0.03^{a}	0.01^{a}	0.02	0.42
Methane (mL g^{-1} DM)	19.1^{a}	17.0^{b}	16.8^{c}	9.5^{d}	15.6	0.53

^{abc} Values with different superscripts in the same row are statistically different (P<0.05).

A= Asymptotic/potential gas production from the fermentable fraction; Kd= Fractional rate of gas production from the slowly fermentable feed fraction (A); AGPR= Average gas production rate when half of "A" occurred; L= Discrete lag time prior to gas production

Table 3: True degradability of dry matter, microbial biomass synthesis (MBS) and efficiency microbial biomass synthesis of experimental treatments.

	Treatments				Mean	SEM
	T1	T2	T3	T4		
TDMD, g Kg^{-1} DM+	582^{d}	603^{c}	622^{b}	642^{a}	612.2	3.4
GP_{24}, h mL g^{-1} DM	89.3^{d}	99.3^{c}	110.5^{b}	112.8^{a}	103	1.78
MBS mg $^{-1}$ g DM	160.2^{d}	164.4^{c}	178.3^{b}	182.2^{a}	171.2	2.18
EMBS (%)	27.5^{b}	27.2^{b}	28.6^{a}	28.3^{a}	27.9	0.051
Partition Factor mg TDMD/mL gas	6.5^{a}	6.0^{b}	5.6^{c}	5.6^{c}	5.9	1.055

^{abc} Values with different superscripts in the same row are statistically different (P<0.05).

TDMD= True degradability dry matter; GP_{24}= Gas production at 24 h; MBS= Microbial biomass synthesis; EMBS= Efficiency of microbial biomass synthesis; PF= Partition factor.

SEM: Standard error of the mean.

Table 4: Ruminal fermentation patterns of experimental treatments.

	Treatments				Mean	SEM
	T1	T2	T3	T4		
pH	6.7^{a}	6.7^{a}	6.6^{a}	6.5^{a}	6.6	0.07
Ammonia N, mg dL^{-1}	15.1^{c}	17.1^{b}	17.5^{b}	18.1^{a}	16.7	0.03
Total VFA, mM	86.6^{b}	104.4^{a}	104.6^{a}	104.8^{a}	100	0.36
Acetate VFA, mol100 mol^{-1}	52.8^{a}	50.5^{b}	50.8^{b}	48.1^{c}	50.5	0.10
Propionate mol100 mol^{-1}	24.8^{c}	28.0^{b}	27.8^{b}	33.0^{a}	28.4	0.08
Butyrate mol100 mol^{-1}	12.2^{a}	12.6^{a}	13.5^{a}	13.2^{a}	12.8	0.02
A:P ratio	2.1^{a}	1.8^{a}	1.8^{a}	1.4^{b}	1.7	0.03

^{abc} Values with different superscripts in the same row are statistically different (P<0.05).

SEM: Standard error of the mean.

Fermentation ruminal patterns

The fermentation ruminal patterns and methane production are presented in Table 4. The highest NH_3-N concentration was recorded in T4 and the lowest in T1 (P<0.05). There was no difference between T2, T3 and T4 in TVFA (P>0.05); but both treatments were different to T1 (P<0.05). The highest acetate concentration was recorded in T1 and the lowest in T4 (P<0.05); whereas, the highest propionate concentration was recorded in T4 and the lowest in T1 (P<0.05). Ruminal NH_3-N concentrations recorded in all experimental treatments evaluated, were maintained within range of 15 to 30 mg/100 mL suggested for optimal microbial growth (Wanapat and Pimpa, 1999). In contrast, with the results obtained in this study, several studies *in vitro* and *in vivo* report that garlic oil or garlic powder reduced or have no effect on ruminal fluid NH_3-N concentration (Cardozo *et al*., 2014). The TVFA concentrations obtained with T2, T3 and T4 are consistent with other *in vitro* studies where did not differ with addition of garlic oils (Klevenhusen *et al*., 2011). The propionate concentration observed in T4 was higher than in the other treatments; this is evidenced with decreased ratio of acetate:propionate. A lower acetate:propionate ratio improves ruminal fermentation efficiency as well as energy is available for rumen microbes activities.

CONCLUSION

These results indicated that the substitution of alfalfa hay by raw garlic leaves improved the *in vitro* gas production parameters, ruminal ferrmentation patterns, microbial protein synthesis and decreased the methane production when measured *in vitro*.

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