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Degradabilidad Ruminal In Vitro De Alfalfa Con Diferentes Niveles De Inclusión De Larrea Tridentata In Vitro Ruminal Degradability Of Alfalfa With Different Levels Of Inclusion Of Larrea Tridentata

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RESUMEN
El objetivo del presente trabajo fue evaluar parámetros de degradabilidad ruminal in vitro del substrato alfalfa con Larrea t., en concentraciones de 1, 3 y 5 %. Las tres concentraciones de inclusión de Larrea t. al 1 % (T 2), 3 % (T 3), y 5 % (T 4), y un testigo de alfalfa al 100 % (T 1) como control. La degradabilidad ruminal in vitro de realizó durante 48 h. Los resultados se analizaron mediante PROC MIXED con prueba de poder de Tukey y correlaciones de Pearson. Los resultados mostraron que una DIVV inferior para tratamiento de 100 % alfalfa y el tratamiento con mayor porcentaje de DIVV fue el tratamiento 2 alfalfa con el 1 % de Larrea t., entre el tratamiento 1 y los demás tratamientos se observaron diferencias significativas ($p<0.05$). Los resultados de la degradabilidad in vitro de FDA entre tratamientos y control fueron diferentes ($p<0.05$). Se observaron correlaciones positivas significativas entre los AGV ($p<0.001$), y volúmenes de gas ($p<0.05$). Se observó una correlación negativa entre la adición de Larrea t., al sustrato de alfalfa y los volúmenes de gas y correlaciones positivas entre degradabilidades de fibras, AGV y metano. La inclusión de Larrea tridentata al 1 % mejora la degradabilidad de fibras.

Palabras clave: Larrea tridentata, degradabilidad ruminal, AGV, CH_4

ABSTRACT
The objective of this work was to evaluate in vitro ruminal degradability parameters of the alfalfa substrate with Larrea t., in concentrations of 1, 3 and 5 %. The three inclusion concentrations of Larrea t. at 1% (T 2), 3% (T 3), and 5% (T 4), and a 100% alfalfa control (T 1) as a control. Rumen degradability in vitro was carried out for 48 h. The results were analyzed using PROC MIXED with Tukey's power test and Pearson correlations. The results showed that a lower DIVV for 100% alfalfa treatment and the treatment with the highest percentage of DIVV was treatment 2 alfalfa with 1% Larrea t., between treatment 1 and the other treatments significant differences were observed ($p < 0.05$). The results of the in-vitro degradability of FDA between treatments and control were different ($p<0.05$). Significant positive correlations were observed between VFA ($p<0.001$) and gas volumes ($p<0.05$). A negative correlation will be observed between the addition of Larrea t., to the alfalfa substrate and the gas volumes and positive correlations between fiber degradabilities, VFA and methane. The inclusion of 1% Larrea tridentata improves the degradability of the fibers.

Keywords: Larrea tridentata, ruminal degradability, VFA, CH_4.

INTRODUCTION

The greenhouse effect caused by the gases resulting from the combustion of fossil fuels, mainly used for agricultural production, transport, conversion and/or generation of energy, has affected the chemical composition of the atmosphere in recent decades, together with the agricultural sector in the production systems of milk or ruminant meat. According to FAO, livestock contributes up to ~37% of total anthropogenic methane (CH_4) emissions, mostly from rumen and enteric fermentation of ruminants (Glatzle, 2014). Total enteric methane emissions represent energy losses of between 2 and 12% of gross energy consumption in ruminants, depending on the amount of feed intake and the type or composition of the diet (Johnson and Johnson, 1995; Boadi et al., 2004; Nevels and Demeyer, 1996). That is why feeding strategies are implemented

that have as their main objectives to reduce methane emissions by ruminants, but on the other hand, to redirect that potential energy for the benefit of the ruminant. Beauchemin et al. (2009) state that a 25% reduction in methane production in ruminants can result in increases of 1 L of milk per day in high-producing cows, and a 75 g/d increase in weight in beef cattle. The main strategies to mitigate methane production are the selection of the most efficient animals according to their residual feed intake (Muro et al. 2011), chemical additives such as nitro-components (Martins et al., 2024), Ionophores (Marques et al., 2021; Sun et al., 2023), lipids (Beauchemin et al., 2022 and Fouts et al., 2022), and secondary plant components (Ku-Vera et al., 2020), among others.

Plants are already part of the diet of herbivores, but there are some that are generally not consumed by livestock or their consumption is low, and that contain bio-active compounds such as EO (essential oils), saponins and tannins, which have antimicrobial properties that can be used in animal nutrition with the aim of improving food utilization and animal health (Ju et al., 2022). Extracts from some plants have been found to possess antimicrobial properties against a wide variety of microorganisms, including bacteria, fungi, and viruses (Efenberger et al., 2021; Pateiro et al., 2021; Tako et al., 2020).

In the past decades, chemical additives such as antibiotics, ionophores and other chemical agents were developed for protozoan defaunation, with the aim of modulating rumen fermentation, maximizing growth, increasing milk production, improving consumption and feed efficiency. However, the use of these as growth promoters in livestock production has no longer been allowed in Europe since January 2006 (OJEU, 2003). The main reason for the ban on the use of antimicrobials in animal feed is based on reducing the risks of antimicrobial resistance to antibiotics used in the treatment of infections in humans (PCIFAP, 2008). As a result, research on the use of antimicrobial agents of synthetic or natural origin as growth promoters is intensifying, such as secondary plant metabolites. The plant known as gobernadora, creosote, chaparral or hedion (Larrea tridentata), has properties such as the reduction of edema, and decrease of the replication and transcription of the human immunodeficiency virus, is hypoglycemic, is antioxidant and induces an anti-apoptotic action, has protective action of keratinocytes against the action of ultraviolet rays, and has cytotoxic action for various cancer cells (Morales et al., 2021). The leaves of the Larrea produce a phenolic resin composed of numerous flavonoids and flavones partially methylated with the nordihydroguayaretic acid that comprises 40% of the dry mass of the total resin (Martínez et al., 2020).

MATERIAL AND METHODS

10 samples were randomly collected per time of year, obtaining a total of 40. The samplings were carried out from January to December 2015, within an area of 2 ha. NDF and ADF analyses were performed on the samples before and after in vitro incubations by gas production, to determine the degradability of these nutrients. The analysis of these nutrients was used to determine DIVV (true in vitro degradability), DIVVBS (in vitro degradability on a dry basis), DIVFDN (in vitro degradability of neutral detergent fiber), DIVFDA (in vitro degradability of acid detergent fiber). To perform these analyses, the samples were dehydrated at 60°C for 48 h and ground in a mill with a 1mm sieve. Dry matter (DM) and organic matter (OM) were determined. The fiber analysis was performed by the Van Soest method, using the ANKOM 2000 fiber analyzer (Ankom Technology). The in vitro PG procedure was performed according to the Menke et al., (1979) with some modifications for incubations in ANKOM bottles. The amount of sample used and recorded within 25 jars (5 blanks, 5 controls 5 and 5 per Larrea level t.) was 1000 mg of sample, 150 ml of the artificial saliva solution and fluid were added to each vial (in a 2:1 ratio, respectively). They were introduced to the incubator, where they remained for 48 h. In the present work, the final gas volume was measured at 48 h of the three inclusion levels of Larrea tridentata: alfalfa at 1, 3 and 5 %, white and control. The substrates used were for treatment 1, alfalfa 100%, treatment 2, alfalfa 99% with Larrea t. at 1%, treatment 3, alfalfa 97% with Larrea t. 3% and treatment 4 alfalfa 95% with Larrea t., 5%. 1 g of substrate was added per Ankom jar for a total of 5 incubation jars per treatment. After in vitro degradability by gas production, the nutrient, DM and fiber profile was analysed as already indicated to determine DIVV, DIVVBS, DIVFDN, DIVFDA. After in vitro incubations, the three main volatile fatty acids, acetic, propionic and butyric, were quantified in the residual liquid of the in vitro incubation. gas chromatography and methane were calculated based on the equation of Moos et al., (2000) incubation. For the comparison analysis of degradability, AGVs and methane, the design was randomized blocks, and the statistical analysis was performed using PROC MIXED with Tukey's test. A Pearson correlation analysis was also performed between the evaluated variables, and the SAS 9.0 statistical package was used.

RESULTS AND DISCUSSION

Degradability of different substrates

Table 1 shows the results of the DIVV (True in vitro degradability), DIBS (in vitro degradability on a dry basis), DIFDN (in vitro degradability of neutral detergent fibre) and DIFDA (in vitro degradability of acid detergent fibre), of the four substrates evaluated.

Table 1. In vitro degradability and degradability of alfalfa inoculum fibers with different concentration levels of Larrea t., at 48 h.

Treatment	DIVV %	DIVBS %	DIVFDN %	DIVFDA %
1	78.77[a]	77.22[a]	36.89[a]	25.66
2	83.14[b]	81.90[b]	47.31[bc]	36.25
3	81.16[ab]	79.78[ab]	41.60[ab]	30.99
4	81.03[ab]	79.66[ab]	46.35[c]	31.17
EE	0.9619	1.0320	2.8575	3.7928

Columns with different literals showed significant differences.

The results showed that IVD was lower for control treatment of 100% alfalfa and that the treatment with the highest percentage of IVDL was treatment 2 or alfalfa substrate with 1% Larrea t., between treatment 1 and the other treatments significant differences were observed ($p<0.05$), however, all treatments containing Larrea t. in different proportions they did not show significant differences in this variable. The DVIV estimates obtained can be interpreted as estimates of the actual digestibility of forages (Tassone et al., 2020). The residue resulting from in vitro incubation for 48 h is a mixture of undigested forage and rumen microorganisms and its subsequent passage through neutral detergent solubilizes the microorganisms and the remains of cellular content of the forages (Gursoy et al., 2020), so that the cell wall content and the time of its degradation determine the value of the in vitro degradability of the substrate (Perrusquía Tejeida, 2021, Tassone et al., 2020). Boschini-Figueroa and Chacón-Hernández (2017), They evaluated the degradability of four varieties of alfalfa and found effective degradability percentages between 59.06 % and 67.09 % however, the procedure used was through in bag degradability.

Very similar results were observed in the DIVBS variable, showing that treatment 1 with 100 % alfalfa was the one in which the lowest percentage of degradability was observed and was significantly different from treatments containing another percentage of Larrea t. ($p<0.05$), it was also recorded that treatment 2 of alfalfa with 1% of Larrea t., was the one that revealed the highest percentage of DIVBS. However, this assessment is fully correlated with IVF. Among arid plants, Larrea tridentata extract has been used commercially as an antioxidant in the United States between 1943 and 1970 (Lara and Francisco, 2023). Currently, the nordihydroguaiaretic acid of this species is used as an antioxidant for the storage of synthetic and natural rubber (Reyes Melo et al., 2021), but its effect on the environment or the rumen microbiota and therefore also on the modifications of rumen fermentation is unknown.

The results of the DIVFNA showed the highest percentage of degradability for treatment 2, followed by treatment 4 and the Control treatments and treatment 3 showed the lowest degrees of degradability. Significant differences were observed between the control treatment with treatment 2 and 3, and similarities between treatment 2 and 3, and 2 and 4. ($p<0.05$). In the present study, non-significant differences were observed in the variable of DIVFDN ($p>0.05$). Arias et al., (2013) In vitro degradability studies of alfalfa added with different grain percentages were carried out and observed that the increase in ground corn caused a linear decrease in NDF and FDA at 48 h ($p<0.05$), concluding that the use of the starch provided by ground corn as an energy source produced a decrease in rumen pH and lower use of NDF and FDA of alfalfa. However, in the present experiment, contrary results were observed with the addition of Larrea t. in different percentages, favoring the degradability of the fibers, even though alfalfa is considered a forage with an excellent nutritional profile.

Volatile fatty acid and methane profiles

Table 2 shows the results of the analysis of volatile fatty acids in residual rumen fluid after 48 h of in vitro incubation. VFAs are end products of rumen fermentation of food and mainly carbohydrates. These by-

products of microbial metabolism in an anaerobic fermentation system are important precursors for the metabolic processes of ruminants (Wang et al., 2020).

Table 2. Profile of AVG and CH_4 in incubations of different concentrations of Larrea t., at 48 h.

Treatment	Acetic	Propionic	Butyric	CH_4
1	40.48	7.51	0.75	16.45
2	45.70	9.07	0.84	18.41
3	51.07	10.38	1.05	20.54
4	34.33	5.97	0.40	13.90
EE	5.0294	1.3460	0.2366	1.9878

Columns with different literals showed significant differences.

In the present study, non-significant differences were found in VFA production, as well as in the estimated methane production, this by stoichiometry (Moos et al., 2000). The amount and molar ratio of acetate (a), propionate (p), and butyrate (b) determines the moles of CO_2 and CH_4 produced. The stoichiometry of Wolin (1960) assumes that the net oxidation balance of all products is equal to zero (a+p+b+CO2+CH4=0), that CO_2 and CH_4 are produced only from acetate and butyrate, twice the amount of CO_2 and CH_4 is generated from butyrate formation (CO_2+CH_4=a+2b). After a few substitutions, the resulting equation is: CO_2=a/2+p/4+1.5*b. Thus, the moles of CO_2 produced can be calculated from the molar amount and proportion of acetate, propionate and butyrate, and once the moles of CO_2 are known, the moles of CH_4 can be calculated according to: CH_4=(a+2b)-CO_2 (Cattani et al., 2014). On the other hand, it has been shown that the disappearance of the NDF is related to gas production (Arbabi et al., 2017). Also Menke et al. (1979) report high correlation between in vivo OM digestibility and cumulative 24-hour in vitro gas production . Although in the present work no significant differences were found in terms of the methane profile, numerically it was observed that the substrate with alfalfa and 5 % of Larrea t., is the one that presented the lowest amount in methane production, however, the lowest amount of VFA profile was also logically observed. The goals of improving animal efficiency are aimed at favoring or not affecting the production of VFAs and reducing methane production, that is, only directing the energy that is lost as methane towards other metabolites usable by the ruminant, this through the inclusion of secondary components of plants and/or nitro components mainly.

Cattle emit methane because different types of microorganisms participate in the anaerobic digestive process, which degrade the ingested cellulose to glucose, which they then ferment to acetic acid and reduce carbon dioxide to form methane (Carmona et al., 2005). For this reason, it is considered that activities such as agriculture and livestock production contribute to the emissions of methane (CH_4), carbon dioxide (CO_2) and nitrous oxide (N_2O) into the atmosphere, whose increase in the concentrations of these gases causes a warming of the earth's surface and the destruction of the ozone layer (Hernández, 2020). This work used the In vitro technique of gas production in order to evaluate gas production at 48 h in in vitro rumen cultures, including a plant that has been reported with properties to optimize animal efficiency and reduce the emission of gases that generate the greatest impact on the environment.

It is known that the ruminants that produce the most methane are usually those that consume tropical grasslands, as they are of fair to low quality (high in lignocellulose and low in other nutrients, mainly protein and soluble carbohydrates (Archimède et al., 2011), the opposite happens when legumes are included in the diet (Boadi et al., 2004), because they have high protein content, low NDF content and sometimes considerable amounts of secondary compounds such as tannins.

Table 3 shows the results of Pearson's correlation analysis. Pearson's correlation analysis showed positive correlations between DIVV, with DIVVBS, DIVFDN, and DIVDFA ($p<0.001$). DIVBS showed positive correlations with DIVFDN and DIVFDA ($p<0.001$).

Table 3. Results of Pearson Correlation analysis of the variables evaluated.

	DIVVBS	DIVFDN	DIVFDA	Propionate	Butirato	Methane
Treatment	0.2200	0.3587	0.1488	-0.1168	-0.2647	-0.1320
DIVV	**0.9999***	**0.9395***	**0.8571***	0.0408	-0.0247	0.0274
DIVVBS		**0.9408***	**0.8574***	0.0375	-0.0282	0.0240
DIVFDN			**0.8686***	-0.0653	-0.1350	-0.0827

DIVFDA	0.2185	0.1255	0.1862
ACETATE	**0.9978***	**0.9745***	**0.9998***
PROPIONATE		**0.9667***	**0.9966***
BUTIRATO			**0.9778***

DIVV = True in vitro digestibility, DIVVBS = True in vitro digestibility on a dry basis, DIVFDN = In vitro digestibility of neutral detergent fibre, DIVFDA = In vitro digestibility of acid detergent fibre. * = < 0.0001.

The results also showed highly significant positive correlations of acetate with the other volatile fatty acids, propionate and butyrate, as well as with methane ($p<0.001$). Similarly, propionate showed a highly significant positive correlation with butyrate and methane ($p<0.001$). Likewise, butyrate showed a positive and highly significant correlation with methane ($p<0.001$). The production of methane by ruminants has its natural origin in the digestive process in ruminants, but it constitutes a loss of energy and contributes to greenhouse gas emissions. This is influenced by feed consumption, composition and digestibility of the components of the diet, mainly fibers. However, in the present study, no correlations were observed between the degradability evaluated and methane production. The results of the present study show that the three VFAs evaluated had a positive correlation with methane production, specifically that acetate showed the highest degree of correlation with a value of 0.9998 ($p< 0.0001$). These results show what has already been documented in this regard that the amount of propionic acid produced and especially the proportion of this with respect to that of acetic acid, is the factor with the greatest impact on the production of CH_4, especially in diets with a higher concentration of forage in which the cell walls of the fiber result in a high acetic proportion: propionic, and therefore higher CH_4 productions (Danielsson et al., 2017; Kulivand y Kafilzadeh, 2015; Williams et al., 2019).

Table 4 presents the gas production profiles at 48 h for the different concentrations of substrates evaluated in vitro of Larrea t.:alfalfa, T 1 control 100% alfalfa, T 2 at 1%, T 3 at 3% and T 4 at 5%.

Table 4.- Gas production volumes of in vitro incubations of alfalfa and Larrea t. substrates, in different proportions, ml/200 mg DM, and pH values in the medium.

Tx1 (T)	Tx2 (99-1)	Tx3 (97-3)	Tx4 (95-5)
$49.13(\pm3.11)^a$	$49(\pm0.95)^b$	$48.13(\pm1.12)^b$	$44.46(\pm0.88)^b$
7.12	7.1	7.1	7.12

Rows with different bunk beds showed significant differences ($p<0.05$).

In the results of the present study regarding the gas volumes at 48 h, differences were only observed between the alfalfa substrate 100 or treatment 1, with the other treatments added with the different levels of Larrea t inclusion. ($p<0.01$). It is known that fermentation in the rumen is mainly the result of the enzymatic activity of bacteria, protozoa and fungi that inhabit it on the substrates supplied (Liu et al., 2021). Although it has been reported that some plants are generally consumed in small quantities by livestock, and that contain bio-active compounds such as EOs (essential oils) among others, which have antimicrobial properties and that redirect methane production towards the production of VFAs mainly propionate (Seow et al., 2014). The results of this work show that gas production decreases as the percentage of Larrea t increases, as a substrate for in vitro rumen fermentation, with stoichiometric estimation of methane from the production of volatile fatty acids. On the other hand, it has been reported that the fermentation of the substrate up to propionate produces gas only from the neutralisation of the acid; therefore, lower gas production is associated with propionic fermentation (Chen et al., 2020). To corroborate these effects, it is necessary to evaluate the degradability of nutrients before and after in vitro rumen fermentation and to characterise the profiles of gases produced. Evaluation by stoichiometry of methane from the amount of VFAs also showed a lower methane production in the incubation of the substrate with the highest concentration of Larrea t. coinciding with the lowest total gas production at 48 h, as already mentioned.
Table 5 shows the results of Pearson's correlation analysis for significant variables.

Table 5.- Pearson correlation of in vitro gas production volumes and treatments.

	Gas production volume
Treatment	-0.70
	<0.01

The results of Pearson's correlation analysis show a negative correlation between the increase in Larrea's percentage in the substrate evaluated for in vitro degradability by gas production and the gas profiles produced. However, it has been reported that the disappearance of the FDN is related to gas production (Suassuna et al., 2022) and it could have been affected. Gemeda and Hassen (2014) report a correlation between in vivo OM digestibility and cumulative 24-hour in vitro gas production, i.e., incubation of conventional forages with lower gas production indicates decreased OM degradability. However, research on the effects of secondary components of plants aims to mitigate the production of methane gas in the rumen without affecting the degradability of nutrients and, more importantly, to favor the profiles of VFAs mainly. It is important to note that it has been estimated that the energy consumed that is eliminated in the form of methane varies between 2 and 15% (Broucek, 2014). According to Subepang et al., (2019) this reduces the energy efficiency of the animal, since these gases are lost through burping and do not go to productive destinations such as the formation of volatile fatty acids (VFA), this is corroborated Hammond et al. (2019), who confirm that the largest percentage of enteric methane produced is eliminated through the mouth and nose.

CONCLUSIONS

The results obtained with respect to the variables analyzed first show that the DIVV, DIVBS and DIVFDN are increased with the addition of 1 % of Larrea t., to alfalfa substrates in in vitro rumen incubations by gas production, but not when the inoculum only contains alfalfa and/or Larrea t., 3 and 5%. The results also showed that the in vitro degradability of PC is favored with the addition of Larrea t., at 1 % in alfalfa inoculums at 48 h of incubation. There are positive correlations between DIVV, DIVVBS, DIVFDN and DIVFDA. In the same way, there are positive correlations between VFAs, and these with methane production, with respect to the latter, acetic have the highest degree of correlation with methane production. The total gas production in in vitro incubations is negatively correlated with the addition of any concentration of Larrea t. in alfalfa inoculum, although there are differences between treatments of different concentrations of Larrea t., which are not significant. However, there is a positive correlation between the concentration of Larrea t. inoculums with alfalfa inoculum incubated in vitro and the volumes of gas production at 48 h.

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