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Mesquite Extract as Phytogenic Additive to Improve the Nutrition of Sheep

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Abstract

Four concentrations (0, 200, 400, 600 and 800 mg extract per ml of water) of mesquite extract were used as phytogenic additive to verify the potential to increase the nutritional value of the feed, ruminal parameters (primarily propionate production) and nitrogen use efficiency, microbial protein synthesis and quantify the reduction of ciliated protozoa and characterize the ingestive behavior of sheep. Ten adult male sheep were subjected to a 5 × 5 double Latin square design. Prior to feeding, the animals received the mesquite extract. Nutrient intake was estimated from the difference of the amount of feed provided and the total surplus. Rumen content samples were collected to evaluate the profile of short-chain fatty acids, ammonia nitrogen, pH, ciliated protozoa, turnover rate and disappearance rate. To estimate the microbial protein synthesis, the technique of purine derivatives was used. The mesquite extract quadratically increased ($P < 0.05$) the digestibility of dry matter, organic matter, crude protein and total digestible nutrients, as well as increased propionate production, acetate:propionate ratio and microbial protein synthesis. The numbers of ciliate protozoa in the rumen decreased as a result of mesquite extract inclusion in the diet. The use of mesquite pod extract at a concentration of 488 mg/mL is recommended to improve digestibility of dry matter, organic matter, crude protein and total digestible nutrients, and to optimize microbial protein synthesis and increase propionic acid production.

Keywords: bioactive compounds, natural drugs, short-chain fatty acids, small ruminants, tannins

1. Introduction

For decades, growth-promoting antibiotics known as ionophores, have been used in the diet of ruminants to promote increased production and feed conversion, and to reduce diseases. Their impacts on performance are attributed to the manipulation of microorganisms responsible for ruminal fermentation from the control of bacteria, especially those that are Gram-positive (Carvalho et al., 2017; Castillejos, Calsamiglia, Martín-Tereso, & Wijlen, 2008).

Although representing an innovation in terms of improving animal performance, the inclusion of ionophores in the diet increases feed cost and is condemned by many consumer groups, government institutions and research centers, which adopt the banning or the substitution of synthetic drugs commonly used in animal production aiming to keep the final product free from any toxicity (Nisbet, Callaway, Edrington, Anderson, & Krueger, 2009; Oskoueian, Abdullah, & Oskoueian, 2013). Due to this restriction, alternatives to ionophores have been sought. Thus, the use of plant bioactive compounds as additives in ruminant feed (phytogenic additives) becomes an alternative means for improving animal performance that is unrestricted by most markets of animal products.

Bodas et al. (2012), Durmic and Blache (2012), and Flachowsky and Lebzien (2012) reported that bioactive compounds produced through secondary metabolism in plants have the ability to affect rumen microorganisms, including protozoa, fungi and gram-positive bacteria. Therefore it is possible that these compounds can be used to optimize ruminal fermentation. Although gram-positive bacteria are very important for the fermentation of structural carbohydrates (cellulose and hemicellulose), gram-negative bacteria increase their population

proportionally, and consequently decrease the acetate:propionate ratio, while increasing the microbial protein supply to the ruminant animal; Other benefits such as energy utilization and greater dietary protein supply to the animal also exist (Durmic & Blache, 2012).

Using synthetic additives for animal production in the semi-arid region of Northeast Brazil is unfeasible, not only from the financial point of view, but also logistic. In this sense, there is an opportunity to use plants such as Mesquite (*Prosopis juliflora* (Sw.) DC.). They are rich in secondary compounds, whose mode of action on ruminal fermentation is very similar to that of synthetic additives. These compounds also have the advantage of not imparting residues into the final products of animals that consume them. As a result, the use of mesquite represents a viable alternative because it is widespread in the Brazilian semi-arid region and is therefore very well adapted to the climate. It is capable of fruiting in the dry season, producing pods that can be used as a source of secondary compounds for phytogenic additive preparation.

Mesquite (*Prosopis juliflora* (Sw.) DC.) produces compounds that can be used as manipulators of ruminal fermentation, as they exhibit antibacterial, antioxidant, antifungal, antihelminthic and antitumor activity (William & Jafri, 2015). As a result of these activities, they optimize propionate production, decrease the deamination of dietary amino acids, reduce methanogenic bacteria, increase the protein flow to the small intestine and improve digestibility of the diet. Among the secondary compounds found in mesquite, the tannins are highlighted, which despite having negative effects on nutrition, present specific antibacterial action, mainly on Gram-positive bacteria.

The condensed tannins are capable of complexing with enzymes, causing changes in the microbial metabolism (Bodas et al., 2012). They also inhibit the action of cellulolytic and proteolytic bacteria by reducing ruminal proteolysis. Because they bind with dietary proteins their degradation is slower in the rumen, due in part to the difficulty that microbes have acting upon these tannin-protein complexes (Durmic et al., 2008; Morales & Ungerfeld, 2015). Moreover, the condensed tannins bind to the membranes of protozoa causing their death, resulting in lower predation of Gram-negative bacteria and thereby better fermentation of nutrients in the rumen (Bodas et al., 2012; Patra & Saxena, 2011). Patra and Saxena (2011) reported that states that although tannins reduce the availability of nutrients, they cause changes in the partition of nutrients, leading to a greater proportion of nutrients available for microbial synthesis and a lower proportion for the production of short-chain fatty acids.

The objective of this study was to evaluate the use of mesquite extract as a phytogenic additive on intake, digestibility, ingestive activity, ruminal parameters, nitrogen use efficiency, microbial protein synthesis and ciliated protozoa in the rumen of sheep.

2. Materials and Methods

This study was carried out in strict accordance with the recommendations of the Guide of the National Council for the Control of Animal Experimentation (CONCEA). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal Rural University of Pernambuco, Pernambuco State, Brazil (approval no. 005/2014).

2.1 Experiment site, Animals and Feed

The experiment was carried out from January to March 2014, in the Academic Unit of Serra Talhada ("Unidade Acadêmica de Serra Talhada, UAST") of the Federal Rural University of Pernambuco ("Universidade Federal Rural de Pernambuco, UFRPE"; 07°59′31″S and 38°17′54″W). This location has a semiarid climate and annual rainfall of approximately 400 mm. During the experiment, rainfall was 7.28 mm and the average temperature was 30 °C (National Institute of Meteorology [INMET], 2014). Ten adult male sheep of no defined breed (five of those fitted with rumen cannula) with an average body weight of 47.6±4.89 kg were used. The experiment lasted 90 days, which were divided into five 18-day periods, of which seven days was for animal acclimation to the experimental diets and 11 for data collection. The animals were treated for internal and external parasites by administration of doramectin (DECTOMAX®) prior the beginning of the experiment. The animals were housed in individual 2 × 2 m pens, equipped with an individual feeder and water trough.

The feed was composed of 117.21 and 637.64 g/kg crude protein and total digestible nutrients, respectively, and consisted of Tifton 85 grass hay, ground corn, soybean meal, urea, ammonium sulfate and mineral mixture (Table 1). The animals were fed the complete ration twice a day (9:00 and 16:00) and the amount fed was adjusted daily according to the previous day's consumption, allowing orts of 10%.

Table 1. Proportions of ingredients in the feed

Ingredients	Proportions of ingredients in the feed (g/kg)				
Tifton85 grass hay	700				
Corn meal	180				
Soybean meal	100				
Urea + ammonium sulfate	10				
Mineral mixture*	10				
Nutrients	Ration chemical composition (g/kg)				
Dry matter (g/kg of natural matter)	958.74				
Organic matter (g/kg of dry matter)	914.67				
Mineral matter (g/kg of dry matter)	85.33				
Crude protein (g/kg of dry matter)	117.21				
Ether extract (g/kg of dry matter)	637.64				
Total carbohydrates (g/kg of dry matter)	24.46				
Neutral detergent fiber (g/kg of dry matter)	772.99				
Non-fibrous carbohydrates (g/kg of dry matter)	601.01				
Total digestible nutrients (g/kg of dry matter)	637.64				
Tannins (g/day)	Extract levels of mesquite pods (mg/mL)				
	0	200	400	600	800
Soluble condensed tannins	0.0	0.74	1.49	2.23	2.97
Protein-bound condensed tannins	0.0	4.19	8.37	12.56	16.74
Fiber-bound condensed tannins	0.0	0.15	0.31	0.46	0.61
Total condensed tannins	0.0	5.08	10.17	15.25	20.33

Note. * Composition of the mineral mixture (nutrients/kg of product): Ca = 140 g/kg; P = 70 g/kg; Mg = 1.320 mg/kg; Fe = 2.200 mg/kg; Co = 140 mg/kg; Mn = 3.690 mg/kg; Zn = 4.700 mg/kg; I = 61 mg/kg; Se = 45 mg/kg; S = 12 g/kg; Na = 148 g/kg; F = 700 mg/kg.

2.2 Experimental Treatments

The treatments consisted of 6 mL (3 mL prior to the first feeding and 3 mL prior to the second feeding) of five concentrations (0, 200, 400, 600, and 800 mg/mL water) of mesquite pod extract, provided orally with a syringe.

To obtain the aqueous extract from the mesquite pods, 20, 40, 60 and 80 g of the material (mesquite pods) were weighed on a semi-analytical balance, macerated and diluted in 100 mL of boiling distilled water at 100 °C. Subsequently, the crude extract was stored in airtight containers for 40 minutes and labeled with the concentrations of 0, 200, 400, 600 and 800 mg extract/ml of water, respectively, for obtaining the extract.

2.3 Intake Determination and Nutrient Digestibility

Dry matter intake (DMI) and other nutrients were estimated by the difference between the amount of feed provided and the total surplus. Fecal dry matter production was determined by total feces collection. In this procedure, collection bags made from unbleached cotton coated with napa were utilized. The nutrient digestibility was calculated according to the quantity of nutrient absorbed, by taking the value of excreted nutrient divided by the value of ingested nutrient.

For three consecutive days of the collection period, samples from Tifton 85 grass hay, ground corn, soybean meal, mineral mix, orts and feces were collected, which were weighed, placed in plastic bags previously identified and stored in a freezer at -20 °C. Subsequently, samples consisting of period and treatment were made. All samples were dried in a forced air oven at 55±5 °C for 72 hours and ground in a Wiley mill to pass through sieves with 1 mm diameter mesh for analysis of dry matter (DM) (method 967.03), mineral matter (MM) (method 942.05), and crude protein (CP) (method 988.05), following recommendations of the AOAC (1990). The neutral detergent fiber (NDF) was determined according to Van Soest, Robertson and Lewis (1991) using alpha-amylase as recommended by AOAC (1990). The ether extract (method 920.29) was determined using an ANKOM XT-15 Extractor, in which extraction was conducted at a temperature of 90 °C in a closed system for 60 minutes, using hexane as an organic solvent. The equations proposed by Sniffen, O'Connor, Van Soest, Fox

and Russell (1992), Hall (2000) and Weiss (1999) were used to estimate total carbohydrates, non-fibrous carbohydrates and total digestible nutrients (TDN), respectively.

2.4 Ingestive Behavior

The ingestive behavior was assessed by simultaneous observation of animals by the punctual instant sweep method every five minutes over a 24-hour period. An observer was used for each animal to increase the observation efficiency and take notes of data individually. The following activities were observed: total feeding, rumination, chewing (sum of total feeding time plus total rumination time) and idle time. With these data it was possible to determine feed efficiency and rumination efficiency:

Feed efficiency in DM = DM intake (kg/day)/feeding time (min/day);

Feed efficiency in NDF = NDF intake (kg/day)/feeding time (min/day);

Rumination efficiency in DM = DM intake (kg/day)/rumination time (min/day) and Rumination efficiency in NDF = NDF intake (kg/day)/rumination time (min/day).

2.5 Determination of the Ruminal Parameters

Rumen content samples (± 300 ml) were manually collected from four different points in the ventral region of the rumen, after the homogenization of the rumen content. The first sample was taken before feeding, at 09h00, with subsequent samples taken at the following times: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 hours after feeding. The content was filtered through four layers of cheesecloth, then the solid part was returned to the rumen, the liquid was immediately homogenized and the pH was measured by direct reading with a digital potentiometer (Handylab 1-SCHOTT).

After measuring the pH, a 20 mL aliquot was packed in a glass bottle containing one milliliter of hydrochloric acid (6 N) and stored at -20 °C for determination of ruminal short-chain fatty acids (SCFA) and ammonia nitrogen ($N\text{-}NH_3$). Ruminal SCFA (i.e., acetic, propionic and butyric acids) were determined using a gas chromatograph (GC-Master, GC Analitica, Ltda, Brazil) equipped with a 30 m corbowax 20Mfused silica capillary column. Column temperature was fixed at 150 °C for a run time of two min. Injector and detector temperatures were 250 °C and 270 °C, respectively. Gas flows were 30, 300, and 25 ml/min for He, air and H_2, respectively. Isocaproic acid was used as an internal standard. For the determination of $N\text{-}NH_3$, the samples were thawed and centrifuged at 3000 rpm for 15 minutes, according to the technique described by Fenner (1965).

The total rumen content and the ruminal fluid density were calculated by the technique of complete emptying of the rumen, before the first feeding (time zero) and four hours after. 500-g samples were collected for subsequent analysis of dry matter (DM) (method 967.03) and crude protein (CP) (method 988.05), following recommendations of the AOAC (1990), and NDF according to Van Soest et al. (1991). The turnover rate (kg/h) and the disappearance rate of rumen content (h) were calculated according to Cannas, Van Soest and Pell (2003), taking into account the DM, NDF and CP.

2.6 Nitrogen Use Efficiency and Estimation of Microbial Protein Synthesis

Total excreted urine over a 24-h period was used to determine the nitrogen use efficiency and estimate the microbial protein synthesis. Samples were collected with the help of funnels fixed in the animals, and the urine was collected in a carboy, which contained 100 ml of 40% v/v sulfuric acid (H_2SO_4). The samples were pH adjusted, when necessary, to values below three, with small drops of concentrated sulfuric acid, to prevent the bacterial destruction of purine bases in the urine and the precipitation of uric acid.

After measuring the total amount of urine, a 10-ml sample was separated, centrifuged at 2000 × g for 20 minutes at 4 °C and frozen at - 20 °C for further analysis. Urine samples were analyzed for total nitrogen by the Kjeldahl method, according to the methodology described by method 988.05 (AOAC, 1990). The nitrogen use efficiency (NE) was calculated considering the amount of nitrogen offered less the sum of nitrogen on the remains, feces and urine: NE = Nitrogen intake – (Nitrogen on the feces + Nitrogen on the urine).

The purine derivatives (*i.e.*, allantoin, xanthine, hypoxanthine and uric acid) were determined according to Chen and Gomez (1992). The amount of absorbed microbial purine (X mmol/day) corresponding to the purine derivatives excreted (Y mmol/day) was calculated according to Chen and Gomez (1992):

$$Y = 0.84X + (0.15BW^{0.75}\ e^{-0.25X}) \quad (1)$$

Where, BW is the body weight, and 0.84 the recovery of purines absorbed as purine derivatives in the urine. The microbial nitrogen supplied to the small intestine was calculated from the absorbed microbial purine (X) according to the equation of Chen and Gomez (1992):

$$\text{Microbial N (g/day)} = (70X)/(0.83 \times 0.116 \times 1000) \quad (2)$$

Where, 70 represents the content of N in the purines (mgN/mmol), and 0.83 the digestibility of microbial purines.

2.7 Count of Ciliated Protozoa in the Rumen

The count of ciliated protozoa in the rumen was performed according to the technique described by Dehority (1984). For this, four hours after feeding, 10 ml of rumen fluid filtered through cheesecloth were collected and preserved in 10 ml of 18.5% formalin. The quantification of ciliate genera was performed in a Sedgewick-Rafter chamber, according to Dehority (1984), with modifications proposed by D'agosto and Carneiro (1999).

2.8 Determination of the condensed Tannins Extraction

The extraction of the tannins of the mesquite pod was made according to Terrill, Rowan, Douglas and Barry (1992), which uses purification standards for the analysis of condensed tannins (Table 1).

2.9 Experimental Design and Statistical Analysis

The experiment was analyzed in a double balanced Latin square design. Data related to intake (n = 10), nutrient digestibility (n = 10), feeding behavior (n = 10), N balance (n = 10) and ruminal parameters (n = 5, only fistulated animals) underwent analysis of variance for a double Latin square using the GLM procedure of the SAS package, version 9.1 (SAS, 2009), with the following model:

$$Y_{ijk} = \mu + T_i + P_j + C_k + e_{ijk} \quad (3)$$

Where, Y_{ijk} is the observation, μ is the average popuation, T_i is the treatment, P_j stands for the period, C_k is the random effect of the animal, and e_{ijk} is the residual error. Data related to ruminal pH, ammonia-N and SCFA (n = 5, only fistulated animals) were analyzed as repeated measures using the SAS PROC MIXED procedure, with the following model:

$$Y_{ijk} = \mu + T_i + P_j + C_k + S_j + T_iS_j + e_{ijk} \quad (4)$$

Where, Y_{ijk} is the observation, μ is the average population, T_i is the treatment, P_j stands for the period, C_k is the random effect of the animal, S_j is the collection time, T_iS_j is the interaction between treatment and collection time, and e_{ijk} is the residual error. The comparisons between the different concentrations of the mesquite extract were conducted by decomposing the treatment sum of squares into contractions relative to the linear, quadratic and cubic effects, with subsequent adjustment of regression equations. Contrast analysis was performed on CONTRAST-encoded SAS. The carryover effects were tested on the SAS-encoded CARRY. The standard error of the mean was obtained from original data. Treatment effects were considered significant when $P < 0.05$.

3. Results

3.1 Nutrient Intake, Digestibility and Ingestive Behavior

The use of mesquite extract resulted in no significant effect ($P > 0.05$) on dry matter intake (DMI), organic matter (OM), neutral detergent fiber (NDF), non-fibrous carbohydrates (NFC), crude protein (CP) and total digestible nutrients (TDN). Additionally, there was no change in total rumination, chewing and idle time or in the feeding and rumination efficiencies (Table 2). The digestibility of dry matter, organic matter, crude protein and total digestible nutrients had a quadratic effect ($P < 0.05$) with the addition of the extract, showing maximum response when the extract was provided in the concentrations of 502, 482, 458 and 466 mg/ml. There was no effect ($P > 0.05$) on the digestibility of NDF and NFC (Table 2).

Table 2. Nutrient intake, digestibility and animal behavior of sheep fed increasing levels of mesquite pods extract

Items	Extract levels of mesquite pods (mg/mL)					S.E.M.	Ŷ	Polynomial contrasts		
	0	200	400	600	800			L	Q	C
Dry matter										
Intake (kg/day)	1.22	1.35	1.33	1.34	1.29	0.043	1.31	0.32	0.37	0.60
Digestibility (g/kg)	666	709	713	711	685	0.790	*[1]*	0.48	0.03	0.98
Organic matter										
Intake (kg/day)	1.12	1.24	1.22	1.22	1.18	0.040	1.20	0.33	0.37	0.58
Digestibility (g/kg)	685	725	729	726	700	0.756	*[2]*	0.54	0.03	0.91
Neutral detergent fiber										
Intake (kg/day)	0.69	0.78	0.76	0.77	0.75	0.026	0.75	0.30	0.37	0.33
Digestibility (g/kg)	630	651	654	656	619	1.345	642	0.86	0.30	0.62
Non-fibrous carbohydrates										
Intake (kg/day)	0.23	0.25	0.24	0.25	0.24	0.008	0.24	0.59	0.60	0.56
Digestibility (g/kg)	864	949	939	934	943	1.917	926	0.27	0.37	0.43
Crude protein										
Intake (kg/day)	0.16	0.17	0.16	0.17	0.16	0.005	16.4	0.57	0.59	0.64
Digestibility (g/kg)	730	765	787	779	738	1.077	*[3]*	0.69	0.04	0.55
Total digestible nutrientes										
Intake (kg/day)	0.79	0.86	0.91	0.91	0.86	0.035	86.6	0.20	0.27	0.69
Digestibility (g/kg)	637	677	680	677	653	0.734	*[4]*	0.53	0.03	0.92
Feeding behavior										
Rumination (min)	513	561	529	528	526	12.09	531	0.98	0.53	0.13
Feeding (min)	309	280	282	290	282	6.82	289	0.34	0.42	0.12
Chewing (min)	822	841	811	818	808	13.07	820	0.60	0.87	0.47
Idleness (min)	618	599	629	622	632	13.07	620	0.60	0.87	0.46
FEF_{DM} (g/min)	4.69	4.34	4.98	4.77	4.03	0.182	4.56	0.39	0.35	0.12
FEF_{NDF} (g/min)	2.66	2.59	2.84	2.75	2.32	0.107	2.63	0.44	0.28	0.20
REF_{DM} (g/min)	2.44	2.47	2.32	2.76	2.22	0.106	2.44	0.75	0.57	0.14
REF_{NDF} (g/min)	1.39	1.45	1.32	1.58	1.28	0.064	1.40	0.85	0.54	0.24

Note. S.E.M. = Standard error mean; FEF_{DM} = Feed efficiency in dry matter; FEF_{NDF} = Feed efficiency in neutral detergent fiber; REF_{DM} = Rumination efficiency in dry matter; REF_{NDF} = Rumination efficiency in neutral detergente fiber; L = Linear; Q = Quadratic; C=Cubic; $^{[1]}\hat{Y} = -0.0002x^2 + 0.2006x + 670.09$, $R^2 = 0.88$; $^{[2]}\hat{Y} = -0.0002x^2 + 0.1929x + 688.51$, $R^2 = 0.91$; $^{[3]}\hat{Y} = -0.0003x^2 + 0.275x + 727.8$, $R^2 = 0.98$; $^{[4]}\ \hat{Y} = -0.0002x^2 + 0.1862x + 640.83$, $R^2 = 0.88$.

3.2 Ruminal Parameters

There was no effect ($P > 0.05$) of mesquite extract on the production of short-chain fatty acids (SCFA), acetic acid, butyric acid, molar ratio of butyric acid, ruminal pH, ammonia nitrogen, rumen content, ruminal fluid density, turnover rate and disappearance rate (Table 3). Notwithstanding, the inclusion of mesquite extract in the diet of sheep caused quadratic responses ($P < 0.05$) in the production of propionic acid, molar ratio of acetic acid, molar ratio of propionic acid and acetic acid:propionic acid ratio (Table 3). The extract increased production of propionic acid with the concentration of 509 mg/ml, the lowest molar ratio of acetic acid in the concentration of 504 mg/ml, the highest molar ratio of propionic acid in the concentration of 505 mg/ml and the lowest acetate:propionate ratio in the concentration of 506 mg/ml.

Table 3. Ruminal parameters of sheep receiving mesquite pods extract

Items	Extract levels of mesquite pods (mg/mL)					S.E.M.	Ŷ	Polynomial contrasts		
	0	200	400	600	800			L	Q	C
Production										
SCFA (µm/ml)	37.83	38.5	38.48	40.8	38.85	1.02	38.88	0.64	0.75	0.58
Acetic acid	28.8	28.2	27.03	27.3	28.41	0.72	27.9	0.40	0.44	0.76
Propionic acid	6.71	8.03	9.04	9.94	8.13	0.49	*[1]*	0.02	0.01	0.16
Butyric acid	2.32	2.26	2.41	2.59	2.31	0.13	2.37	0.65	0.71	0.42
Molar ratio										
Acetic acid	76.32	73.3	70.66	67.1	73.05	1.01	*[2]*	0.01	0.03	0.14
Propionic acid	17.57	20.8	23.16	26.6	20.96	0.90	*[3]*	0.00	0.01	0.11
Butyric acid	6.10	5.86	6.17	6.25	5.98	0.24	6.07	0.88	0.89	0.59
Acetate:propionate	4.37	3.54	3.13	2.66	3.54	0.16	*[4]*	0.01	0.01	0.26
Ruminal pH	6.07	6.02	6.14	6.07	6.08	0.02	6.07	0.63	0.68	0.48
N-ammoniated (mg/dl)	5.76	5.79	6.52	5.20	6.47	0.18	5.94	0.94	0.80	0.12
Rumen contents (kg)										
Dry matter										
Before feeding	0.96	0.99	1.00	0.95	0.84	0.04	0.95	0.37	0.39	0.91
After feeding	1.36	1.17	1.22	1.38	1.39	0.05	1.30	0.47	0.26	0.32
Neutral detergent fiber										
Before feeding	0.33	0.33	0.34	0.31	0.28	0.01	0.32	0.37	0.44	0.91
After feeding	0.45	0.37	0.39	0.46	0.46	0.02	0.42	0.47	0.23	0.26
Crude protein										
Before feeding	0.12	0.12	0.12	0.12	0.10	0.01	0.11	0.45	0.59	0.93
After feeding	0.18	0.15	0.18	0.16	0.20	0.01	0.17	0.65	0.51	0.95
Density (kg/cm³)										
Before feeding	0.84	0.87	0.89	0.88	0.89	0.01	0.87	0.10	0.27	0.63
After feeding	0.93	0.90	0.91	0.93	0.92	0.01	0.92	1.00	0.46	0.15
Turnover rate										
Dry matter (%/h)	17.27	17.6	17.21	13.8	14.47	1.13	16.07	0.25	0.81	0.57
NDF (%/h)	11.12	10.3	10.36	8.34	8.53	0.76	9.74	0.19	0.99	0.80
Crude protein (%/h)	17.93	18.1	15.59	13.7	14.21	1.59	15.89	0.30	0.93	0.67
Disappearance rate										
Dry matter (%/h)	6.74	5.77	6.22	8.42	7.36	0.43	6.90	0.21	0.67	0.13
NDF (%/h)	10.80	9.88	10.53	15.2	12.44	0.89	11.77	0.18	0.96	0.16
Crude protein (%/h)	8.54	6.42	6.54	8.65	9.06	0.76	7.84	0.55	0.28	0.48

Note. S.E.M. = Standard error mean; L = Linear; Q = Quadratic; C = Cubic; SCFA = Short-chain fatty acids; NDF = Neutral detergent fiber; *[1]*$\hat{Y} = -0.00001318x^2 + 0.01342x + 6.36651$, $R^2 = 0.73$; *[2]*$\hat{Y} = 0.00003046x^2 - 0.03072x + 77.06057$, $R^2 = 0.78$; *[3]*$\hat{Y} = -0.00002993x^2 + 0.03023x + 16.93381$, $R^2 = 0.80$; *[4]*$\hat{Y} = 0.00000598x^2 - 0.00605x + 4.43879$, $R^2 = 0.91$.

3.3 Nitrogen Use Efficiency, Microbial Protein Synthesis and Ciliated Protozoa

Nitrogen use efficiency was not affected ($P > 0.05$) by the mesquite extract (Table 4). Microbial nitrogen, microbial protein synthesis, microbial protein synthesis efficiency and number of ciliated protozoa in the rumen showed quadratic behavior ($P < 0.05$) with the inclusion of mesquite extract in the diet of sheep. Microbial nitrogen, microbial protein synthesis and microbial protein synthesis efficiency had greater values in the concentrations of 483, 487 and 460 mg/ml, respectively. The ciliated protozoa in the rumen showed lower counts in the concentration of 496 mg/ml.

Table 4. Nitrogen use efficiency, microbial protein synthesis and ciliated protozoa in sheep submitted to extract intake of mesquite pods

Variables	Extract levels (mg/mL)					Ŷ	S.E.M.	Polynomial contrasts		
	0	200	400	600	800			L	Q	C
Nitrogen (g/day)										
Consumed	29.09	30.83	30.37	29.71	30.46	30.09	1.148	0.62	0.91	0.32
Digested	21.80	24.12	22.75	23.50	23.42	23.12	1.092	0.53	0.91	0.42
Excreted in feces	7.29	6.70	6.94	6.21	7.04	6.83	0.276	0.66	0.38	0.66
Excreted in urine	0.58	0.65	0.89	0.71	0.74	0.72	0.099	0.59	0.71	0.11
Retained	21.21	23.05	21.86	22.78	22.68	23.32	1.079	0.57	0.94	0.58
Retained:ingested	0.71	0.74	0.71	0.77	0.74	0.74	0.012	0.33	0.53	0.74
Microbial nitrogen	7.51	8.65	10.90	10.77	8.92	*[1]*	0.538	0.20	0.05	0.44
Microbial protein	46.93	54.07	68.13	67.30	55.78	*[2]*	3.360	0.20	0.05	0.44
MPSE (g/kg TDN)	46.28	51.91	66.94	64.39	51.75	*[3]*	3.380	0.34	0.01	0.38
Protozoa (x10^4/mL)	189.1	170.0	160.2	128.2	178.9	*[4]*	11.87	0.47	0.01	0.40

Note. S.E.M. = Standard error mean; L = Linear; Q = Quadratic; C = Cubic; *[1]*$\hat{Y} = -0.00001492x2 + 0.01441x + 7.16691$, $R^2 = 0.88$; *[2]*$\hat{Y} = -0.00009323x^2 + 0.09006x + 44.79207$, $R^2 = 0.87$; *[3]*$\hat{Y} = -0.00009665x^2 + 0.08903x + 43.83851$, $R^2 = 0.83$; *[4]*$\hat{Y} = 0.0002x^2 – 0.1987x + 194.49$, $R^2 = 0.63$.

4. Discussion

The presence of secondary plant compounds in the diet of ruminants can cause a number of disorders in the animal's metabolism, and can act directly on other body functions as the central nervous system (Ali, Tudsri, Rungmekarat, & Kaewtrakulpong, 2012; Kingori, Odero-Waitituh, & Guliye, 2011). However, the use of mesquite extract did not cause any effect on the nutrient intake and caused no changes in the behavioral patterns of the animals. This result is considered satisfactory, since the presence of secondary plant metabolites can affect the acceptability of the feed and consequently cause decreased consumption (Bonfim et al., 2012). NRC (2007) reported 1.15 kg/day of dry matter requirement for sheep with a 60 kg body weight and an average daily gain of 26 g/day. In this study, dry matter intake showed mean values of 1.31 kg/day, respectively (Table 2).

The ingestive behavior is influenced negatively by the presence of secondary plant metabolites, due to reduced palatability and digestibility of the diet. Gabbi, Moraes, Skonieski, and Viegas (2009) found changes in the behavior of heifers when fed with a phytogenic additive. Nevertheless, the way in which these secondary compounds were used in the present study was not sufficient to cause changes in the feeding behavior of sheep, which highlights the use of plant extracts as phytogenic additives.

The tannins found in the mesquite extract (Table 1) increased propionic acid production and microbial protein synthesis (Tables 3 and 4) and consequently increased flow to the small intestine. This increase in the microbial protein flow to the small intestine resulted in increased digestibility of crude protein and thus increased digestibility of dry matter, organic matter and total digestible nutrients (Table 3). The mesquite extract acted on Gram-positive bacteria, as these are more sensitive to the penetration of bioactive compounds, due to the absence of an outer membrane for protection, present only in Gram-negative bacteria (Thao, Wanapat, Kang, & Cherdthong, 2015). With the reduction of the activity of Gram-positive bacteria by the action of the extract, the growth and degradation of feed by Gram-negative bacteria was favored. These bacteria have an increased rate of multiplication when compared to the Gram-positive ones.

Jayanegara, Goel, Makkar, and Becker (2015), Bodas et al. (2012), Durmic and Blache (2012) reported that the increase of Gram-negative bacteria decrease the acetate:propionate ratio, increase the microbial protein supply to the ruminant animal, due to its multiplication speed being more accelerated, besides other benefits such as the energy and higher supply use of dietary protein to the animal.

Patra and Saxena (2011) reported that ruminal Gram-positive bacteria have a low molecular weight, becoming more susceptible to the action of tannins; consequently, the inhibitory effect of tannins on these microorganisms would be greater. Francisco et al. (2015) and Morales and Ungerfeld (2015) reported that tannins antimicrobial properties have opened the possibility of using them to manipulate ruminal microbial activity in favorable directions, *e.g.* slow down protein digestion, increase microbial protein synthesis, decrease methanogenesis, modify fatty acids biohydrogenation and prevent bloat. Condensed tannins also have the ability to complex with

enzymes, thereby causing changes in the microbial metabolism (Bodas et al., 2012; Mandal, Roy, & Patra, 2014).

The increased microbial protein synthesis was also influenced by the reduction in the number of protozoa present in the rumen (Table 4). More specifically, the condensed tannins act directly on the cell wall of protozoa, causing cell lysis, leading to cell death. With this, the number of bacteria becomes greater, as the predation by protists, especially ciliates, also decreases; consequently, the feed degradation in the rumen becomes more efficient, due to the existence of a larger number of bacteria acting directly on the feed consumed (Bodas et al., 2012). García-González, González, and López (2010) and Anantasook, Wanapat, Cherdthong, and Gunun (2013) reported that the increase of Gram-negative bacteria improves the fermentation of nutrients in the rumen, consequently causing an increase in the digestibility of dry matter and organic matter.

The reduction in the Gram-positive bacteria population (major acetate producer), caused by the use of mesquite extract, led the reduced cofactors to be oxidized in the production of propionic acid during the fermentation of carbohydrates, stimulating increased propionic acid production in the rumen, as well as increased molar ratio of propionic acid, decreased molar ratio of acetic acid and decreased acetate:propionate ratio (Table 3), increasing the retention of energy by the animal and stimulating gluconeogenesis. Beauchemin, McGinn, Martinez, and McAllister (2007) and Castro-Montoya, Makkar, and Becker (2011) reported a reduction in the production of acetic acid and an increase in the production of propionic acid in ruminants fed with condensed tannins.

Normally, the increase in propionate production is associated with the addition of a concentrate in the diet and therefore decreased ruminal pH. Nonetheless, the mesquite extract increased the production of such short-chain fatty acid without altering the ruminal pH (Table 3). The ammonia nitrogen can vary in a negative way when the tannins bind to the protein in the rumen, consequently reducing the release of ammonia nitrogen, however, in spite of the extract having condensed tannins in its constitution (Table 1), this characteristic did not alter the ammonia nitrogen content (Table 3) and did not impair the microbial protein synthesis (Table 4).

5. Conclusion

The use of mesquite extract at a concentration of 488 mg/ml is recommended to improve digestibility of dry matter, organic matter, crude protein and total digestible nutrients, in an effort to optimize microbial protein synthesis and increase propionic acid production.

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