Mesquite (*Prosopis Juliflora*) Pod Meal to Goats Feed: Ruminal Parameters and Molecular Diversity of Ruminal Bacteria and Methanogenic Archaea

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Abstract**—** *This study aimed to evaluate the effects of mesquite (Prosopis juliflora) pod meal (MPM) replacing corn in concentrate feeds on ruminal parameters and microbial diversity. MPM was used in 0.0, 33.3, 66.7 and 100% levels in isonitrogenous diets, and elephant grass (Pennisetum purpureum) silage as forage. For the experiment we divided the animals into 4x4 Latin square. The intake of dry matter, crude protein, neutral detergent fiber and acid detergent fiber were not affected by the MPM levels. The pH varied linearly, increasing according to the levels of MPM and remained at adequate range between 6.32 and 6.85 for 8 hours after feeding. The ammonia concentration showed a peak of 14.01 mmol L^{-1} 2 hours after the morning feeding and the acetate, propionate and butyrate concentrations did not show any effect. The genetic diversity of bacteria and archaea was determined by PCR-DGGE. The analyses showed variations in banding pattern, indicating changes in the populations studied as a result of the treatments and a reduction in methanogenic after the addition of up to 66.7% of MPM. MPM can be used at levels of 33.3% and 66.7% of corn replacing without reducing the nutrients intake. The reduction of archaea has a possible contribution in reducing methanogenesis, since it also reduces the acetate:propionate ratio. Mesquite is a source of food for goats in small holdings, with potential reduction in methanogenesis.*

Keywords**— ecology, microbial, multivariate analysis, PCR-DGGE, ruminal fermentation.***

I. INTRODUCTION

Frequently there is needed to search for alternative feeds as substitutes for those commonly used in the ruminants' diets. According to [1], the mesquite tree (*Prosopis juliflora*), introduced in Northeast Brazil in the 1940's to serve as animals feed during dry season, is highly palatable and productive, and its chemical composition reveals 25-28% glucose, 11-17% starch, 7-11% protein and 14-20% organic acids and pectins. These qualities render the mesquite an important species with high potential as animal feed in the semi-arid regions.

The use of mesquite in concentrate feeds for ruminants is limited due to the presence of toxins and anti-nutritional factors, such as polyphenols, nitrogenated compounds and lectin [2]. Mesquite pods have low tannins levels that are toxic to animals [3]. However, alkaloids have been isolated, such as julifloricine, which has significant antimicrobial activity, especially on Gram-positive bacteria. This effect was compared to the action of benzyl penicillin, gentamicin and trimethoprim [4, 5].

In studies by [6], some ruminal fermentation parameters were analyzed non-fistulated lactating Saanen goats fed with different levels of mesquite pod meal (MPM). Their results evidenced that the intestinal flow of microbial nitrogenous

compounds decreased linearly with the increase of MPM levels in the diets with no change to the intake of dry matter and milk production by the animals, although the assessed feed efficiency had a linear reduction.

The study of ruminal microbial diversity tries to clarify the transformations that occur in the rumen, to explain the nature of fermentation and how it affects the ruminant nutrition, as well as the fluctuations in the degree of feed supply, for example the changes in the levels of a concentrate diet. The quality and quantity of fermentation products depends on the type and activity of microorganisms that are part of the population and these, in turn, depends on the diet [7].

Therefore, our objective for this research was to assess the effect on the intake of nutrients after substituting corn for MPM in the concentrate feeds, as well as on ruminal parameters and microbial diversity of bacteria and archaea.

II. MATERIAL AND METHODS

Four crossbreds Anglo-Nubian adult goats were used for this study. They were fistulated, non-lactating and average live weight of 31 kg, confined at individual raised sheds on slatted floors measuring 1.5 x 1.0 m. The animals were distributed into a 4 x 4 Latin square for assessment of the effects of different levels of mesquite pod meal (MPM) in isonitrogenous diets, based on elephant grass silage forage at 43% of the dry matter (DM). The substitution of corn for MPM in the concentrate was the independent variable used to characterize the treatments, consisting of four levels of substitution (0, 33.3, 66.7 and 100% of natural matter) (Table 1). Sources of protein were soy meal and cotton meal.

TABLE 1
PROPORTION OF NUTRITIONAL INGREDIENTS OF CONCENTRATE FEED (g kg^{-1}).

Ingredients	Replacement level of milled corn by mesquite pod meal (%)			
	0	**33.3**	**66.7**	**100**
Mesquite pod meal	0.0	254.2	512.8	776.0
Milled corn	781.0	525.1	264.8	0.0
Soybean meal	133.0	132.4	131.9	131.3
Cottonseed meal	49.2	49.6	50.0	50.5
Mineral mixture	36.8*	38.5†	40.3‡	42.0§
Total	1000.0	1000.0	1000.0	1000.0

*Dicalcium phosphate 399.0 g kg^{-1}, Common salt 201.0 g kg^{-1}, Commercial mineral salt 400.0 g kg^{-1}. †Dicalcium phosphate 423.0 g kg^{-1}, Common salt 192.0 g kg^{-1}, Commercial mineral salt 385.0 g kg^{-1}. ‡Dicalcium phosphate 444.0 g kg^{-1}, Common salt 186.0 g kg^{-1}, Commercial mineral salt 370.0 g kg^{-1}. §Dicalcium phosphate 464.0 g kg^{-1}, Common salt 179.0 g kg^{-1}, Commercial mineral salt 357.0 g kg^{-1}.

The experiment was performed in four experimental periods, each one lasting 14 days: nine days for adaptation and the last five days to collect data. The goats had free access to food and water. They were fed at 8 am and 4 pm. The diet supplied and the leftovers of all animals were daily weighed in order to intake estimate. The leftovers were kept at around 10% of the total feed offered.

During the data collection period, from the 10th to the 14th day of each experimental period, samples of forage, concentrate feed and the leftovers of each animal were collected daily, packaged into plastic bags and stored at -20°C for further analysis and determination of the nutrients intake.

The chemical composition of the food offered (Table 2) and leftovers was determined according to methods described by [8]. For the determination of neutral detergent fibers (NDF) level we used thermostable alpha-amylase prior to extraction with neutral detergent. The total carbohydrates (TCH) levels in the feed offered and leftovers were calculated according to [9]: TCH = 100 - (Crude Protein (%) + ethereal extract (%) + ash (%)) and the levels of non-fibrous carbohydrates (NFC) were estimated by subtracting the concentrations of NDF from TCH, according to [10]: NFC = TCH – NDF (Table 2).

TABLE 2
CHEMICAL COMPOSITION OF ELEPHANT GRASS SILAGE, MESQUITE POD MEAL, CONCENTRATES AND DIETS (g kg^{-1} of DM).

	EGS*	MPM†	Concentrate			
			Replacement level of milled corn by MPM (%)			
			0	33.3	66.7	100
Dry Matter	281.0	927.5	727.0	849.0	843.0	830.0
Organic Matter	906.0	958.3	950.0	935.0	935.0	925.0
Crude Protein	40.9	78.2	135.4	134.7	134.5	133.0
Ethereal Extract	38.7	16.4	40.0	35.0	28.0	23.0
Neutral Detergent Fiber	789.0	296.5	340.0	314.0	332.0	346.0
Non-Fibrous Carbohydrates	106.4	56.72	464.6	496.3	480.5	458.0
Total Carbohydrates	895.4	86.37	804.6	810.3	812.5	804.0
Acid Detergent Fiber	488.5	24.15	133.0	141.0	148.0	191.0
Mineral Matter	25.0	4.17	20.0	20.0	25.0	40.0
			Diet			
Dry Matter			535.2	604.8	601.3	593.9
Organic Matter			931.1	922.5	922.5	916.8
Crude Protein			94.8	94.4	94.3	93.4
Ethereal Extract			39.4	36.6	32.6	29.8
Neutral detergent fiber			533.1	518.3	528.5	536.5
Non-Fibrous Carbohydrates			310.6	328.6	319.6	306.8
Total Carbohydrates			843.6	846.9	848.1	843.3
Acid Detergent Fiber			285.9	290.4	294.4	318.9
Mineral Matter			22.2	22.2	25.0	33.6

*EGS - Elephant grass silage. †MPM - Mesquite pod meal.

At the 14th day of each experimental period the ruminal liquid was collected via fistula, in periods of 0, 2, 4, 6 and 8 hours after the morning feed. Time 0 (zero) refers to the sampling that preceded the feed supply. The ruminal fluid collected from its ventral sac was filtered in four layers of gauze. The pH was measured immediately after collect. To determine the concentration of ammonia (N-NH3) and volatile fatty acids (VFA) in the rumen, the samples were fractioned and immediately acidified with sulfuric acid 20% and phosphoric acid 25% (1 mL acid: 10 mL ruminal fluid), respectively. A portion of the samples collected four hours after the morning feed was immediately frozen at -20ºC for further microbial diversity analyses. A portion of the samples were defrosted and centrifuged at 2,000 x g during 10 minutes and 2 mL of the supernatant were collected to determine the N-NH3 concentration after distillation with 5 mL of KOH 2N. The other samples were centrifuged at 800 x g for 15 minutes for determination and quantification of VFA. This experiment was carried out by gas chromatography according to the method by [11]. A gas chromatograph model GC-2010 (Shimadzu Corporation) equipped with an Rtx-Wax 30 m x 0.25 mm x 0.25 mm column was used. The temperatures used for operation of the injector, separation column and flame ionization detector were 210, 90 to 170 and 230ºC, respectively. Solutions with 20 mM of acetic, propionic and butyric acids were prepared as standard VFA solution. For each determination 1.0 µL of sample was injected and the result was obtained through an integrator that used the standard solution as the base to calculate the VFA concentrations in the sample.

Total DNA was extracted and isolated using the methodology described by [12, 13] of the rumen content collected 4 hours after morning feeding, on the 14th day of each experimental period. Briefly, the samples were submitted to a rinsing process with 1x PBS (Buffered Saline Solution). 600 µL of TESC (10 M Tris base, 1 M EDTA, 0.1 M NaCl, pH 8.3) and 30 µL of Tween 80 (Merck®) were added. The sample was submitted to a Magiclean 1600 ultrasonic bath (Unique®) for 2 minutes to promote the release of bacterial cells, due to the possibility of being adhered to organic matter. Then, the samples were again centrifuged for 3 minutes at 50 x g and the supernatant was collected and later centrifuged at 7,000 x g for 5 minutes. The sample was resuspended in 700 µL of Cell Lysis Buffer (50 mM Tris-HCl (pH 8.5) containing 500 mM NaCl) with 12 µL of proteinase K (20 mg mL^{-1}) and 12 µL of 10% SDS (Sodium Dodecyl Sulfate). The samples were incubated at 65ºC for 30 minutes.

Physical lysis was promoted by thermal shock in three cycles of 10 minutes at -80ºC and 5 minutes in a water bath at 80ºC. An equivalent volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added and the mixture was gently homogenized. The mixture was centrifuged for 10 minutes at 1800 x g and the aqueous phase recovered. DNA was precipitated by the addition of 0.7 volume of cold isopropanol and 0.1 volume of 3M sodium acetate. The solution was gently mixed and kept at -20°C. The pellet was resuspended in 50-100 µl of TE buffer (10 M Tris-HCl, 0.1 M EDTA, pH 8.0). The extracted DNA was purified on Sephadex G-200 mini columns according to [14].

For analysis of microbial diversity, the polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) were performed. Approximately 200 bp of the bacterial 16S rDNA was amplified using primers 357F (5'-CCCGGGGTACGGGAGGCAGCAG -3') and 518R (5'- ATTGCTGCTACCGCGGG-3') with a GC clamp on the 5' end of the forward primer [15]. The primers 1100F (5' - AACCGTCGACAGTCAGGYAA CGAGCGAG - 3') with the GC clamp and 1400R (5' - CGGCGAATTCGTCGTA GGAGCAGG GAC -3') described by [16] were used for identification of members of domain Archaea.

The PCR mixture for domain Bacteria consisted of 17.65 µL of sterile Milli-Q water, 1 x of Taq polymerase buffer, 2.5 mM of MgCl2, 10 pmol of each primer, 200 µM of each dNTP, 0.5 U of Platinum Taq DNA Polymerase (Invitrogen) and 2 µL of DNA (c. 20 ng) in a final volume of 25 µL. The cycling conditions were 5 min at 95°C, plus 35 cycles of 60 s at 95°C, 60 s at 55°C, and 60 s at 72°C, ending with an extension step at 72°C for 3 min. The reagents concentrations for domain Archaea in a final 25 µL reaction were 16.85 µL sterile Milli-Q water, 1 x Taq polymerase buffer, 2.1 mM of MgCl2, 280 µM of each primer (1100F with GC-clamp and 1400R), 280 µM of each dNTP and 2.5 U of Platinum Taq DNA Polymerase (Invitrogen), and 2 µL of DNA (c. 20ng). The cycling conditions were 2 min at 94ºC, plus 35 cycles of 30 s at 94ºC, 30 s at 55ºC, 90 s at 72ºC, and a final extension at 72ºC for 3 minutes. The reactions were analyzed in agarose gel at 2% (w/v), stained with ethidium bromide, and photographed on UV transillumination.

The DGGE was performed using the Mutations Analysis System CDC 20 x 20 cm (Bio-Rad). 20 µL of the PCR products were placed in 8% (w/v) polyacrylamide gel in TAE buffer (2 M Tris-base, 1 M Acetic acid, 0.5 M EDTA, pH 8.0) 0.5 mmol L^{-1} with denaturing gradient ranging between 30 and 55% for domain Bacteria and between 35 and 65% for domain Archaea. 100% denaturant is defined as 7 M urea and 40% (v/v) formamide [15]. Gels were electrophoresed at 85 V for 20 minutes and then at 200 V for 5 h at 60ºC. The gels were visualized by silver staining.

The data analysis was performed using MIXED procedure by SAS [17]. The treatment effects and the experimental times were decomposed in linear polynomial contrasts at 4th degree. Furthermore, we examined the interaction of the treatments along time using the MIXED slice option and contrasts. The lowest configuration of the Akaike information criterion (AIC) was obtained by using the variance component (VC):

$$Y_{ijk} = \mu + Tr_i + T_j + Tr_iT_j + \varepsilon_{ijk} \; ; \; NID\,(0; \sigma^2) \quad (1)$$

Where: Tr = levels of substitution (0; 33.3; 66.7 and 100%) and T = time (0; 2; 4; 6 and 8 h).

In order to choose the best equation, factors Tr and T as well as non-significant interactions in $p > 0.05$ were removed from the model. The interaction contrasts were used to compare the effect of levels of extracts and interactions along time in pH, N-NH3, VFA.

Multivariate statistical techniques were performed to evaluate the multivariate structure contained in the original data. From the DGGE profiles, binary matrixes of presence and absence of bands were performed [18]. From these matrixes we performed a cluster analysis in dendrograms for which we used the calculation of similarity among individuals and the clustering method UPGMA (average distances method) with the Raup-Crick similarity index [19] using statistical program PAST.

The Biplot analysis applied to the Principal Components (PC) was made to verify correlations between the assessed variables and to identify which had more influence from the diets. The PAST program was used for this analysis. The Microsoft Excel 2010 was used to prepare the species richness graph and the Venn diagrams, using the banding profile generated in the DGGE and considering only the levels of substitution of corn by mesquite pod meal.

III. RESULTS AND DISCUSSION

No effect was observed ($p > 0.05$) for replacement levels of corn by MPM in the intake of dry matter (DM), organic matter (OM), mineral matter (MM), Crude Protein (CP), Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) or Total

Carbohydrates (TCH) according the linear (L), quadratic (Q) and cubic (C) contrasts analysis (Table 3). However, the non-fiber carbohydrates intake (NFCI) showed a quadratic effect ($p < 0.05$) regarding levels of MPM. The maximum estimated relative NFCI was 0.382 kg day^{-1} at the level of 35.4% of corn replacement by MPM. The ether extract (EE) intake showed a linear decrease. Replacing corn by MPM also obtained a significant effect ($p < 0.0001$, with quadratic fit) on NDF intake. The data was described as a percentage of dry matter intakes (NDFI-DMI). The estimation of a minimum relative intake was 47.7% at 26.8% of corn replacement level (Table 3).

TABLE 3
AVERAGE INTAKE RESULTING FROM THE REPLACEMENT LEVELS OF CORN BY MESQUITE POD MEAL IN CONCENTRATED FEED FOR GOATS

	Levels of mesquite mod meal (%)				SEM	Effect*		
	0	33.3	66.7	100		L	Q	C
	Intake (kg day^{-1})							
Dry Matter	0.713	0.728	0.742	0.753	0.060	0.77	0.98	0.99
Organic Matter	0.667	0.673	0.685	0.690	0.056	0.84	0.99	0.97
Crude Protein	0.072	0.074	0.077	0.077	0.005	0.62	0.87	0.95
Ethereal Extract	0.028	0.028	0.027	0.026	0.002	0.73†	0.90	0.95
Mineral Matter	0.028	0.026	0.023	0.021	0.002	0.23	0.97	0.85
NDF	0.356	0.349	0.355	0.395	0.031	0.61	0.66	0.93
ADF	0.201	0.194	0.196	0.230	0.017	0.48	0.46	0.84
NFC	0.365	0.315	0.326	0.350	0.012	0.57	0.03‡	0.43
NDFI-DMI (%)	49.82	47.80	46.72	52.37	0.830	0.30	0.02§	0.36

SEM - Standard Error of Mean; NDF - Neutral Detergent Fiber; ADF - Acid Detergent Fiber; NFC - Non-Fibrous Carbohydrates; NDFI-DMI - Percentage of NDF intake in relation to DM intake.
*L - linear, Q - quadratic and C - cubic effects.
† $\hat{Y} = (0.032 \pm 0.0028)^{+} - (0.00013 \pm 0.000045)Tr^{+++}$
‡ $\hat{Y} = (0.3761 \pm 0.0072)^{+} - (2.81^{-6} \pm 1.31^{-6})Tr^{2+++}$
§ $\hat{Y} = (48.38 \pm 0.6)^{++} - (0.054 \pm 0.029)Tr^{ns} + (0.0010 \pm 0.00028)Tr^{2+++}$
$^{+}(p < 0.0001)$; $^{++}(p < 0.001)$; $^{+++}(p < 0.05)$.

The DM intake was not influenced by the diets (Table 3). This result was expected because the similarity between nutrient levels (Table 2). In another study, with unpublished data, we also observed this behavior when assessing the same levels of MPM in the concentrate supplied to lactating Saanen goats. The explanation for the reported results is that the use of MPM did not increase NDF to levels that restrict the food intake. Furthermore, isonitrogenous diets also does not affect DM intake by animals [20].

The CP intake was not affected ($p > 0.05$) when corn replaced by MPM for all replacement levels used due isonitrogenous diets used. Just as there was no significant variation in the DM intake (Table 3).

The non-fiber carbohydrates intake (NFCI) showed a quadratic effect ($p < 0.05$) regarding levels of MPM. The maximum estimated relative NFCI was 0.382 kg day^{-1} at the level of 35.4% of corn replacement by MPM. Sugars and organic acids belong to fraction 'A' of rapid ruminal degradation, according to the Cornell Net Carbohydrate and Protein System [21]. The starch represents 91.45% of non-structural carbohydrates in corn and belongs to fraction 'B1' of intermediate degradation, likewise pectin. However, the main components of MPM are mono-and oligosaccharides (28%) followed by organic acids and pectin (20%). Starch with only 11-17% is not the main energy component. [22] reported that the value of fraction A + B1 of MPM (59.92%) was lower than corn (72.20%).

The ether extract (EE) intake showed a linear decrease. This behavior is possibly due to the lower concentration of this nutrient in MPM. Other authors also observed the same behavior [6, 23].

Replacing corn by MPM obtained significant effect ($p < 0.0001$, with quadratic fit) on NDF intake. The data was described as a percentage of dry matter intake (NDFI-DMI). The estimation of a minimum relative intake was 47.7% at 26.8% of corn substitution level. This increase in ingestion is possibly due to the higher concentration of NDF in MPM when compared to corn. The maximum level of NDF in diets that does not have inhibitory effect on intake is not well defined. But, in this work, at the level of fiber ingestion, it seems that the intake control mechanism was not caused by the ruminal fill.

The principal components analysis (PCA) allowed us to resize the space of the original information into a new space formed by two latent variables named principal components 1 and 2 (PC1 and PC2). It was created by linear combinations of the original variables in the region that had the higher concentration of the original variance. These new variables arranged orthogonally generated a bidimensional distribution of variables as can be observed in Fig. 1. The biplot analysis applied to the principal components explained 97.29% (PC1 = 72.61% and PC2 = 24.68%) of the total variability observed in intake of nutrients data (Fig. 1). Fig. 1 presents the correlations between each variable and their respective principal components, indicating those variables that discriminated the most in each axis. PC1 (horizontal axis or X axis) explained 72.61% of the total variation, showing a clear discrimination of levels 66.7 and 100% of substitution of corn by MPM in relation to the others 0.0 and 33.3%.

The variables with positive correlations were responsible for the discrimination of treatments in right PC1 and those with negative correlations were responsible for the discrimination of treatments in left PC1 (Fig. 1). Therefore, variables DMI, OMI, CPI, NDFI, ADFI, TCI and NDFI-DMI, in this order, were responsible for the discrimination of levels 66.7 and 100% of corn substitution by MPM, whereas variable EEI was responsible for the discrimination of levels 0.0 and 33.3% of substitution. The variables with positive correlations were responsible for the discrimination of treatments above the zero and those with negative correlations were responsible for the discrimination of treatments below the zero in PC2 (vertical axis or Y axis). Therefore, variable CPI was responsible for the discrimination of level of substitution 66.7%, whereas variables NFCI and NDFI-DMI discriminated level 100% of substitution of corn by MPM, in this order (Fig. 1).

For lactating Saanen goats fed a similar diet, [6] did not observe a significant effect of collection time and levels of MPM substitution on rumen pH, having found a mean of 6.95. [24] Working with non-pregnant and non-lactating Alpine goats fed with diets consisting of different ratios of forage and concentrate, observed pH values of 6.5 to 6.9 in the animals that received 40 and 20% of concentrate, respectively. The authors observed that there was a quadratic decrease ($p > 0.05$) of the concentrate level in relation to pH and that it achieved its minimum point between 2 and 4 hours following each feed.

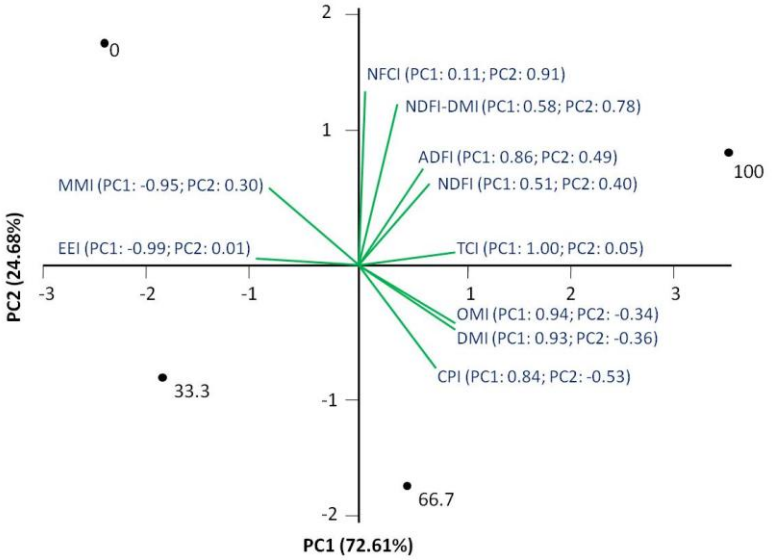

FIGURE 1: Biplot graph resulting from the Principal Components (PC) Analysis showing the distribution of treatments and nutrients intake and its correlations. DMI – dry matter intake; OMI – organic matter intake; CPI – crude protein intake; EEI –ethereal extract intake; MMI – mineral matter intake; NDFI – neutral detergent fiber intake; ADFI – acid detergent fiber intake; TCI – total carbohydrates intake; NFCI – non-fibrous carbohydrates intake; NDFI-DMI -percentage of NDF intake in relation to DM intake.

According to [25], this is due to the higher rate of volatile fatty acid production from the fermentation of the fibrous fraction of the feed. However, in the present study the rumen pH showed minimum estimated values for treatments 0; 33.3; 66.7; and 100% of MPM of 6.33, 6.34, 6.46 and 6.52, respectively, at 2.1 h and maximum values of 6.68, 6.74, 6.80 and 6.86 between 6.5 at 7 h after feeding (Fig. 2). The high pH value observed for treatment 100%, almost 7 h after ingestion, owes to the rumination time, which increased linearly between diets. The rumination increases both the superficial area and the rate of fermentation of the feed. It also increases the flow of saliva, which keeps the pH favorable for microorganisms and animals [7].

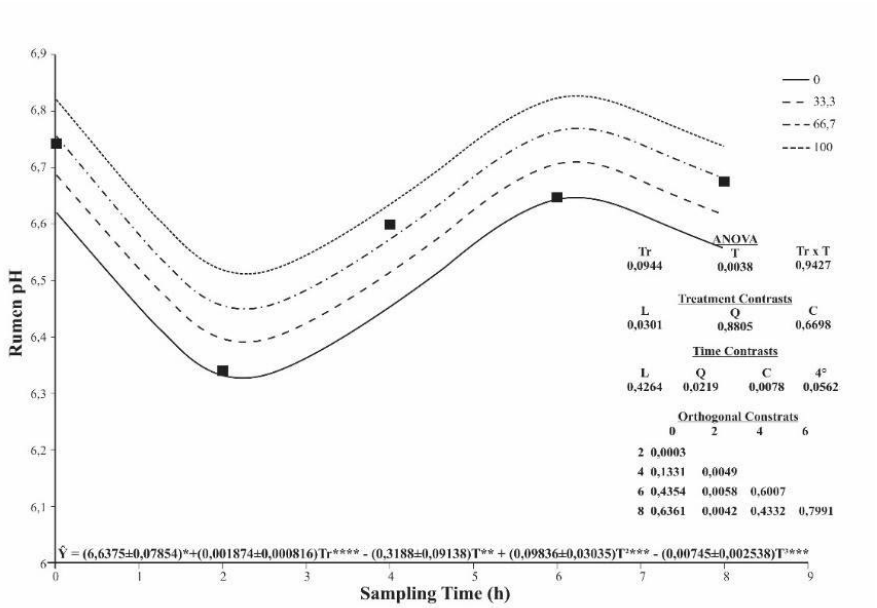

FIGURE 2: Ruminal pH estimative in non-lactating fistulated goats, fed a concentrate containing mesquite pod meal as substitute for corn in different levels. Lines correspond to replacement levels (%) of mesquite pod meal. *(p < 0.0001); **(p < 0.001); *(p < 0.01); ****(p < 0.05).**

The concentration of N-NH3 showed a quartic behavior ($p < 0.05$) as result of sampling times. There was no influence of the diets on the concentration of N-NH3, with means of 7.95, 7.68, 8.88 and 8.36 mmol L^{-1} for treatments 0, 33.3, 66.7 and 100% of MPM as substitute for corn. A peak of N-NH3 production was observed 2 h after feeding (Fig. 3). The values of N-NH3, for all levels of MPM, were higher than 5 mmol L^{-1} of rumen liquid, which is the minimum level necessary to keep the ruminal normal function [26]. However, the level of ammonia should be higher than 10 mmol L^{-1} to increasing ruminal dry matter digestion, and higher than 20 mmol L^{-1} to increasing dry matter intake [27]. The concentration of ruminal ammonia usually varies according to the time elapsed since feeding, the sampling spot in the rumen, the balance between protein and energy in the diet, the solubility, and the level of protein in the feed [28].

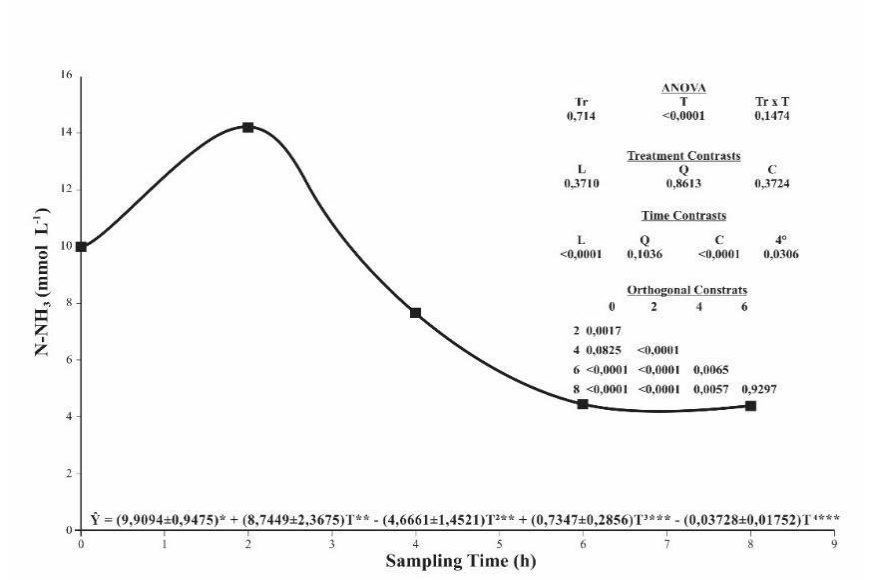

FIGURE 3: Concentration of N-NH_3 in fistulated, non-lactating goats, fed concentrate containing mesquite pod meal as substitute for corn. *(p < 0.0001); **(p < 0.001); *(p < 0.05).**

The relative concentrations of acetate, propionate and butyrate (Table 4) indicate that diets "with added" MPM resulted in higher values of acetate in relation to propionate. The concentrations of total or individual volatile fatty acids (VFA) in the rumen are highly variable and depend on feed frequency, time after feeding and diet composition. In the present work, we obtained a relative proportion of VFA produced of 69:20:11 (mols of acetate:propionate:butyrate). This proportion is near to

65:15:10 for diets with high proportions of forage [29]. There was significant cubic effect of replacement levels by MPM on acetate:propionate ratio, being the lowest ratio found for the replacement level of 33.3%. This report corroborates the hypothesis of that mesquite use reduces the acetate:propionate ratio in ruminal liquid.

TABLE 4
LEAST SQUARE MEANS, MEAN STANDARD ERROR AND SIGNIFICANCE INDICATORS FOR THE EFFECTS OF CONTRASTS OF THE CONCENTRATIONS OF ACETATE, PROPIONATE AND BUTYRATE IN GOATS FED CONCENTRATE CONTAINING MESQUITE POD MEAL AS SUBSTITUTE FOR CORN.

Item	Levels of Mesquite pod meal (%NM)				SEM	Effect♦		
	0	33.3	66.7	100		L	Q	C
Acetate (mM)	17.96	13.43	17.54	14.84	1.45	0.70	0.77	0.29
Propionate (mM)	4.52	4.66	5.27	4.35	0.40	0.98	0.55	0.61
Butyrate (mM)	2.40	2.69	2.60	2.55	0.24	0.85	0.76	0.89
Acetate:Propionate (mM)	4.04	2.85	3.28	3.47	0.18	0.52	0.06	0.05[1]

♦L, Q and C – linear, quadratic and cubic effect.
[1]$\hat{Y} = 4.0418^{*} - 0.07894Tr^{***} + 0.0016Tr^{2***} - 8.43^{-6}Tr^{3***}$
*significant ($p < 0.0001$); **significant ($p < 0.001$); ***significant ($p < 0.05$).

The sum of the two initial components shows the 86.57% of data variation for fermentation parameters, showing that the horizontal axis (PC1) is the most important for the results interpretation, since it cluster the most data variation as show in Fig. 4. There was a shift of treatment 0% to the negative part of the graph in PC1, indicating that this treatment shows differences in relation to the others. PC1 shows a separation of treatments with MPM (33.3, 66.7 and 100%) and without MPM (0%) as replacement of corn. The variables with positive correlations that most influenced the discrimination of treatments to the right of the graph in PC1 were, in this order Butyrate, pH, N-NH_3 and Propionate. Those with negative correlations and responsible for the discrimination of treatment 0% to the left of PC1 were acetate:propionate and acetate, in this order (Fig. 4). The treatment with 66.7% of corn replacing by MPM appears distant from the other treatments, shifting positively along the Y axis, with the other levels of substitution (0, 33.3 and 100%) shifting in the opposite direction. PC2 was responsible for 33.2% of data variation, especially for the shift in treatment 66.7% of MPM, influenced by variables with positive correlation, Propionate, Acetate and N-NH_3, in this order.

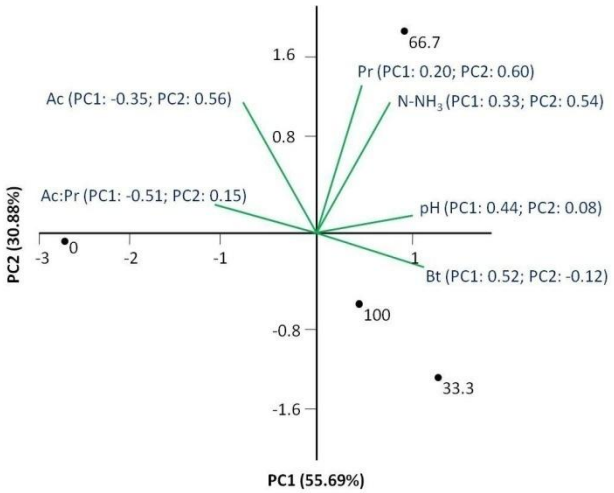

FIGURE 4: Biplot graph resulting from the Principal Components (PC) Analysis showing the distribution of treatments and nutrients intake and its correlations. N-NH3 – ammoniacal nitrogen; Ac – acetate; Pr – propionate; Bt – butyrate; A:P – acetate: propionate ratio.

DGGE is normally used to determine the number or differences between genus and species of microorganisms present in the sample. In the present work, it was possible to observe differences between the patterns of PCR products in DGGE banding, both for domain Bacteria and Archaea demonstrated by the different number of bands, which possibly indicate different genus or species. The DGGE banding patterns obtained were used to analysis of microorganism community structure. The microbial diversity was estimated by cluster analysis based on a data matrix of bands presence and absence verified in each sample (Fig. 5 and 6).

In the Raup-Crick dendrogram (Fig. 5A) generated from DGGE considering the replacement levels of corn by mesquite pod meal (0, 33.3, 66.7 and 100%) shows that the point of union of all groups has similarity of approximately 50%. The high similarity (88%) was observed between treatments 0 and 33.3% of MPM which can be explained due the similarity between both ruminal environments. The other treatments showed a lower similarity rate. The Venn diagram clearly demonstrates the clustering of bands (supposed species) that are shared or that are specific to each "sampled group" (Fig. 5B). It is observed that treatments 0, 33.3%, 66.7 and 100% share the same number of bands (3 bands), demonstrating possible similarity in terms of species distribution.

Diet components and changes to ingestive behavior can cause changes to gastro-intestinal microbial ecology, which plays a fundamental role on the animal's health and productivity. With the inclusion of MPM in the diets of crossbred Anglo-Nubian goats, an increase in NDF was observed in the diets because of the increase in substitution levels (51.83; 52.85 and 53.65%). Although there was no evidence of this increase in NDF intake, it is suggested that increments to the level of NDF in the diet increased time spent chewing (TSC), leading to high rumen pH because of the higher flow of saliva to the rumen, and high flow of buffering substances.

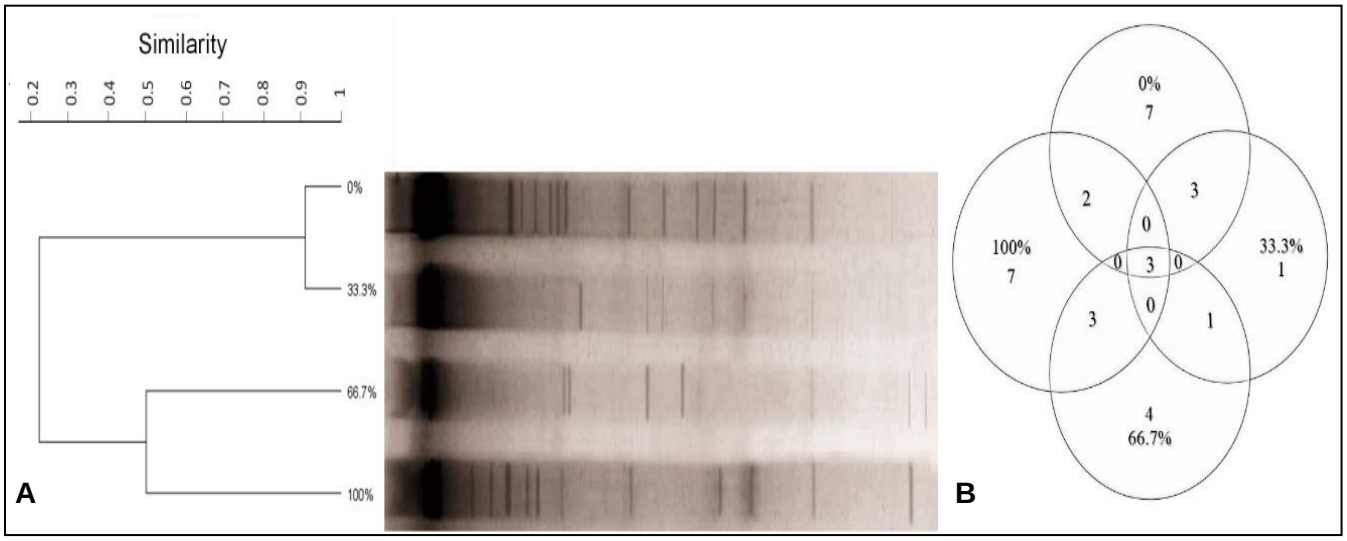

FIGURE 5: Assessment of Bacteria ruminal communities in four levels of corn replacement by mesquite pod meal (0, 33.3, 66.7 and 100%) determined by PCR-DGGE. (A) Raup-Crick dendrogram generated from PCR-DGGE analyses of fragments amplified with specific Bacteria primers (V3 region from 16S rRNA). (B) Venn diagram generated from the DGGE banding profile indicating numbers of shared bands across different treatments.

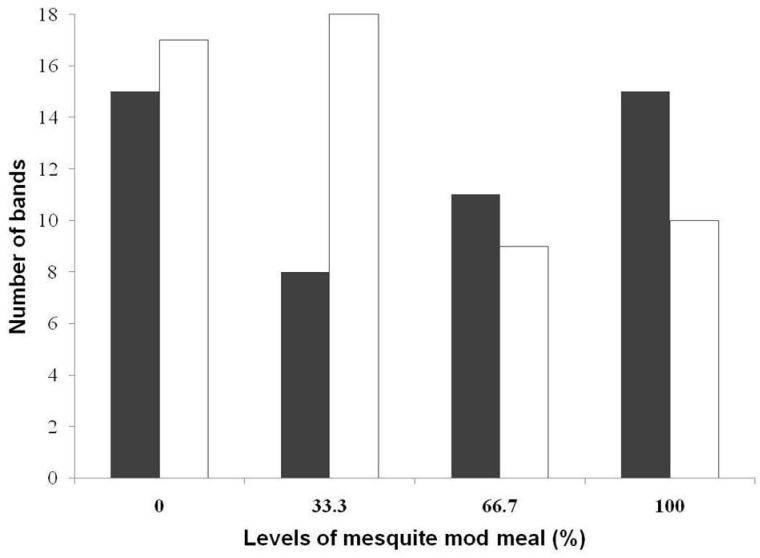

FIGURE 6: Richness of bands for Bacteria (■) and Archaea (□) considering the levels of corn substitution by mesquite pod meal.

Ruminal pH is entirely related with the final products of fermentation, as well as with the growth rate of rumen microorganisms [30]. Thus, pH is the main environmental factor in the distribution of microorganisms, playing a regulating role in the definition of their environment and diversity. It is positively correlated with ammoniacal nitrogen and acetate:propionate ratio. This reflected clearly on the richness of bands for bacteria, where it is possible observe that treatment with 33.3% of MPM, which has the lowest pH value 4 h after feeding, is also the one that shows the lowest diversity of bacteria (Fig. 7) and the lowest number of rare bands (Fig. 5).

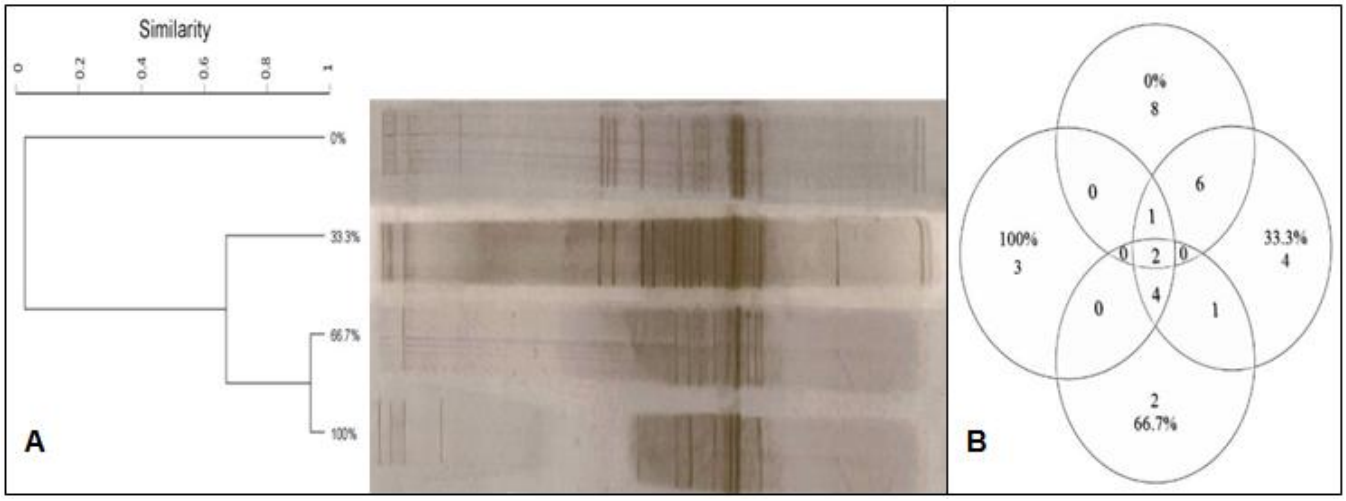

FIGURE 7: Assessment of Archaea ruminal communities in four levels of corn replacement by mesquite pod meal (0, 33.3, 66.7 and 100%) determined by PCR-DGGE. (A) Raup-Crick dendrogram generated from PCR-DGGE analyses of fragments amplified with specific Archaea primers (1100 and 1400 from 16S rRNA). (B) Venn diagram generated from the DGGE banding profile indicating numbers of shared bands across different treatments.

As done to domain Bacteria, a Raup-Crick dendrogram and Venn diagram were generated for domain Archaea from DGGE. The point of union of all groups showed low similarity around 0.08% (Fig. 6). We can see that there was more similarity between levels 66.7 and 100% of MPM (96.2%) than other treatments. This observation is clearly confirmed by Biplot analyses applied to the Principal Components, discussed earlier, that evidenced the same variables exerting influence on the ruminal environment. This influence can have reflected on the richness of archaea species viewed on DGGE banding profile, generating a lower diversity of species for these two substitution levels (Fig. 6 and 7).

The diet supplied with 66.7 and 100% of corn replacement by MPM were the treatments that showed the least number of methanogenic Archaea. This can have occurred due to a reduction of protozoa which has led to a reduction in methanogens associated with these microorganisms, either by adherence or endosymbiosis [31, 32]. These also were the treatments for which we observed the highest levels of $N\text{-}NH_3$ (10.24 and 8.32 mmol L^{-1}, respectively) 4 h after feeding, still exist synergy between levels of acidity (pH). Through them it is possible to increase microbial efficiency, biomass production and degradation of fiber in the feed, thus obtaining increased of feed intake. Such changes to the ruminal environment were sufficient to reduce the population of methanogenic Archaea, also suggesting a hydrogen deviation in the medium towards the production of propionate, as visualized in the treatment that received 66.7% of MPM (Table 4).

IV. CONCLUSION

The use of mesquite pod meal at levels of 33.3% and 66.7% as a replacement for corn does not reduce nutrient intake but alters the bacterial population in the rumen of crossbred goats and decreases the population of archaea present in the fluid. This replacement reduces methanogenesis, contributing to production efficiency and reducing methane emissions into the environment.

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