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PHYSICAL-CHEMICAL AND MICROBIOLOGICAL PARAMETERS OF RUMEN FLUID OF CONFINED SHEEP SUBMITTED TO INCREASING LEVELS OF SUPPLEMENTATION

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ABSTRACT

The objective of this study was to evaluate rumen microbiota of four adult male sheep, castrated, with rumen cannula, alloted in a randomized split-plot block design. The animals were fed *Brachiaria decumbens* hay diet, with increasing levels (0.5, 1.0 and 2.0 g/kg BW) of supplement with maize, soybean meal, urea and mineral mixture. The rumen fluid samples were collected from animals during fasting and at 2, 4 and 6h after feed, and analyzed immediately after the collection, for the parameters pH, methylene-blue reduction, cellulose digestion, activity and counting of ciliate protozoa (cel/mL). The pH values lower and higher than 7.0 were found during fasting (0h) and 2h after feed, respectively. The population of ciliated protozoa and its activity were higher at the highest supplementation level ($P<0.05$). At 0.5 g/kg BW level of supplementation, the population of protozoa decreased with the increase of pH, and greater number of protozoa /mL in rumen fluid was observed during fasting. Cellulose digestion and methylene-blue reduction were not affected by the treatments or after-feed hours ($P<0.05$). It was concluded that, during fasting, the increasing levels of supplementation affected positively the population of ciliated protozoa in the rumen but did not influence the bacterial activity.

KEYWORDS: feeding; infusorians; pH; rumen; sheep.

PARÂMETROS FÍSICO-QUÍMICOS E MICROBIOLÓGICOS DO FLUIDO RUMINAL DE OVINOS CONFINADOS SUBMETIDOS A CRESCENTES NÍVEIS DE MISTURA MINERAL ENERGÉTICO-PROTÉICA

RESUMO

Com objetivo de avaliar a microbiota ruminal de ovinos confinados submetidos a diferentes níveis de suplementação, quatro animais machos adultos, castrados, dotados de cânula ruminal, foram distribuídos em um delineamento de blocos casualizados num esquema de parcelas subdivididas. Foram alimentados com feno de braquiária e níveis crescentes (0,5; 1,0 e 2,0 g/kg de PV) de uma mistura à base de milho, farelo de soja, uréia e

núcleo mineral. Procedeu-se a colheita de amostras de fluido ruminal nos tempos 0 (jejum), 2, 4 e 6h após alimentação, sendo analisados: pH, redução do azul de metileno, digestão de celulose, atividade e contagem de protozoários ciliados (cel/mL). Valores de pH menores que 7,0 foram obtidos no jejum (0h) e os maiores, 2h após alimentação. A população de protozoários ciliados e sua atividade foram superiores no nível mais alto de suplementação (P<0,05). No nível de suplementação 0,5 g/kg de PV, a população de protozoários reduziu-se com aumento do pH, registrando no jejum maior número de protozoários por mL de fluido ruminal. Os parâmetros digestão de celulose e tempo de redução do azul de metileno não foram influenciadas pelos tratamentos ou tempo após a alimentação (P>0,05). Concluiu-se que, no jejum, os níveis crescentes de suplementação influenciaram positivamente a população de protozoários ciliados do rúmen, mas não influenciaram a atividade bacteriana.

PALAVRAS-CHAVE: alimentação; carneiros; infusórios; pH; rúmen.

INTRODUCTION

Rumen environment allows the continuous development of microbial population, acting as a fermentation chamber, due to the following factors: average ideal temperature of 39°C; anaerobiosis; buffer mean pH 6.8; presence of bacteria, protozoa and fungi; nutrient supplement and continuous removal of digesta and fermentation products; dry matter between 10 and 15% and constant osmotic pressure (LANA, 2005).

Bacteria and ciliated protozoa present in the rumen are responsible for digestion of 70% to 85% of the digestible dry matter of the ration. These microorganisms are adapted to grow in the absence of oxygen at a temperature ranging from 39°C to 40°C, and, from food digestion process, they produce volatile fatty acids, which serve as an energy source, besides carbon dioxide, methane and ammonia. Moreover, such microorganisms are a source of natural protein for ruminants. The microbial protein synthesis in the rumen depends on the growth of microorganisms and on the efficiency of using energy and nitrogen substrates, which is the main constituent of animal's body and, therefore, vital to maintenance, growth and reproduction processes (SILVA et al. 2002; OLIVEIRA et al., 2007).

It is estimated that the ciliated protozoa, which constitute the majority of protozoa in the rumen, account for about 2% of rumen content weight, 40% of microbial nitrogen and provide 60% of the microbial fermentation products in this organ (YOKOYAMA & JOHNSON 1988). The pH of the rumen content is a determining factor in the concentration and composition of bacteria and protozoa population (FRANZOLIN et al. 2000; KAMRA, 2005). Therefore, the excess or the deficiency of food substrates causes an imbalance in microbial population and rumen. These nutritional abnormalities may lead to extremely fast changes, lacking time for microbial adaptation. Consequently, there is an excessive growth of certain types of bacteria, with the overproduction of certain final icrobial digestion products, an insufficient substrate degradation and lack of microflora activity. The effects such abnormalities on the animal range from rumen dysfunctional motility, impaired growth and performance, to total toxicity and injury in different organisms (DIRSEN, 1993; FRAZOLIN et al., 2000).

According to BORGES et al. (2002) and COSTA et al. (2008), rumen fluid analysis has unquestionable value in the diagnosis of diseases related to the digestive tract of ruminants, especially those of pre-gastric compartments, because rumen microbiota is highly sensitive to external and internal changes, which the animals are routinely submitted to. Rumen manipulation, considering the multiple interrelationships between diet and microorganisms, has been studied aiming at finding ways to improve the efficiency of production systems (PEREIRA et *al.*2000; KAMRA, 2005; VIEIRA et al., 2007; SOUZA et al. 2007; MATOS et al., 2008). According to DONATO et al. (1999), rumen contents may be odserved as for the physical aspects (color, odor, consistency, sedimentation and flotation time) and the chemical characteristics (pH, glucose fermentation, nitrite reduction and methylene blue reduction test). The biological parameters should be included in the assessment of bacteria and protozoa.

This paper aimed at studying the physicochemical and microbiological changes in rumen fluid of the pre-gastric compartment in feedlot sheep submitted to *Brachiaria decumbens hay* diet and supplemented with increasing levels of mineral-protein-energy mixture.

MATERIAL AND METHODS

This study was conducted at the Veterinary and Animal Science School, of Universidade Federal

de Goiás, in three consecutive periods of 17 days, being 16 days for diet adaptation and one day for rumen fluid collection. Four adult castrated sheep with rumen cannula were used. The animals were distributed in a randomized block design in split plots, in which treatments were the plots and collection time the subplots.

The animals were treated with heavy endoparasiticides and kept in individual stalls equipped with feeder and drinker, and were fed with *Brachiaria decumbens* hay supplemented with mineral-protein-energy mixture. Supplements, which constituted the treatments, were prepared with corn, soybean meal, urea and mineral core, given in amounts of 0.5, 1.0 and 2.0 g / kg body weight, which provided similar levels of minerals and non-protein nitrogen, but different levels of true protein and energy (Table 1).

Table 1. Centesimal and chemical composition of *Brachiaria decumbens* hay and of the energy protein mixtures which constituted the treatments, offered to confined, cannulated sheep, used to determine the physical-chemical and microbiological parameters of rumen fluid

Ingredients	Supplement 1	Supplement 2	Supplement 3	Hay
(%)	(0.5 g / kg BW)	(1.0 g / kg BW)	(2.0 g / kg BW)	
Corn	3. 50	46.177	69.98	-
Soybean meal	3.29	7.712	6.96	-
Mineral Core	62.35	31.111	15.56	-
Urea	30.00	15.000	7.50	-
Sulfur	0.87	-	-	-
DM	90.00	90.00	90.00	80.00
CP (%)	86.06	49.32	29.80	8. 22
DM (kcal / kg) *	160.52	1439.48	2091.61	1645.61
TDN (%) *	5.40	43.41	62.11	45.61
NDF (%)	-	-	-	65.35
ADF (%)	-	-	-	46.23

* Calculated values

We used a vacuum pump for rumen fluid collection through the implanted cannula, resulting in individual samples of approximately 200 mL. Samples were collected on the 17th day after the beginning of each experimental period, at times 0h (fasting), 2, 4 and 6 hours post-feeding, and were immediately filtered and analyzed. Four samples per day of collection were obtained from each animal, totaling 12 samples per animal. The following parameters were analyzed: pH (measured by potentiometer); organoleptic tests (color, odor, and consistency); methylene blue reduction test (MBRT); protozoa density, motility and viability; cellulose digestion; morphological and staining evaluation of bacteria by Gram's method; count (cells/mL of rumen fluid) and classification of small, medium and large infusorians, according to the methodology described by DEHORITY (1977).

Data were interpreted to determine the analysis of variance and means were compared by Tukey test at 5% significance level. Some variables were expressed as absolute values and percentages and others by means of descriptive statistics (SAMPAIO, 2007).

RESULTS AND DISCUSSION

The organoleptic tests in the rumen fluid of the pre-gastric compartment of feedlot sheep submitted to basic *Brachiaria decumbens* hay diet and supplemented with increasing levels of mineral-energy-protein mixture revealed similarities among the samples: the color ranged from shades of green, the predominant odor was aromatic and consistency was slightly viscous (Table 2). Therefore, these findings are within normal ranges and are supported by reports by DIRKSEN (1993).

Table 2. Physical aspects of the rumen fluid of cannulated sheep in feedlot receiving different levels of mineral protein-energy mixture (0.5, 1.0 and 2.0 g of mixture / kg of BW)

Treatment / Time		Variables		
		Color	Odor	Consistency
T1	0h	Yellowish green	Slightly foul	Viscous
	2h	Yellowish green	Aromatic	Slightly viscous
	4h	Brownish green	Aromatic	Slightly viscous
	6h	Olive Green	Aromatic	Slightly viscous
T2	0h	Brownish green	Slightly foul	Viscous
	2h	Brownish green	Aromatic	Slightly viscous
	4h	Brownish green	Aromatic	Slightly viscous
	6h	Brownish green	Aromatic	Slightly viscous
T3	0h	Brownish green	Slightly foul	Viscous
	2h	Brownish green	Slightly foul	Slightly viscous
	4h	Brownish green	Aromatic	Slightly viscous
	6h	Olive Green	Aromatic	Slightly viscous

VIEIRA et al. (2007) studied the rumen fluid of sheep reared on *Brachiaria decumbens* pasture and verified that in the dry season the predominant color of the fluid was brown, and in the rainy season it was olive green, being the fluid collection carried out from 4 to 6 hours after morning feeding. Also according to these authors, the smell was aromatic, being more accentuated during the rainy season, and the consistency was slightly viscous in both seasons, though more intensified during the rainy season. The similarities between the results found by VIEIRA et al. (2007), which partly corroborate the findings of this study, are understandable because of the roughage-based feeding, and the differences in color are explained by the concentrate supply.

The pH remained close to neutrality (6.74 to 7.40) with no differences among treatments (P> 0.05), as shown in Table 3. HOFIREK & HAAS (2001), comparing two methods of rumen fluid collection, by oroesophagic tube and percutaneous punction, found higher pH values when using the first method and related this finding to the fact that the oral tube can only reach the cranial sac of the rumen, richer in saliva and buffering solutions. This change was not observed in this study, probably because the collection was made in cannulated animals. There was no difference (P> 0.05) among treatments after feeding, regarding cellulose digestion and methylene blue reduction tests; however, a larger methylene blue reduction time was observed at the lowest supplemetation level, in the most acid pH (fasting), and it decreased at the alkalinity peak (17 to 7 minutes), which occurred about two hours after feeding. The moment of greatest acidity affected negatively the bacterial activity according to the findings by FRANZOLIN et al. (2000).

SOUZA et al. (2007) also observed no differences in mean pH of rumen fluid, at times 0, 2, 8 and 10 hours after feeding, in buffaloes submitted to increasing levels of phosphorus (8, 12, 15 and 18 g/day/animal , keeping the forage:concentrate ratio at 85:15) added to the total sugar cane-based diet.

Although the rumen pH values observed remained close to neutrality, with no differences among treatments (P> 0.05), at the lowest level of supplementation (0.5 g/kg BW), at time 0h (fasting) and at 2h the most acid and the most alkaline pH (P <0.05) were verified, coinciding with the increased protozoa viability, which remained stable up to six hours post-feeding (Table 3). At the highest level of supplementation (2.0 g/kg BW), although there were no differences among collection times (P> 0.05), at the time of fasting, this level showed better percentage (%) of viable protozoa (P <0.05) than the other treatments.

Table 3. Influence of the levels of mineral protein-energy mixture (0.5, 1.0 and 2.0 g of the mixture / kg of BW) and time of collection of rumen fluid on the physical, chemical and microbiological aspects of the rumen fluid of confined sheep

Level of supplementation (g / kg body weight)	Collection Time			
	0h	2h	4h	6h
		pH		
0.5	6.74^{B}	7.23^{A}	7.02^{AB}	6.78^{B}
1.0	6.80	7.17	7.09	7.03
2.0	7.00	7.40	7.40	7.32
		Viable protozoa (%)		
0.5	77.50^{Bb}	98.80^{A}	99.50^{A}	99.80^{A}
1.0	77.50^{Bb}	86.20^{A}	91.80^{A}	93.80^{A}
2.0	92.80^{a}	96.00	98.20	94.50
		Time to digest the cellulose in hours		
0.5	48.50	45.00	47.00	47.00
1.0	44.80	43.00	41.00	41.80
2.0	54.00	59.50	48.50	51.00
		Time for methylene blue reduction in minutes		
0.5	17.98^{Aa}	7.40^{B}	7.50^{B}	6.80^{B}
1.0	8.50^{b}	5.98	7.80	6.50
2.0	11.20^{b}	11.70	10.20	12.60
		Average number of protozoa per ml rumen fluid		
0.5	17968.80^{c}	10937.50^{b}	20312.50^{b}	46093.80
1.0	146093.80^{b}	42968.80^{b}	76562.50^{ba}	48437.50
2.0	375781.20^{Aa}	267968.80^{Ba}	128906.20^{Ca}	101562.50^{C}
		Small Infusorian (%)		
0.5	92.50	86.20	86.20	90.50
1.0	99.20	88.20	92.00	90.00
2.0	98.00	92.80	93.50	92.00
		Medium Infusorian (%)		
0.5	6.20	12.80^{a}	12.00^{a}	7.00
1.0	1.20	7.80^{ab}	5.20^{b}	7.20
2.0	1.80	3.80^{b}	4.00^{b}	3.80
		Large Infusorian (%)		
0.5	2.00	2.50	2.00	3.20
1.0	1.20	4.00	3.00	3.50
2.0	1.20	3.50	3.00	4.20

* Means in the same column followed by same lowercase letters and in the same line followed by same capital letters do not differ by Tukey test (P <0.05).

Uniformity in 100% of the samples was observed by Gram test, with a predominance of Gram-negative cocci and bacillus, regardless of the level of supplementation. Some of the common characteristics of bacteria found in the rumen of animals consuming diets with high-forage rates are the following (KAMRA, 2005): most bacteria are Gram-negative; the number of Gram-positive bacteria tends to increase with increasing enegy levels in the diet; and great pH growth between 6.0 and 6.9. UNDERWOOD (1992) reported that in cases of acute rumen acidosis, severe pH reductions, decrease or not in protozoa activity, predominance of Gram-positive bacteria over Gram-negative bacteria and decrease in sedimentation and flotation time may be noted. However, in this study, pH remained close to neutrality, and the patterns found in the morphological and bacteriological examination by the rumen fluid smear stained by Gram's method, were in agreement with the descriptions by DISKSEN (1993) for grazing cattle.

The bacteriological profile found for the sheep in this study, although the animals were confined and fed *Brachiaria decumbens hay*, is similar to what was described for goats reared on pasture, in a study conducted by SILVA et *al.* (1994). The authors reported average pH value around 7.0, and the microbiological control of rumen fluid revealed the predominance of Gram-negative microorganismas over Gram-positive ones in 100% of the samples, and the presence of isolated cocci, streptococci, rods, sarcina, rosettes and bacilli. Time

for cellulose digestion and methylene blue reduction ranged, respectively, from 41 to 59.5 hours and 6 to 12 minutes. In these authors´s study on goats, methylene blue reduction time was 6.21 ± 0.65 minutes and cellulose digestion occurred at 50.84 ± 2.58 hours, similar to those values observed in this study using confined sheep.

The population of ciliated protozoa was higher at the highest level of supplementation (P <0.05), at the order of 100,000, while it was in the order of 10,000 (cells/mL) in the other treatments. Small protozoa prevailed over medium and large ones, showing, in most cases, frequence lower than 90%, suggesting the predominance of the *Entodinium genus.* The predominance of small protozoa was also reported by FRANZOLIN et al. (2000). The large size of protozoa may affect their flow in the rumen, because the smallest species are probably more resistant to rumen fermentation. According to LENG (1982), this aspect involves the low use of microbial protein produced by the host protozoan, due to the lower flow to the inferior portions of the gastrointestinal tract.

In the present study, the results related to protozoa population corroborate the observations by MATOS et al. (2008), who studied the population of protozoa in the rumen of sheep reared on pasture of native "caatinga" of Pernambuco, and found predominance of *Entodinium* around 90%. According to these autors, there was significant difference in the total number and average concentration of protozoa of the genus *Entodinium*, regarding the time of collection of rumen samples, being higher before feeding, similar to that observed in this study.

MARTINELE et al. (2008) did not find any significant difference in the total number of ciliated protozoa in the rumen of cows fed elephant-grass and two inmcreasing levels of concentrate (only elephant-grass; 80% elephant-grass and 20% concentrate; and 60% elephant-grass and 40% concentrate). In such study, the genus *Entodinium* was predominant in the three diets in 73.5%, 76.9% and 72.2% of the total of ciliated protozoa, respectively, and the levels of 20 and 40% of concentrate (based on corn grain, cotton and wheat bran and urea) in the diet also produced no effects on the rumen fluid pH.

The largest number of protozoa per mL of rumen fluid was withdrawn at the time of fasting in 100% of treatments, and the population grew significantly with the increase of the supplement provided (P <0.05). However, the population tended to decrease as time passed after feeding, which was observed at the highest level of supplementation. FRANZOLIN NETO et al. (1991) found higher levels of ciliated protozoa at the time of feeding, decreasing to 12 hours after feeding, but it increased subsequently until the point immediately before to the offer of the diet. As it can be observed in Table 4, the analyses of protozoa motility and density revealed a gradual change for the levels 0.5 and 1.0 g/kg BW, suggesting increased activity of protozoa, from fasting to 4h, followed by a reduction at 6h after feeding. However, at the level 2.0 g/kg BW, the protozoa population was more stable and active.

TABLE 4. Evaluation of rumen fluid of sheep submited to different levels of mineral protein-energy mixture (0.5, 1.0 and 2.0 g of the mixture / kg of BW) and aspects of protozoa motility and density

Treatment	Hours	VARIABLES	
		Motility of Protozoa	Density of Protozoa
0.5	0h	+ +	+ ± [1]
	2h	+ + ±	+ + [2]
	4h	+ + +	+ + ± [3]
	6h	+ + ±	+ +
1.0	0h	+ ±	+ ±
	2h	+ +	+ +
	4h	+ + +	+ + + [4]
	6h	+ + ±	+ +
2.0	0h	+ +	+ + ±
	2h	+ + +	+ + ±
	4h	+ + +	+ + +
	6h	+ +	+ + ±

[1] + ± → weak; [2] + + → good; [3] + + ± → very good; [4] + + + → excellent.

By comparing the levels of supplementation of 0.5 g/kg of BW and 1.0 g/kg BW, a similar behavior of protozoa may be observed even when dealing with different amounts of supplement. However, as for the treatment 2.0 g / kg of BW, it seems that the amount of supplement supplied affected the protozoa population, which is reinforced when one considers that the same animals were assessed at T1, T2 and T3. As it can be seen in Tables 2, 3 and 4, the values of the variables tended to be superior for the treatment with 2.0 g / kg BW, at the four collection times. According to SIQUEIRA & D'AGOSTO (2003), behavior and distribution of ciliated protozoa may be influenced by several factors such as diet ingested by the host, rumen pH, time interval after feeding and the relations

established both among them and between them and bacteria and fungi.

Apparently, when the sheep received the largest amount of supplement (2.0 g / kg BW), the rumen protozoa population was more significantly altered, which can be verified by the increase in the number of infusorian (per mL of rumen fluid), and motility and density of protozoa. Researches on corn and sorghum based diets carried out by TOWNE et al. (1990) and FRANZOLIN & DEHORITY (1996) showed high concentrations of protozoa in the rumen under these dietary conditions. In contrast, FRANZOLIN et al. (2000) replaced corn silage by sugar cane in diets for sheep and observed a linear increase in pH and a reduction in the number of ciliated protozoa as the percentage of sugar cane silage increased. When the sugar cane was supplied as the only roughage, the authors found a protozoa population in the rumen in the order of 2.0×10^4/mL of fluid at pH 6.9, and the number of protozoa decreased with the increase of energy content. Similar results were observed by LYLE et al. (1981) and DENNIS et al. (1983), who found 1.5, 2.5, and 4.1×10^5 protozoa/mL rumen fluid for diets with high, medium and low energy, respectively.

A final evaluation of the results obtained from confined sheep supplemented with increasing levels of energy and true protein allows us to infer that the population of ciliated protozoa responded positively. At the highest level of supplementation (T3), pH values remained around 7.0 and the number of infusorian (cells / mL) was higher than in other treatments (T1 and T2), being initially in the order of $3x10^5$ and reducing to the order of $1x10^5$ six hours after feeding. FRANZOLIN et al. (2000) observed the largest number of protozoa when the animals received lower energy in the diet, that is, corn silage as the only roughage, and the population of protozoa was in the order of 5.0×10^4/ mL of rumen fluid at pH 6.7, thus, differing from the findings by BIRD et al. (1979), who provided sugar cane based diet to sheep and found 5.0×10^5 protozoa/mL rumen fluid.

CONCLUSION

Methylene blue reduction test, pH and cellulose digestion were not influenced by the level of supplementation and the organoleptic characteristics - color, odor and consistency - were similar for the increasing levels of supplementation. The frequency of ciliated protozoa rating remained stable with a predominance of small protozoa. The increasing levels of supplementation influenced positively the population of ciliated protozoa in the rumen, but did not affect bacterial activity.

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