Tropical and Subtropical Agroecosystems

RUMEN MICROBIAL GROWTH IN SHEEP FED THREE DISTINCT QUALITY HAYS

[SÍNTESIS MICROBIANA RUMINAL EN OVINOS ALIMENTADOS CON HENO DE TRES CALIDADES DIFERENTES]

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SUMMARY

Forages usually fed to sheep in tropics have low values of crude protein (CP) content and/or low availability of protein. The aim of this work was to identify different profiles of microbial protein (MP) yield for sheep fed hays with distinct CP levels. Three hays (LUC - Lucerne, SIG - signalgrass and TIF - Tifton-85), six animals (Santa Inês wethers fitted with rumen and duodenum cannulas) placed in a double Latin square were used to evaluate transit rates of microbial protein and in vivo microbial synthesis. In vitro microbial protein synthesis was evaluated in a complete factorial statistical design. The parameters studied were rumen pH and ammonia-N, microbial protein transit using ^{15}N as marker and microbial protein synthesis by in vivo (purine derivatives urinary excretion) and in vitro (^{32}P incorporation) techniques. Rumen pH values did not present significant differences for treatments (hays) and these values were in a level considered as satisfactory to good microbial development. As studied hays presented great variations on CP levels, the concentration of ammonia-N was higher for animals fed LUC (191 g CP/kg DM) and lower for animals fed SIG (29 g/kg DM), therefore presented no difference for ammonia-N concentration ($P > 0.05$) between SIG and TIF (75 g CP/kg DM). The nitrogen assimilation rate (k_1) by rumen microorganisms was much faster ($P < 0.01$) for treatment LUC, which showed that, in this treatment, nitrogen was more available than in others. MP produced from LUC had a faster passage (k_2) from rumen to duodenum ($P < 0.01$). Animals fed LUC were those that presented higher MP supply ($P < 0.05$). Although there was no difference ($P = 0.12$) between both treatments with lower protein levels (TIF and SIG), there is a visible trend of that with lower protein content, the results were even more unsatisfactory, regarding the MP flow. The analysis of variance of the results of net MP production after 8 h incubation with ^{32}P showed that there was an effect of inoculum ($P = 0.0089$), but there was no effect neither of substratum nor of substratum*inoculum interaction ($P > 0.05$). Feeds with low protein content had negative effect on utilization of nutrients by sheep and on maintenance of a healthy rumen environment. This fact was highlighted by variations on rumen ammonia-N concentrations. The microbial synthesis capacity was identified more efficiently by purine derivatives urinary excretion technique. The in vitro technique of ^{32}P incorporation did not distinguish microbial growths from tested feeds degradation. *In vitro* technique showed strong dependency of inoculum quality.

Key words: Lucerne; microbial synthesis; protein; ruminants; signalgrass.

RESUMEN

Los forrajes que usualmente alimentan a las ovejas en los trópicos tienen bajos valores de proteína cruda (PC) y/o baja disponibilidad de proteína. El objetivo de este trabajo fue identificar diferentes perfiles de producción de proteína microbiana (PM) en los ovinos alimentados con henos de distintos niveles de PC. Tres henos (LUC - Lucerna, SIG - Brachiaria y TIF - Tifton-85), seis animales (corderos Santa Inés con cánulas en el rumen y en el duodeno) se organizaron en un doble cuadrado latino para evaluar las tasas de tránsito de proteína microbiana y la síntesis microbiana *in vivo*. La síntesis *in vitro* de la proteína microbiana se evaluó en un diseño estadístico factorial. Los parámetros estudiados fueron el pH ruminal, el N amoniacal, el tránsito de proteína microbiana utilizando el ^{15}N como marcador y la síntesis de proteína microbiana por técnicas *in vivo* (la excreción urinaria de derivados de purinas) e *in vitro* (incorporación de ^{32}P). Valores de pH ruminal no presentaron diferencias significativas para los tratamientos (henos) y estos valores se encontraban en un nivel considerado como satisfactorio para el desarrollo microbiano. Como los henos estudiados

presentan grandes variaciones en los niveles de PC, la concentración de amoníaco-N fue mayor para los animales alimentados con LUC (191 g CP/kg MS) y menor para los animales alimentados con SIG (29 g / kg MS), por lo tanto, no presentaron diferencia de concentración de amoníaco-N (P>0.05) entre SIG y TIF (75 g PC / kg MS). La tasa de asimilación de nitrógeno (k_1) por los microorganismos del rumen fue mucho más rápida (P<0.01) para el tratamiento LUC, que demostró que el nitrógeno es más disponible que en otros. La proteína microbiana producida a partir del LUC tiene un paso más rápido (k_2) de rumen a duodeno (P<0.01). Animales alimentados con LUC son los que presentan mayor producción de PM (P<0.05). Aunque no hubo diferencias (P=0.12) entre ambos tratamientos con menores niveles de proteína (TIF y SIG), hay una visible tendencia de que, con menor contenido de proteína, los resultados fueron aún más insatisfactorios, en relación a el flujo de PM. El análisis de varianza de los resultados de la producción neta de PM después de 8 h de incubación con ^{32}P mostró que había un efecto de inóculo (P=0.0089), pero no hubo efecto de ninguno de sustrato ni de la interacción sustrato*inóculo (P>0.05). Alimentos con bajo contenido proteico tuvieron efectos perjudiciales sobre la utilización de nutrientes por los corderos y en el mantenimiento de un medio ambiente saludable del rumen. Este hecho fue destacado por las variaciones en las concentraciones de amoníaco-N en el rumen. La capacidad de síntesis microbiana se determinó de manera más eficiente por la técnica de excreción urinaria de derivados de purinas. La técnica *in vitro* de la incorporación del ^{32}P no distinguía los crecimientos microbianos a partir de la degradación de los alimentos evaluados. La técnica *in vitro* mostró fuerte dependencia de la calidad del inóculo.

Palabras clave: Alfalfa; síntesis microbiana; proteína; rumiantes; Brachiaria.

INTRODUCTION

Forages are a huge range of feeds to yield animal products (meat, milk, wool, leather) with lower costs. However, as mentioned by Beever and Mould (2000), the diversity of forages represents at the same time opportunities and challenges to the use of those feeds in ruminant diets. Forages usually fed to sheep in tropics have low values of crude protein content and/or low availability of protein. Nitrogen requirements for ruminants are the results of microbial protein (MP) synthesis from protein degradation in the rumen, endogenous nitrogen recycled via saliva, dietetic protein non-degraded in the rumen and animal protein (Boer *et al.*, 1987). Thus, the knowledge about MP yield potential from the feed is important to understand protein requirements of ruminants.

The quantity of dietetic rumen undegradable protein supply to the gut depends on rumen degradation. The amount of MP synthesised in the rumen, as the result of microbial fermentation, is interesting because there are evidences that MP can be influenced by diet characteristics (Dove and Milne, 1994). According to Chen and Gomes (1992), MP contribution on intestinal flow of protein is usually considered constant based on feed intake but great variations have been observed. Studies to determine MP contribution as protein source for the host animal use microbial markers, which can be internal (e.g. DAPA, D-alanine, AEP, DNA, RNA and ATP) or external (e.g. ^{15}N, ^{32}S, ^{32}P and ^{33}P). Another possibility to estimate MP synthesis is the use of indirect techniques like the urinary excretion of purine derivatives (Broderick and Merchen, 1992; Chen and Gomes, 1992 and Csapó *et al.*, 2001).

The aim of this work was to identify different profiles of MP yield in sheep fed three hays with distinct protein levels.

MATERIAL AND METHODS

Location and animals

The experiment was carried out in south east of Brazil at the Animal Nutrition Laboratory, Centre for Nuclear Energy in Agriculture, University of Sao Paulo. Previously the experiment, six Santa Inês (a Brazilian breed) wethers were submitted to surgery to set the animals with rumen and duodenum cannulas. They had 40 ± 5.7 kg of live weight (LW) at the beginning of the experiment.

Experimental period

The experiment comprised three consecutive periods of 28 days each. The first 21 days were used to change over and adaptation. Between days 9 and 18, dry matter voluntary intake (DMVI) were measured. At 8:00 A.M. of the day 22, markers were introduced directly into the rumen through the cannulas. During the days 22 and 27, samples were collected for the *in vivo* assays. At the day 28 of each period, the animals fastened and at 8:00 A.M. of the next day rumen liquor samples were taken for the *in vitro* assays.

Treatments and feeding

Three hays were chosen for this experiment, having as main criteria their crude protein (CP) content: Lucerne (*Medicago sativa*), signalgrass (*Brachiaria decumbens*

cv. Basilinsk) and Tifton-85 (*Cynodon* sp.), here denominated as LUC, SIG and TIF, respectively. Animals were fed twice a day (50 % at 8:30 A.M. and 50 % at 4:30 P.M.) based on their dry matter voluntary intake (90 % DMVI), trying, this way, to avoid refusals.

All hays were chemically characterised (Table 1) according to AOAC (1995) (DM – dry matter, OM – organic matter and CP – crude protein) and Van Soest and Wine (1967) (NDF – neutral-detergent fibre, ADF – acid-detergent fibre and ADL – acid-detergent lignin). Also acid-detergent insoluble protein (ADIP) was determined and hemicellulose and cellulose were estimated.

Rumen environment

Samples of rumen liquor were collected to characterise the rumen environment through pH and ammonia-N values. The collections were made at different times: 0, 0.5, 1, 2, 3, 4, 6, 8, 8.5, 9, 10, 11, 12, 14, 18, 24 h (days 22 and 23). Collections made at 0 and 8 h correspond to meal offering (8:30 A.M. and 4:30 P.M., respectively).

Rumen pH was determined immediately after collection. For ammonia-N content, the collected sample was immediately acidified with 3-4 drops of H_2SO_4 and frozen for further analysis of ammonia-N according to Preston (1995).

Microbial protein transit assay

For this assay, $(^{15}NH_4)_2SO_4$ salt, with isotopic enrichment of 90 %, was used as rumen microorganism marker. A pulse dose of 1.0 mg ^{15}N per kg LW previously diluted in 40 ml of distillated water was injected directly into the rumen, immediately before the first meal.

Samples of duodenum digesta were taken after 0, 4, 8, 12, 16, 20, 24, 26, 30, 34, 38, 42 and 46 h after infusion of ^{15}N. Nitrogen-15 was determined by mass spectrometry technique and the results were fitted using the bi-compartmental model of Grovum and Williams (1973), as following presented:

$$y = 0 \rightarrow t \leq TT \quad (1)$$

$$y = A_1 \cdot e^{-k1 \cdot (t-TT)} - A_2 \cdot e^{-k2 \cdot (t-TT)} \rightarrow t > TT \quad (2)$$

where y is the concentration of marker at time t; A_1 and A_2, model constants without defined biological value; k_1 and k_2, passage rates for pools 1 and 2; and TT, transit time.

From equations (1) and (2), we have:

$$TT = (\ln A_2 - \ln A_1)/(k_2 - k_1) \quad (3)$$

$$MRT_{pool1} = 1/k_1 \quad (4)$$

$$MRT_{pool2} = 1/k_2 \quad (4)$$

$$TMRT = TT + MRT_{pool1} + MRT_{pool2} \quad (5)$$

where, MRT_{pool1} and MRT_{pool2} are, respectively, mean retention time of marker in pools 1 and 2; and $TMRT$ is total mean retention time of marker.

This model is indicated to whole digestive tract, but in this experiment only a fraction of this tract was studied (rumen $\rightarrow$ duodenum). Thus, the presented model can be defined as:

$$Marker \rightarrow pool_1 \rightarrow pool_2 \quad (6)$$

Interpreting the results, $pool_1$ represents the microbial population in the rumen and $pool_2$, the microbial population in the duodenum digesta. Thus, k_1 would represent the rate of marker incorporation by bacteria and k_2, the rate of passage of the MP from the rumen to the duodenum.

Table 1. Chemical composition† (g/kg dry matter) of Lucerne (LUC), signalgrass (SIG) and Tifton-85 (TIF) hays.

Feeds	DM‡	OM	NDF	ADF	ADL	HCEL	CEL	ADIP	CP
LUC	841	900	521	418	106	103	307	21	191
SIG	852	926	778	470	61	308	382	8	29
TIF	849	907	804	461	66	343	361	16	75
s.e.d.	1.3	2.9	11·0	8.3	4.0	6.6	4.9	0.7	2.9

† DM: dry matter; OM: organic matter; NDF: neutral-detergent fibre; ADF: acid-detergent fibre; ADL: acid-detergent lignin; HCEL: hemicellulose; CEL: cellulose; ADIP: acid-detergent insoluble protein (ADIN×6.25); CP: crude protein

‡ in g/kg fresh matter

In vivo microbial protein synthesis assay

The urinary excretion of purine derivatives (PD) was used to estimate daily MP supply. Daily urine excretions between days 23 and 27 of each period were collected in plastic flasks containing 100 ml of sulphuric acid 10% solution, to keep pH of collected urine between 2 and 3. After measurement of total excretion volume, 20 ml were taken and frozen. After the experiment, they were unfrozen and homogenised in an ultrasonic bath, for 5 min. A composed sample were made by each animal, with proportional quantities according to the daily excretion and diluted to an equivalent volume of 3 litres per day. From this pre-diluted sample, another two samples were prepared by dilution according to Chen and Gomes (1992). Allantoin, uric acid, xanthine and hypoxanthine were determined using a colorimetric methodology as described by Chen and Gomes (1992).

In vitro microbial protein synthesis assay

Microbial synthesis was also estimated *in vitro* using the radiophosphorous (^{32}P) incorporation technique, according to Van Nevel and Demeyer (1979) with the modification proposed by Bueno (1998) and Gobbo (2001). As inoculum, rumen content of the same animals fed the experimental treatments was used.

Inocula from animals fed different treatments were prepared separately. All treatments were inoculated with all inocula to test the specificity of inocula.

The quantity of ^{32}P used for each 1 g DM of sample was 25 µl, corresponding to an activity of 3700 Bq (0.1 µCi). After incubation for 8 h, the radioactivity in soluble and insoluble fractions was determined by Cerenkov effect in a liquid scintillator.

All exceeding ^{32}P compared to the natural abundance was considered as applied ^{32}P. The quantity of phosphorous incorporated in microbial biomass was determined according to Van Nevel and Demeyer (1979).

Statistical design

The statistical design of the *in vivo* studies was a double Latin square (3 treatments, 3 periods, 6 animals) and the analysis of variance (ANOVA) was made using the following model:

$$y_{ijk} = \mu + T_i + A_j + P_k + e_{ijk}$$

where: y_{ijk} = dependent variable;
μ = general mean;
T_i = treatment effect (i = 1 to 3);
A_j = animal effect (j = 1 to 6)
P_k = period effect (k = 1 to 3)
e_{ijk} = error.

For the *in vitro* assay, a complete factorial design was used and the ANOVA was made using the following model:

$$y_{ijk} = \mu + S_i + I_j + P_k + S_i \times I_j + e_{ijk}$$

where: y_{ijk} = dependent variable;
μ = general mean;
S_i = substratum effect (i = 1 to 3);
I_j = inoculum effect (j = 1 to 3)
P_k = period effect (k = 1 to 3)
$S_i \times I_j$ = substratum*inoculum interaction effect
e_{ijk} = error.

For the statistical analysis, SAS software (SAS, 2000) was used and the probability level to accept or to reject the hypothesis test was 5 %. Means were compared by Student-t test and standard error of difference (s.e.d.).

RESULTS

Rumen environment

Rumen environment evaluation by modifications on pH and ammonia-N are graphically demonstrated in Figures 1 and 2. There is a significant difference ($P < 0.05$) only at 0 h collections (made immediately before the first meal). For this time, rumen pH of animals fed Lucerne hay (LUC) was higher than the others. There was no difference ($P > 0.05$) for pH values at other time collections (Figure 1).

The greatest variations in pH values were noticed for animals fed LUC. Maximum pH values were 7.1, 6.8 and 6.8 and minimum values were 6.3, 6.4 and 6.3, respectively for treatments LUC, SIG and TIF. Ammonia-N concentration (Figure 2) was higher in rumen liquor from animals fed LUC. The concentration was lower for animals fed SIG. The treatments showed differences ($P < 0.05$) for all collections except for 6 and 8 h, in which LUC and TIF did not differ among them ($P > 0.05$) but they were different ($P < 0.05$) than SIG.·

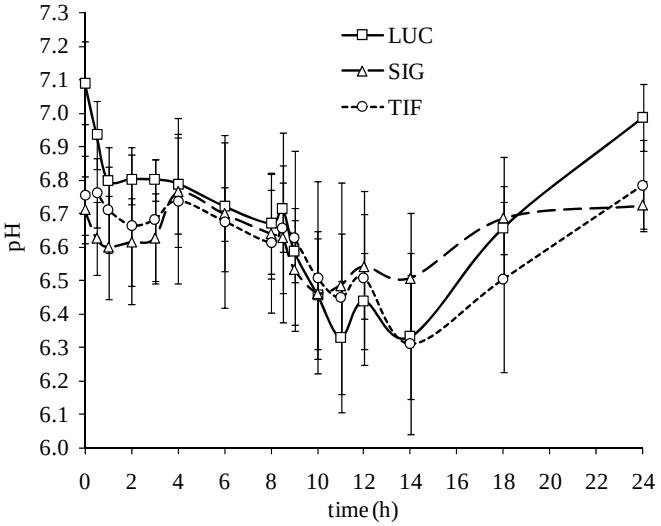

Figure 1. Rumen pH modifications, along 24 h, for sheep fed Lucerne (LUC), signalgrass (SIG) or Tifton-85 (TIF) hays. (note: 0 and 8 h correspond to two daily meals).

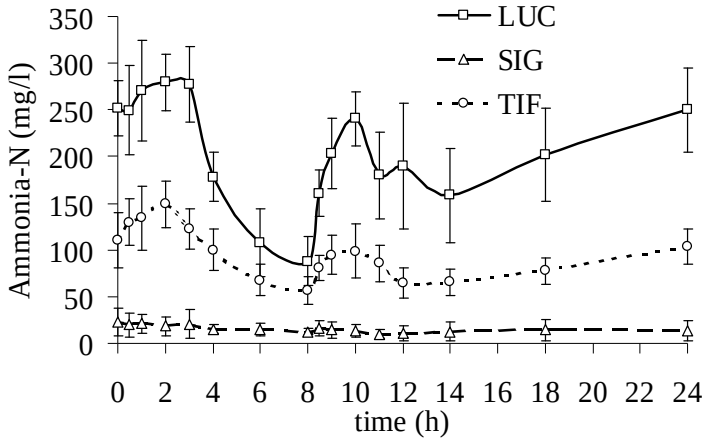

Figure 2. Rumen ammonia-N content modifications, along 24 h, for sheep fed Lucerne (LUC), signalgrass (SIG) or Tifton-85 (TIF) hays. (note: 0 and 8 h correspond to two daily meals).

Microbial protein transit

The main differences were found between treatments SIG or TIF and LUC. SIG and TIF promoted very similar microbial growth kinetics and microbial protein transit from rumen to duodenum (Table 2).

***In vivo* microbial protein synthesis**

The urinary excretion of PD presented the profiles described on Table 3 and the gut absorption of puric bases is presented on Table 4.

Table 2. Parameters of microbial protein transit from rumen to duodenum for sheep fed Lucerne (LUC), signalgrass (SIG) or Tifton-85 (TIF) hays.

parameters	treatments			s.e.d.
	LUC	SIG	TIF	
k_1	0.048	0.027	0.027	0.0053
k_2	0.518	0.280	0.354	0.0419
TT	9.84	16.11	14.11	1.775
MRT_{pool1}	21.87	39.73	44.06	4.041
MRT_{pool2}	1.94	3.74	3.24	0.444
TMRT	33.65	59.58	61.41	3.333

Table 3. Proportions of purine derivatives (PD) excreted via urine by sheep fed Lucerne (LUC), signalgrass (SIG) or Tifton-85 (TIF) hays.

PD[†]	treatments			s.e.d.
	LUC	SIG	TIF	
hypoxanthine + xanthine	5.77	6.28	8.41	0.779
uric acid	42.32	40.85	34.39	3.959
allantoin	51.91	52.87	57.20	3.888
total (mmol/d)	16.23	4.79	6.34	-

[†] proportion (in %) related to total excretion of PD

Animals fed LUC were those that presented higher MP supply ($P < 0.05$). Although there was no difference ($P = 0.12$) between both treatments with lower protein levels (TIF and SIG), there is a visible trend of that with lower protein content, the results were even more unsatisfactory, regarding the MP flow

***In vitro* microbial protein synthesis**

The analysis of variance of the results of net MP production after 8 h incubation with ^{32}P showed that there was an effect of inoculum ($P = 0.0089$), but there was no effect neither of substratum nor of substratum*inoculum interaction ($P > 0.05$) (Table 5).

Table 4. Profiles of purine derivatives (PD) excreted via urine by sheep fed Lucerne (LUC), signalgrass (SIG) or Tifton-85 (TIF) hays.

variables	treatments			s.e.d.
	LUC	SIG	TIF	
purine absorption				
mmol/d	19.3	4.9	7.0	1.67
µmol/kg $LW^{0.75}$/d	1172.6	332.0	438.3	75.11
microbial synthesis				
g/d	14.0	3.6	5.1	1.21
mg/kg $LW^{0.75}$/d	852.5	234.1	318.7	54.60
mg/kg OMD_R	35.2	20.3	24.8	2.61

Table 5. Microbial nitrogen synthesis (in mg MN/g DM) from Lucerne (LUC), signalgrass (SIG) or Tifton-85 (TIF) hays estimated by *in vitro* ^{32}P incorporation, using as inoculum rumen liquor from sheep fed correspondent hays.

inocula	substrata			means
	LUC	SIG	TIF	
LUC	2.36	2.83	3.05	2.75 [a]
SIG	1.77	1.26	1.28	1.44 [b]
TIF	1.33	1.59	1.49	1.47 [b]
means	1.82	1.89	1.94	-

[a, b] means with different superscript are significantly different (Student·-t test; $P < 0·01$)

DISCUSSION

Rumen environment

The results of rumen pH (Figure 1) are compatible to the recommendations of Mould and Ørskov (1983) to keep rumen pH between 6.2 and 7.0, guaranteeing a good rumen microbial development (regarding to cellulolitic bacteria).

The pH values were similar to those obtained by Korndörfer (1999) who also did not find differences in rumen pH for sheep fed signalgrass or Lucerne hays. Although rumen pH has not been a limiting factor to the development of microbial population, the environment was affected negatively by the lowest protein treatment (SIG). This can be observed on the alterations of rumen ammonia-N (Figure 2).

The results illustrated that low N level feeds are unable to supply nutrients to rumen microorganisms and meeting their requirements to a satisfactory development. Satter and Slyter (1974) indicate that ammonia-N values between 50 and 80 mg per litre as minimum concentration to not diminish the microbial growth. NRC (1985) recommends that, although the ideal concentration of ammonia-N necessary to maximum microbial growth is not clear, concentrations above 50-100 mg per litre do not have effect on MP production. Based on that, SIG did not supply enough N to healthy metabolism of microorganisms, even just after the meals (Figure 2). The other treatments did not follow down the critical concentration during all studied times.

Microbial protein transit

The approach used in this paper is not very common. Usually, data using ^{15}N measured protein flow but for that, it is necessary to use continuous infusion, normally with a peristaltic pump, for several days. In this study, a pulse dose was used, thus this approach was not possible. The approach used was to determine MP transit instead of flow.

The nitrogen assimilation rate (k_1) by rumen microorganisms was much faster ($P < 0.01$) for treatment LUC, what shows that this treatment nitrogen is more available than others. MP produced from LUC had a faster passage (k_2) from rumen to duodenum ($P < 0.01$).

The other parameters (Table 2) also showed a better availability of LUC nitrogen when compared to the others.

In vivo microbial protein synthesis

For sheep, Chen and Gomes (1992) presented as approximate proportions of PD, expressed in percentage of the sum, values about 60-80 % for allantoin, 10-30 % for uric acid and 5-10 % for hypoxanthine plus xanthine. Data obtained (Table 3) for allantoin were, therefore, below expected and uric acid content, a little above.

The low volume of urine excreted is a clue to the low percentage of allantoin and higher concentration of uric acid, as highlighted by Chen and Gomes (1992). The absorption of puric bases in gut (Table 4) showed once again that feed CP content had a strong impact on MP yield and, consequently, on the MP supply to the host animal.

Results of MP obtained for LUC were higher than those obtained by Jetana *et al*. (2000), working with sheep fed guinea grass (*Panicum maximum*) (106 g CP/kg DM) and concentrate (185 g CP/kg DM), that varied from 7·8 to 10·8 g MN/d. The probable reason for that superiority is the lower proteic content of their final diet, for animals were fed restrict quantity of concentrate and forage *ad libitum*.

In vitro microbial protein synthesis

The best inoculum was obtained from animals fed LUC. As the incubation period is short (only 8 h), differently of other *in vitro* techniques, the importance of inoculum quality is very great. To guarantee an adequate and fast microbial activity, the inoculum must supply a microbial population in ideal quantity and quality. Besides that, the inoculum, even the donor animals being in fastening before the collection, still contains a quantity of substratum (soluble and particulate) that can supply initial energy to microbial growth during the lag time. This fact probably occurred for inoculum prepared with rumen liquor from those animals fed LUC. The animals fed SIG or TIF, probably as these feeds presented lower contents of nutrients, supplied rumen fluid energetically poorer and, therefore, of lower initial microbial activity.

CONCLUSIONS

Feeds with low protein content had negative effect on utilization of nutrients by sheep and on maintenance of a healthy rumen environment. This fact was highlighted by variations on rumen ammonia-N concentrations. The microbial synthesis capacity was identified more efficiently by purine derivatives urinary excretion technique. The *in vitro* technique of ^{32}P incorporation did not distinguish microbial growths from degradation of tested feeds. *In vitro* technique showed strong dependency of inoculum quality.

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