

Full Length Research Paper

# A study of the dietary quality of grass-diets with urea and cassava meal

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Ten Polwarth × Texel lambs (30±1kg live weight (LW)), housed in metabolic cages and fed *ad libitum* a low-quality grass hay (*Cynodon ssp.*) were used in a replicated 5 × 5 Latin Square experiment to evaluate effects of non-protein N (NPN) and levels of a non-fibre carbohydrate (NFC) source (cassava meal) supplementation (0, 5, 10 and 15 g/kg of LW) on intake, digestibility, N retention, microbial protein synthesis and rumen fermentation. Hay intake and digestibility were not affected by NPN addition. Organic matter, N and digestible energy intake, as well as rumen microbial protein synthesis and N retention increased linearly ( $P<0.05$ ) but, fibre intake and digestibility, decreased linearly ( $P<0.05$ ) as NFC supplementation increased. Rumen pH, as well as rumen concentrations of ammonia, sugars, amino acids and peptides was significantly affected by supplementation and time after feeding ( $P<0.05$ ). Hay utilization was not improved by N addition showing that it was not limited due a lack of N for rumen bacteria. Supplementing both NPN plus a NFC source improved nutrients intake but reduced forage use by ruminants. Although variations of rumen pH and sugars concentrations play an important role, the detailed mechanisms by which fibre digestibility is negatively affected by NFC supplementation needs to be elucidated.

**Key words:** Digestibility, Intake, Non-fibre carbohydrate, Non-protein nitrogen, Rumen fermentation, Tropical grass.

## INTRODUCTION

In comparison to other herbivores, ruminants have the highest potential to obtain nutrients from fibrous feeds. This characteristic is particularly important in developing tropical areas, where the challenge is to improve natural resources utilization. Most organic matter (OM) in tropical forage is present as cell wall carbohydrates, mainly cellulose and hemicellulose. Compared to sucrose or starch, rumen degradation of these carbohydrates is slower and less extensive (Van Soest, 1994). Moreover, N content of mature tropical

grasses is usually low, which limits cellulolytic-bacteria growth and activity. Because of this, N supplementation usually increases low-quality forage intake and digestibility (Paterson et al., 1994). Fibre-degrading micro-organisms, however, also depend of others substrates as sugars and branched-chain volatile fatty acids (Van Soest, 1994). Supplementation with rich-starch feeds, however, usually depresses forage intake and digestibility in ruminants (Paterson et al., 1994). This effect has been associated to a concomitant decreases in rumen fluid pH, which inhibits cellulolytic activity, mainly at pH values lower than about 6,2 (Grant and Weidner, 1992). It has been suggested, in turn, that low levels of non-fibre carbohydrate (NFC) supplementation would Stimulate bacteria growth and

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**Table 1.** Chemical composition<sup>a</sup> and *in vitro*/gas degradation rate of feeds.

	Hay	Cassava meal
DM (g/kg)	848	888
<b>Composition (g/kg of DM):</b>		
OM	904	994
NDF	740	97
ADF	334	29
Lignin	44	Traces
EE	12	03
NFC <sup>b</sup>	113	877
Total N	11	2
<b>Composition (g/kg of N):</b>		
ADIN	63	Traces
NDIN	468	Traces
c (/h) <sup>c</sup>	0.023	0.054

<sup>a</sup>DM, dry matter; OM, organic matter; NDF, neutral detergent fiber (with a heat stable amylase and exclusive of ash); ADF, acid detergent fiber (exclusive of ash); Lignin (sa), lignin determined by the sulphuric acid method; EE, ether extract; NFC, non-fiber carbohydrate; N, nitrogen; ADIN, acid detergent insoluble nitrogen; NDIN, neutral detergent insoluble nitrogen.

<sup>b</sup>NFC = OM - ((N×6.25) + EE + (NDF-(NDIN×6.25)))

<sup>c</sup>Gas production rate obtained from a semi-automated *in vitro*/gas assay (Mauricio et al., 1999) and calculated using the one-pool logistic model of Schofield et al. (1994).

adherence to feed particles and thus, it would improve fibre degradation into the rumen (Morris, 1988; Caton and Dhuyvetter, 1997). At what level of NFC supplementation a stimulatory effect on roughage utilization could be expected is not known.

This experiment was completed to evaluate if additional NPN, associated or not to increased levels of NFC supplementation could improve forage use, as well as to evaluate their effects on intake, digestibility, rumen fermentation, rumen microbial protein synthesis and N retention by lambs fed *ad libitum* a low-quality grass hay.

## MATERIAL AND METHODS

### Feedstuff, animals, housing and experimental design

Ten Polwarth×Texel lambs (30±1kg LW), housed in metabolic cages and fed *ad libitum* a low-quality grass hay (mature *Cynodon ssp.*) were used in a replicated 5 × 5 Latin Square experiment to evaluate the effect of NPN (urea: ammonia sulphate, 9:1) and levels of NFC supplementation (5, 10 and 15 g of cassava meal/kg of LW) on intake, digestibility, N retention, rumen microbial protein synthesis and rumen fermentation. A control treatment (no supplement) was also included. Cassava is a cheap home-grown crop which, depending of cultivar, contain variable levels of cyanide, a toxic compound. However, it is markedly reduced by drying (Van Soest, 1994) at preparing the cassava meal and there is not poisoning risk by its use as animal feed. Five lambs were fitted with rumen cannulae. The chemical composition and degradation rate, obtained from an *in vitro*/gas assay, of the feeds is shown in Table 1. Chopped hay (5-10 cm

length particles) and supplements, except NPN, were fed separately twice daily at 8:00 and 17:00 h. The amount of hay offered was 100 to 200 g/kg in excess to that eaten the previous day. There were not supplement refusals in any experimental period. Non-protein N was added to raise the N content of diets to 24 g/kg on a dry matter (DM) basis. It was dissolved in distilled water (300 g/L) and spread on hay, or was ground (0.5 mm screen) and mixed with cassava meal. A commercial mineral mixture containing (g/kg): Ca: 100, P: 45, S: 4.12, Na: 205, Co: 0.025, Cu: 0.450, Fe: 1.5, I: 0.05, Mn: 1.0, Se: 0.009, Zn: 2.52 and F: 0.45) was fed mixed with the hay at a rate of 10 g/kg DM.

The experimental periods were 16 days, including a 10 day adaptation and 6 day collection period. Feed offered and refused, as well as faeces, were weighted daily, recorded and sampled from day 10 to 15 of each experimental period. All samples were oven-dried at 55°C for at least 72 h and ground through a 1 mm screen for subsequent chemical analysis. Urine was collected daily during the collection period, in buckets containing 100 ml of 7.2 N H<sub>2</sub>SO<sub>4</sub>. Volume was measured and a sample of 10 ml/L was stored frozen until later analysis. On day 16 of each period, samples of rumen fluid were collected at 0, 1, 2, 3, 4, 6 and 8 h after the morning meal and filtered through a 50 µm nylon filter. pH was immediately measured and two 18 ml samples were taken. Two ml of a 7.2 N H<sub>2</sub>SO<sub>4</sub> were added to one sample and 2 ml of a 500 g/L trichloroacetic acid (TCA) to the other. Samples were centrifuged at 4000 × g for 20 minutes at room temperature and the supernatants collected and stored frozen. Pellets were discarded. The supernatant of TCA acidified samples was assumed to contain free amino acids and short chain peptides (< 10 units), while the pellet was comprised of protein and long chain peptides (Greenberg and Shipe, 1979).

### Chemical analysis

Samples of refusals, faeces and urine were pooled on a 5 day basis within each experimental period. Dry matter was determined by dry-

**Table 2.** Intake and digestibility of non-nitrogenous compounds<sup>a</sup> by lambs fed a low- quality grass hay (control) or hay supplemented with non-protein N (NPN) and levels of cassava meal (5, 10 ou 15 g/kg live weight).

	Treatments					SEM <sup>d</sup>	P <sup>c</sup>	
	Control	NPN	5	10	15		NPN	NFC
<b>DM intake:</b>								
g/day	644	648	757	784	809	27	ns	L
g/kg of LW	22	22	24	25	27	0.6	ns	L
<b>OM intake:</b>								
g/day	586	571	681	716	748	25	ns	L
g/kg LW <sup>u,rb</sup>	47	44	53	56	59	1.2	ns	L
<b>Total intake:</b>								
NDF(g/day)	478	470	450	374	310	22	ns	L
NFC (g/day)	75	78	206	318	415	17	ns	L
DE (kj/day) <sup>d</sup>	5791	5443	7004	8548	9845	607	ns	L
<b>Hay intake:</b>								
DM (g/day)	644	630	584	463	361	25	ns	L
OM (g/day)	586	571	530	420	327	23	ns	L
<b>Apparent digestibility:</b>								
OM	0.54	0.53	0.57	0.63	0.71	0.08	ns	L
NDF	0.65	0.64	0.59	0.56	0.57	0.07	ns	L
OMTD <sup>e</sup>	0.72	0.71	0.73	0.77	0.82	0.06	ns	L

<sup>a</sup> See Table 1 for explanations.<sup>b</sup> Standard error of means where n=10 per treatment.<sup>c</sup> Probability of Type I error of NPN and non-fibre carbohydrate (NFC) effects, where: ns = non significant; L = linear ( $P < 0.05$ ).<sup>d</sup> Digestible energy.<sup>e</sup> Organic matter true digestibility.

ing at 105°C during at least 8 hours. Ash was determined by Combusting at 550°C during 2 hours. Total N was assayed by a Kjeldahl method (AOAC, 1995). Acid (ADF) and neutral detergent fibre (NDF) were analysed exclusive of ash and analysis of NDF included amylase but not sodium sulphite. Fibre analysis was according to Robertson and Van Soest (1981) procedures. The sulphuric acid method was used to analyse lignin. Acid detergent insoluble N (ADIN) and neutral detergent insoluble N (NDIN) were analysed according to Licitra et al. (1996). Ether extract (EE) was determined in a reflux system with ethyl ether, at 180°C for 2 hours (Soxtherm, Gerhardt, Germany). Non-fibre carbohydrates (NFC) were calculated as:  $1000 - ((\text{NDF} - (\text{NDIN} \times 6.25)) + (\text{N} \times 6.25) + \text{EE} + \text{ash})$ , according to Van Soest et al. (1991). Organic matter true digestibility (OMTD) was estimated according to Mulligan et al (2001), considering that only the NDF fraction of the faeces originated from the feed (Van Soest, 1994). Heat of combustion (H) was measured, using a calorimetric bomb (Parr, Adiabatic Calorimeter, Moline, Illinois, USA). Digestible energy (DE) intake was calculated as:  $\text{DE (kj/day)} = (\text{DM intake (g/day)} \times \text{hay H (kj/g DM)}) - (\text{faecal DM (g/day)} \times \text{faecal H (kj/g DM)})$ .

Sulphuric-acidified rumen samples were analysed for ammonia (Weatherburn, 1967) and sugars (Dubois et al., 1956) and, the TCA acidified samples, were analysed for amino acids (Palmer and Peters, 1969) before and after hydrolysis with HCl 6 N (2 ml of sample and 2 ml of HCl 6N), at 120°C for 24 hours, in an autoclave. Peptides were calculated as the difference between the amino acids contents before and after hydrolysis. In urine samples, total N was determined as described above, and allantoin and uric acid concentrations were determined

colorimetrically according to Chen and Gomes (1995). Uric acid was determined using a commercial kit (LABTEST, Lagoa Santa, MG, Brazil), after xanthine and hypoxanthine were converted to uric acid with xanthine oxidase. Thus, uric acid values were the sum of uric acid, xanthine and hypoxanthine and, the total purine derivatives as the sum of uric acid and allantoin.

#### Estimation of microbial N supply

The supply of microbial N to the small intestine was calculated from the urinary output of purine derivatives using the method of Chen and Gomes (1995), as previously described (Kozloski et al, 2006a).

#### Statistical analysis

Intake, digestibility, N retention and rumen microbial protein synthesis data were subjected to statistical analysis using the GLM option of SAS (2002) according to the model:

$$Y_{ijkl} = \mu + A_i + S_j + P_k + D_l + (S \times D)_{jl} + e_{ijkl}$$

where:  $A$ ,  $S$ ,  $P$ ,  $D$  are animal, Latin Square, period and fed diet treatment effects and the interaction between Latin Square and diet treatment ( $S \times D$ ), respectively.  $\mu$  is the overall mean and  $e_{ijkl}$  is residual error. Data derived from rumen samples collected at each sampling interval were analysed with the mixed procedure of SAS (2002) for repeated measures according to the model.

**Table 3.** Nitrogen (N) intake, digestibility and retention, and ruminal microbial protein synthesis by lambs fed a low-quality grass hay (control) or hay supplemented with non-protein nitrogen (NPN) and levels of cassava meal (5, 10 ou 15 g/kg of live weight (LW)).

	Treatments					SEM <sup>a</sup>	P <sup>b</sup>	
	Control	NPN	5	10	15		NPN	NFC
<b>Intake (g/day):</b>								
Total	7.7	14.2	17.3	18.4	19.2	0.6	*	L
From hay	7.7	6.2	5.9	4.7	3.6	0.5	*	L
Apparent digestibility	0.49	0.72	0.73	0.74	0.77	0.01	*	L
True digestibility	0.92	0.95	0.95	0.96	0.97	0.005	*	L
Urinary (g/day)	3.5	9.5	8.8	7.9	8.4	0.6	*	ns
Retention (g/day)	0.2	0.8	3.8	5.7	6.4	0.6	ns	L
<b>Ruminal microbial protein synthesis (g microbial N/day):</b>								
	4.1	4.8	6.5	8.4	8.9	0.6	ns	L
<b>Efficiency of ruminal microbial protein synthesis (g microbial N/kg TDOM<sup>c</sup>):</b>								
	13.3	16.1	16.8	17.8	17.1	1.2	ns	ns

<sup>a</sup> Standard error of means where n=10 per treatment.

<sup>b</sup> Probability of Type I error of the non-protein N (NPN) and non-fibre carbohydrate (NFC) effects, where: ns = non significant; \* =  $P < 0.05$  and L = linear ( $P < 0.05$ ).

<sup>c</sup> True digestible organic matter.

$$Y_{ijkl} = \mu + A_i + P_j + D_k + A(P \times D)_{ijk} + T_l + (D \times T)_{kl} + e_{ijkl}$$

where:  $T$  is time,  $D \times T$  is diet treatment by time interaction,  $A(P \times D)$  is the between units random effect and  $e_{ijkl}$  is the within units residual error.

Regression analysis was applied to evaluate the effect of both, NFC level on digestion and time after feeding on rumen variables. Data from non-supplemented and from lambs supplemented with NPN alone were compared by F test.

## RESULTS

### Intake and digestibility of non nitrogenous compounds and energy

Non-protein N addition did not affect any of studied variables (Table 2). Supplementation with NPN associated to increased levels of NFC, however, increased linearly ( $P < 0.05$ ) total DM, OM and DE intake. In opposite, however, hay and NDF intake, as well as NDF digestibility, decreased linearly ( $P < 0.05$ ) with increased NFC supplementation. Cassava meal intake increased from 200 to 540g/kg of total DM intake and forage DM intake decreased from 0.90 to 0.56 of intake in control treatment as cassava meal supplementation increased from 5 to 15 g/kg of lambs LW.

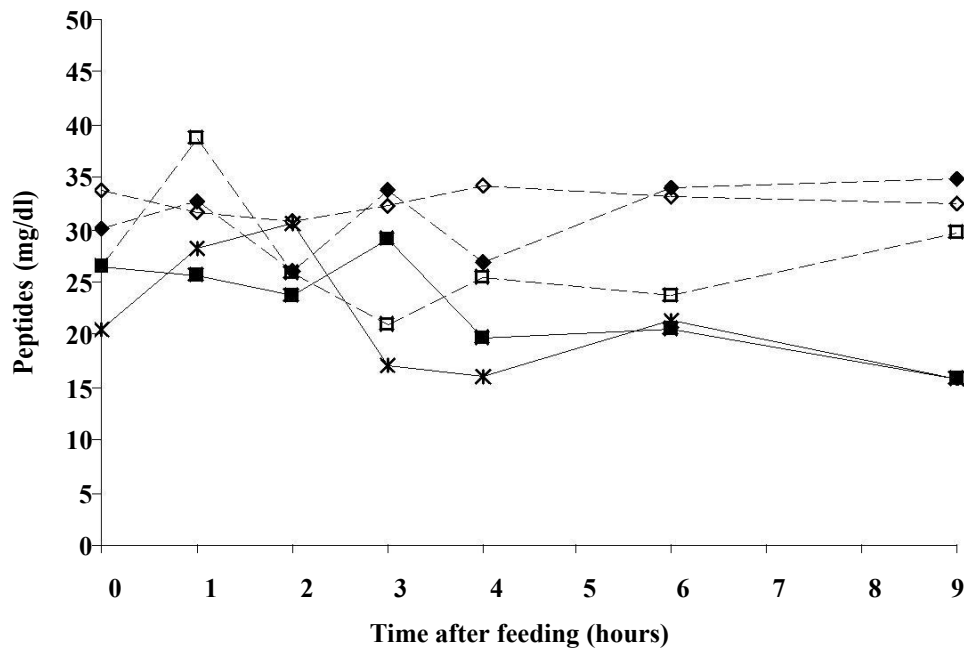
### Nitrogen intake and digestion

NPN alone did not affect neither rumen microbial Protein synthesis nor N retention, but increases

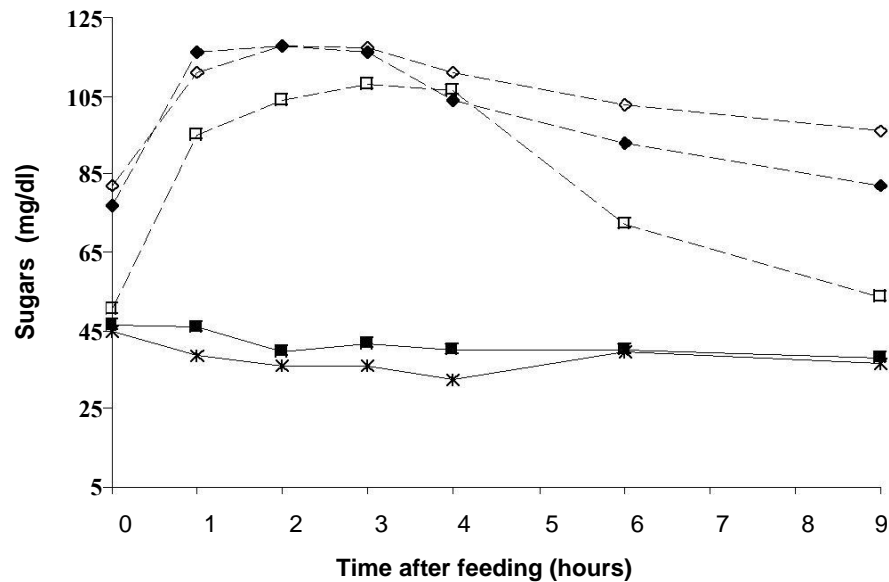
( $P < 0.05$ ) N intake digestibility and urinary excretion (Table 3). Within NPN plus NFC supplemented lambs, N intake from hay decreased ( $P < 0.05$ ), but total N intake and digestibility, as well as rumen microbial protein synthesis and N retention increased linearly ( $P < 0.05$ ) with increased NFC addition. The efficiency of rumen microbial protein synthesis, however, was similar for all treatments.

### Rumen fermentation

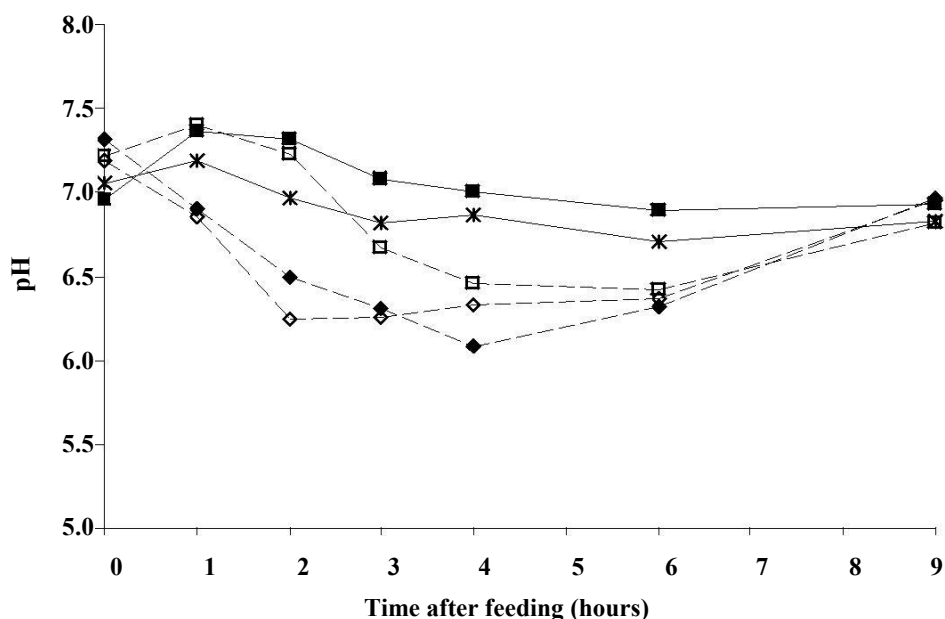
There were treatment by time interactions ( $P < 0.05$ ) for all analysed rumen variables. Supplementation with NPN alone did not affect peptide (Figure 1) and sugars (Figure 2) but affected rumen pH (Figure 3), ammonia-N (Figure 4) and amino acids (Figure 5) concentrations, which increased cubically ( $P < 0.05$ ) to reach a plateau between one and two hours after feeding. As results of increased NFC supplementation, sugars, ammonia-N and amino acids concentrations significantly increased (cubic and quadratic effects,  $P < 0.05$ ) at the first hours after feeding. Rumen pH was also markedly affected by NFC supplementation. Although all values were above 6.0, the declining in rumen pH after feeding was more pronounced for the highest levels of NFC addition. The highest ammonia-N and amino acids values at the plateau level were observed for the lowest level of NFC supplementation. Sugars concentration at the plateau increased as supplementation increased from 5 to 10g/kg of LW, but it was not affected by further addition of NFC. It was not observed a regular variation of rumen peptide



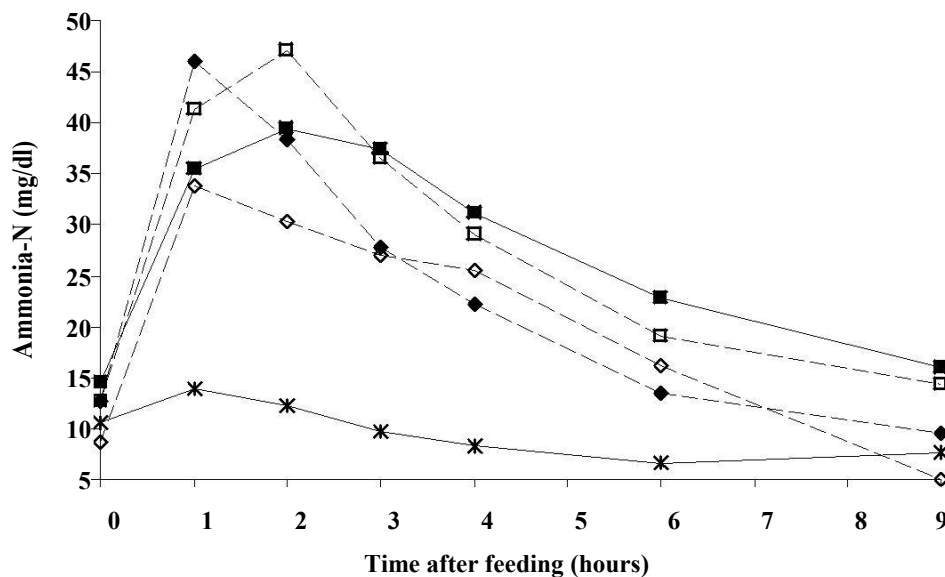
**Figure 1.** Rumen peptides in lambs fed a low-quality grass hay non-supplemented (\*), supplemented with urea alone (□) or with urea plus 5 (■), 10 (♦) or 15 g/kg live weight (○) of cassava meal. Standard error of means (SEM) = 1.9; treatment × time interaction ( $P < 0.05$ ); regression analysis of time effect within treatments: \*, non significant (ns); □, ns; ■, ns; ♦, ns; ○, ns.



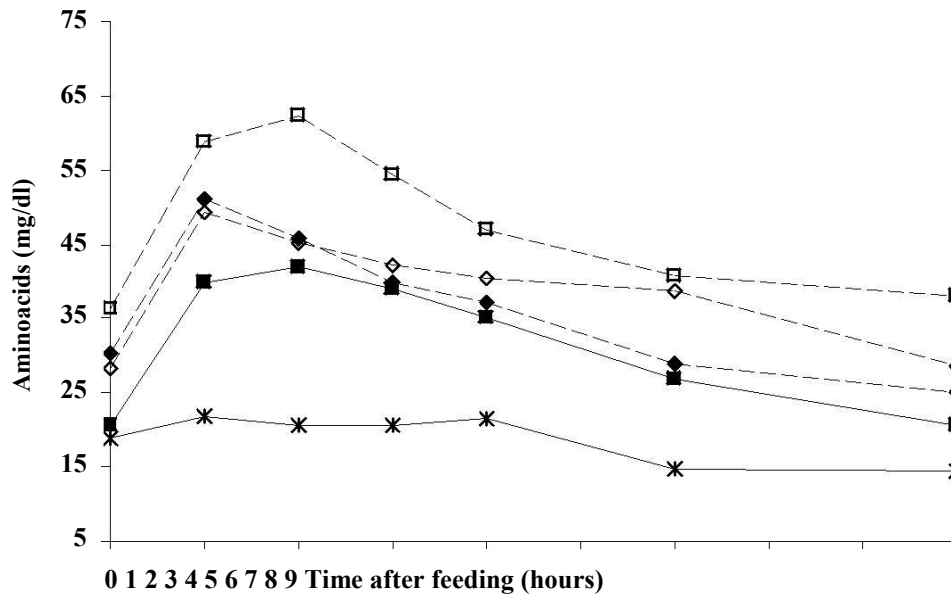
**Figure 2.** Rumen sugars in lambs fed a low-quality grass hay non-supplemented (\*), supplemented with urea alone (□) or with urea plus 5 (■), 10 (♦) or 15 g/kg live weight (○) of cassava meal. SEM = 7.3; treatment × time interaction ( $P < 0.05$ ); regression analysis of time effect within treatments: \*, ns; □, ns; ■, quadratic ( $P < 0.05$ ,  $r^2 = 0.10$ ); ♦, cubic ( $P < 0.05$ ,  $r^2 = 0.26$ ); ○, quadratic ( $P < 0.05$ ,  $r^2 = 0.10$ ).



**Figure 3.** Rumen pH in lambs fed a low-quality grass hay non-supplemented (\*), supplemented with NPN alone (○) or with NPN plus 5 (□), 10 (◆) or 15 g/kg live weight (■) of cassava meal. SEM = 0.06; treatment × time interaction ( $P < 0.05$ ); regression analysis of time effect within treatments: \*, ns; ○, cubic ( $P < 0.05$ ,  $r^2 = 0.21$ ); □, quadratic ( $P < 0.05$ ,  $r^2 = 0.45$ ); ◆, quadratic ( $P < 0.05$ ,  $r^2 = 0.64$ ); ■, quadratic ( $P < 0.05$ ,  $r^2 = 0.41$ ).



**Figure 4.** Rumen ammonia-N in lambs fed a low-quality grass hay non-supplemented (\*), supplemented with urea alone (○) or with urea plus 5 (□), 10 (◆) or 15 g/kg live weight (■) of cassava meal. SEM = 1.36; treatment × time interaction ( $P < 0.05$ ); regression analysis of time effect within treatments: \*, cubic ( $P < 0.05$ ,  $r^2 = 0.55$ ); ○, cubic ( $P < 0.05$ ,  $r^2 = 0.52$ ); □, cubic ( $P < 0.05$ ,  $r^2 = 0.73$ ); ◆, cubic ( $P < 0.05$ ,  $r^2 = 0.59$ ); ■, cubic ( $P < 0.05$ ,  $r^2 = 0.33$ ).



**Figure 5.** Rumen amino acids in lambs fed a low-quality grass hay non-supplemented (\*), supplemented with urea alone ( ) or with urea plus 5 ( ), 10 (◆) or 15 g/kg live weight (□) of cassava meal. SEM = 2.8; treatment × time interaction ( $P < 0.05$ ); regression analysis of time effect within treatments: \*, ns; , cubic ( $P < 0.05$ ,  $r^2 = 0.59$ ); , cubic ( $P < 0.05$ ,  $r^2 = 0.32$ ); ◆, cubic ( $P < 0.05$ ,  $r^2 = 0.52$ ); □, cubic ( $P < 0.05$ ,  $r^2 = 0.39$ ).

concentration, but values tended to decrease throughout the time after feeding in control and NPN-alone treatments.

## DISCUSSION

A major objective of this study was to verify if supplementation could improve forage use by animals. Nitrogen supplementation usually increases low-quality forage intake and digestibility due to increases fibre-degrading bacteria growth and activity (Paterson et al., 1994; Pan et al., 2003). In fact, in the present experiment urea addition markedly increases rumen ammonia concentration but, neither forage digestibility nor microbial protein synthesis was affected, and the N surplus was excreted in urine. Although there has been considerable disagreement concerning the optimal concentration of ruminal ammonia for bacterial growth, *in vitro* studies have yielded values (Satter and Slyter, 1974; Pisulewski et al., 1981) lower than the mean value verified in control treatment (10 mg/dl). Hence, others factors, such as those associated with chemical and/or anatomical characteristics of forage (Nelson and Moser, 1994; Paciullo, 2002), but not a lack of N for rumen bacteria, have limited forage intake and digestion in the present study. The level of urea-N inclusion was relatively high, above of the level usually recommended (up to 0.25 g/kg LW) but, given that no

toxic symptoms were observed, this excessive urea inclusion likely did not result in toxic levels of blood ammonia.

It has been suggested that low levels of non-fibre carbohydrate supplementation would stimulate bacteria growth and adherence and thus, it would improve fibre degradation into the rumen (Morris, 1988; Caton and Dhuyvetter, 1997; Dewhurst et al., 2000). Consequently, it was expected that both, hay intake and fibre digestibility would be enhanced at the lower level of cassava meal supplementation. However, NFC supplementation had a negative effect on NDF digestibility and a linear substitution effect on hay intake. The effect of NFC supplementation on forage use is still more evident when a NPN source is not included (Kozloski et al., 2006b). If the inclusion of lower levels of starch-rich sources could improve roughage intake and digestibility by ruminants, results from the present experiment indicate that these levels must be lower than 5 g/kg of LW. Corn supplementation at a level of 4 g/kg of LW reduced, but at 2 g/kg of LW, enhanced OM digestibility by steers receiving a tropical grass-based diet (Pordomingo et al., 1991). In line with this, Henning et al. (1980) reported that corn supplementation up to a level of 78 g/kg of total DM intake increased maize straw intake by lambs. In our study, however, cassava meal intake varied from 200 to 540 g/kg of total DM intake.

The negative effect of starch supplementation on forage use is usually associated to a concomitant decreases in

rumen fluid pH, which inhibits cellulolytic activity, mainly at pH values lower than about 6.2 (Grant and Weidner, 1992). Indeed, in the present study rumen pH decreased as NFC inclusion increased, but values were almost always higher than 6.2. If the reduction of hay intake and fibre digestibility is due a pH effect, results of this study indicate that there is not a pH threshold value or it is not constant (i.e. 6.2) for all dietetic conditions. *In vitro* studies have demonstrated that fibre degradation is gradually affected by the gradual variation of pH within physiological range (5.5 to 7.5) (Mouriño et al., 2001; Hu et al., 2004).

Although the mechanisms are not still understood, specific induced-starch inhibitory effect on cellulolytic activity, independently of pH value, has also been reported (Lopez et al., 1998; Heldt et al., 1999; Arroquy et al., 2004). Cassava meal exhibits a high gas production rate *in vitro* and, as seen from curves of rumen sugars concentration over time (Figure 2), it is promptly degraded into the rumen. Hence, if there was a specific-starch negative effect on fibre degradation, it occurred only at the first hours after meal, probably affecting bacterial adherence. On the other hand, a specific-sugar negative effect could also have occurred. Excessive sugars availability can be toxic for cellulolytic bacteria (Russel, 1998) and Piwonka & Firkins (1996) verified *in vitro* that glucose fermentation produce a protein which exhibits inhibitory effect on cellulose degradation. The protein structure, as well as the mechanisms of this effect, however, is not known.

As expected, DE intake, rumen microbial protein synthesis and N retention were markedly improved by increasing NFC plus NPN supplement. Compared to control treatment, NPN and NFC supplementation increased rumen ammonia and amino acids. However, within NFC treatments, increased NFC addition decreased rumen ammonia and amino acids, but increased peptide concentration. This is indicative of an increased anabolic activity by rumen bacteria. Concentrate supplementation usually decreases microbial protein synthesis efficiency, mainly due to reduce rumen pH and particle passage rate (Russel et al., 1992; Russel, 1998). In these conditions the bacterial energy expenditure for maintenance is increased. In the present experiment it is probable that rumen pH and particles passage rate changes were not enough to affect the efficiency of rumen microbial protein synthesis.

## CONCLUSIONS

Hay utilization was not improved by N addition showing that it was not limited by lack of N for rumen bacteria. Supplementing both, NPN plus a NFC source improved

nutrients intake but reduced forage use by ruminants. However, although variations of rumen pH and sugars concentrations play an important role, the detailed mechanisms by which fibre digestibility is negatively affected by NFC supplementation needs to be elucidated.

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