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Supplementation with non-fibrous carbohydrates reduced fiber digestibility and did not improve microbial protein synthesis in sheep fed fresh forage of two nutritive values

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To determine whether non-fibrous carbohydrate (NFC) supplementation improves fiber digestibility and microbial protein synthesis, 18 Corriedale ewes with a fixed intake level (40 g dry matter (DM)/kg $BW^{0.75}$) were assigned to three (n = 6) diets: F = 100% fresh temperate forage, FG = 70% forage + 30% barley grain and FGM = 70% forage + 15% barley grain + 15% molasses-based product (MBP, Kalori 3000). Two experimental periods were carried out, with late (P1) and early (P2) vegetative stage forage. For P2, ewes were fitted with ruminal catheters. Forage was distributed at 0900 h, 1300 h, 1800 h and 2300 h, and supplement added at 0900 h and 1800 h meals. Digestibility of the different components of the diets, retained N and rumen microbial protein synthesis were determined. At the end of P2, ruminal pH and N-NH_3 concentration were determined hourly for 24 h. Supplementation increased digestibility of DM ($P < 0.001$) and organic matter (OM; $P < 0.001$) and reduced NDF digestibility ($P = 0.043$) in both periods, with greater values in P2 ($P = 0.008$) for the three diets. Daily mean ruminal pH differed ($P < 0.05$) among treatments: 6.33 (F), 6.15 (FG) and 6.51 (FGM). The high pH in FGM was attributed to $Ca(OH)_2$ in MBP. Therefore, the decreased fiber digestibility in supplemented diets could not be attributed to pH changes. The mean ruminal concentration of N-NH_3 was 18.0 mg/dl, without differences among treatments or sampling hours. Microbial protein synthesis was greater in P2 (8.0 g/day) than in P1 (6.1 g/day; $P = 0.006$), but treatments did not enhance this parameter. The efficiency of protein synthesis tended to be lower in supplemented groups (16.4, 13.9 and 13.4 in P1, and 20.8, 16.7 and 16.2 g N/kg digestible OM ingested in P2, for F, FG and FGM, respectively; $P = 0.07$) without differences between supplements. The same tendency was observed for retained N: 2.55, 1.38 and 1.98 in P1, and 2.28, 1.23 and 1.10 g/day in P2, for F, FG and FGM, respectively; $P = 0.05$). The efficiency of microbial protein synthesis was greater in P2 ($P = 0.007$). In conclusion, addition of feeds containing NFCs to fresh temperate forage reduced the digestibility of cell walls and did not improve microbial protein synthesis or its efficiency. An increase in these parameters was associated to the early phenological stage of the forage.

Keywords: fiber digestibility, fresh temperate forage, microbial protein synthesis, non-fibrous carbohydrate supplementation, sheep

Implications

Semi-intensive grazing systems in southern hemisphere are based on temperate pastures, usually supplemented with different carbohydrate sources to increase their digestibility and the use of N components. In this study, sheep were fed on fresh temperate forage in two stages supplemented or not with grain or a mixture of grain and molasses-based product. Supplementation improved organic matter digestibility of the diet but decreased forage NDF digestibility. Ruminal microbial protein synthesis or its efficiency was not enhanced by supplementation. However, the early phenological stage of the forage, with more protein, soluble sugars and greater cell-wall digestibility, led to higher rumen microbial synthesis.

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Introduction

Supplementation of grazing ruminants with non-fibrous carbohydrates (NFCs) is used to meet high production requirements. Considering the conflict between sustainability of ruminant production systems and the increasing human food requirements, this practice should be revisited and its usefulness should be accurately evaluated. Excessive or inappropriate

Table 1 *Chemical composition (% of DM) of feedstuffs (s.d. in parentheses) and of ingested diets for late (period 1) and early (period 2) forage periods*

Feedstuffs[a]		DM (%)	OM	NDF	ADF	CP	NFC	WSC
Late forage		15.9 (3.1)	89.0 (0.6)	55.4 (4.2)	29.6 (2.5)	11.6 (1.3)	19.0	4.7 (1.0)
Early forage		14.8 (1.7)	87.7 (1.1)	54.6 (3.4)	27.9 (1.8)	14.8 (1.3)	15.3	8.2 (2.0)
Barley		92.4 (0.2)	96.8 (0.3)	16.1 (0.7)	6.0 (0.4)	8.7 (0.1)	69.8	5.3 (0.6)
MBP[b]		97.0 (1.7)	62.7 (0.1)	0.9 (0.1)	0.05 (0.01)	8.5 (1.1)	52.3	25.0 (1.5)
Period	Diet[c]							
	F	–	89.0	55.4	29.6	11.6	19.0	–
1	FG	–	91.3	43.6	22.5	10.8	34.2	–
	FGM	–	86.2	41.4	21.6	10.7	31.6	–
	F	–	87.7	54.6	27.9	14.9	15.3	–
2	FG	–	90.4	43.1	21.3	13.0	31.7	–
	FGM	–	85.3	40.8	20.4	13.0	29.0	–

DM = dry matter; OM = organic matter; NFC = non-fibrous carbohydrates (calculated); WSC = water-soluble carbohydrates; MBP = molasses-based product; F = forage; FG = forage + barley grain; FGM = forage + barley grain + MPB experimental groups.
[a]Forage data = daily (8 days/ period) measurements during the protocol; barley and MBP data = mean of four measurements.
[b]Kalori 3000: sugar cane molasses + soluble condensed molasses + 6.2% $Ca(OH)_2$.
[c]Values of ingested diets were calculated from the data of chemical composition and amount of each feedstuff ingested by each sheep.

energy from carbohydrate sources can adversely affect the cellulolytic flora and fiber digestibility (Martin *et al*., 2006).

Generally, NFC supplementation reduced ruminal NH_3 and enhanced microbial protein synthesis (Bach *et al*., 2005). Nevertheless, reports on NFC supplementation in which the base diet is composed of fresh temperate pastures are scarce and results not consistent with the aforementioned. Aguerre *et al*. (2009), working on pasture supplemented with sorghum at levels of 0.5% to 1.5% of BW, observed a decrease in microbial protein synthesis in wethers but not in heifers. Amaral *et al*. (2011), supplementing ryegrass with cassava meal and corn gluten feed at a level of 7 g/kg in lambs, did not find changes in microbial protein synthesis but observed a decrease in the efficiency of the synthesis. García *et al*. (2000), in dairy heifers grazing oats supplemented with corn or barley grain at 1% of their BW, reported reduced ruminal NH_3 concentration without changes in the production of microbial protein or the efficiency of its synthesis. These different results concerning microbial protein synthesis and its efficiency could be related to the vegetative stage (early or late) of the pasture, the level or the type of supplementation and the intake level. Moreover, in these studies, the ingestion of pasture was not restricted. It seems relevant to explore further the effect of supplementing fresh temperate pasture with NFC on microbial protein synthesis.

The aim of this study was to determine whether NFC supplementation affects fiber digestibility or microbial protein synthesis in restricted-fed sheep offered early or late vegetative fresh temperate forages. This study differed from others in that the sheep were fed with pre-set amounts of each food, including the pasture, and that the same pasture was used in two different periods.

Material and methods

The study was carried out in the Veterinary Faculty Experimental Farm, during mild winter. The experimental protocol was performed in accordance with international ethical guidelines and approved by the Ethical Committee of the Veterinary Faculty of Uruguay (protocol number FV-28.842).

Animals, diets and experimental design

Eighteen adult non-pregnant, non-lactating Corriedale ewes (43 ± 4 kg initial BW) were housed in two lines of nine individual pens (1.10×0.55 m), located in a barn with natural lighting and controlled temperature and hygrometry. The basal diet was temperate pasture (90% *Avena sativa* and 10% *Trifolium repens*) without N fertilization, in early (3040 kg dry matter (DM)/ha, 25 days post-cut) or late (6240 kg DM/ha, 60 days post-cut) vegetative stage, cut fresh every day. A molasses-based product (MBP; Kalori 3000, KK Animal Nutrition Pty Ltd, Umbogintwini, Durban, South Africa) and rolled barley grain (*Hordeum vulgare*) were used as NFC supplements. Water was available *ad libitum*. The chemical composition of feedstuffs is shown in Table 1.

Animals were randomly allocated into one of three groups ($n = 6$ each) receiving the following diets expressed as % of DM: 100% forage (group F), 70% forage + 30% barley grain (group FG) and 70% forage + 15% barley grain + 15% MBP (group FGM). Intake was individually fixed at a level of 40 g DM/kg $BW^{0.75}$ daily (664 ± 99 g DM total intake). For each group two consecutive experimental periods, P1 and P2, were carried out, where P1 represents the late vegetative stage and P2 represents the early vegetative stage. The chemical composition of diets in both periods is shown in Table 1. The forage was distributed four times a day at 0900 h, 1300 h, 1800 h and 2300 h. In groups FG and FGM, the supplement was mixed with forage at 0900 h and 1800 h. For P2 and based on the results observed in P1, sheep were fitted with 30-cm long permanent ruminal polyethylene catheters (inner diameter = 5 mm, outer diameter = 10 mm), with a filter (*in situ* bags, 50 μ pore size nylon, R510, Ankom, NY, USA) around the intraruminal tip, for liquor extraction. The surgery

was performed under local anesthesia (lidocaine at 2%, Fatro Fedagro, Montevideo, Uruguay) after sedation with 0.05 mg/kg i.v. of xylazine (Rompun®, Bayer, Buenos Aires, Argentina). Total duration of the experiment was 42 days as follows: 0 to 10 = adaptation to metabolic cages and P1 diets; 11 to 18 = P1 experimental period (11 to 15 = digestibility and N balance studies, 16 to 18 = microbial protein synthesis determination); 19 = ruminal catheterization; 20 to 33 = post-surgery recovery and adaptation to P2 diets; 34 to 42 = P2 experimental period (34 to 41 = 11 to 18 of P1, 42 = N-NH_3 and pH studies determination of ruminal fluid).

To estimate apparent digestibility of diets, total feces and feed refusals were weighed daily and 10% aliquots taken and frozen until analyzed. Urine was separately collected using a retention catheter placed into the urinary bladder and connected to a plastic bag with 30 ml of 10% H_2SO_4 to maintain the final urine pH below 3.0 (Fujihara *et al.*, 1987). For the microbial protein determination, 30 ml subsamples of urine were collected daily and stored at −20°C for allantoin analyses. For ruminal pH and N-NH_3 determinations, 10 ml of rumen fluid (ventral sac) was individually collected from the ruminal catheter by hourly syringe aspiration during 24 h. The pH was immediately measured (digital pHmeter, Cole Parmer, Vernon Hills, IL, USA) and the samples conserved in 10 ml of 20% NaCl and frozen until N-NH_3 analyses. Animals were weighed at the end of the experiments.

Chemical analyses

After thawing, feed and feces were pre-dried at 60°C to a constant weight, and ground to pass a 1-mm screen (variable-speed rotor mill, Fritsch Pulverisette 14, Idar-Oberstein, Germany, with sieve ring 1-mm trapezoidal perforation). DM (method 934.01), OM (method 942.05), CP (N × 6.25; method 942.01) and ether extract (method 920.39) were determined according to the Association of Official Analytical Chemists (1990) methods, and NDF and ADF according to Robertson and Van Soest (1981). ADF and NDF were assayed sequentially without sulfite and heat stable amylase, and the residual ash content was included in the ADF and NDF content. Each analysis was performed in triplicate. NFC content was calculated as OM − (CP + NDF + ether extract). Water-soluble carbohydrates were determined following the procedure described by Yemm and Willis (1954). Allantoin was analyzed in urine by the colorimetric method proposed by Fujihara *et al.* (1987). Determinations of N-NH_3 were made by direct distillation with sodium tetraborate (Preston, 1995).

Calculations and statistics

Digestibility coefficients for DM, OM, CP, NDF and ADF were calculated as: (g ingested – g feces)/g ingested. The digestible organic matter (OM) ingested (DOMI) was calculated as the OM digestibility coefficient × ingested OM (g). Retained N (g/day) was calculated as daily g of N ingested – g of N excreted (in feces and urine). Microbial protein synthesis was calculated according to the equation $y = e^{(0.830+2.089x)}$, proposed by Puchala and Kulasek (1992), where y = microbial N entering into the duodenum (g/day) and x = urinary excretion of allantoin N (g/day). The efficiency of microbial protein synthesis (EMPS) in the rumen was expressed as g of microbial N/kg of DOMI.

Data were analyzed using the PROC MIXED procedure of SAS (Statistical Analysis System, SAS Institute, Cary, NC, USA). Digestibility, microbial protein production and retained N were analyzed as repeated measures using the sheep as subject for the repeated measurement, according to the model $Y_{ij} = \mu + T_i + P_j + (T \times P)_{ij} + e_{ij}$, where μ was the general mean, T_i the fixed effect of the treatment, P_j the fixed effect of the period of measurement (1 or 2), $(T \times P)_{ij}$ the interaction between treatment and period of measurement and e_{ij} the residual error. The dynamic of pH and N-NH_3 in rumen (measured only in P2) were analyzed also as repeated measures, using the sheep as subject for the repeated measurement, according to the model: $Y_{ij} = \mu + T_i + H_j + (T \times H)_{ij} + e_{ij}$, where μ was the general mean, T_i the fixed effect of the treatment, H_j the fixed effect of the hour of measurement (0000 to 2300 h), $(T \times H)_{ij}$ the interaction between treatment and hour and e_{ij} the residual error. The means of the different treatments were compared using orthogonal contrasts, separating the effect of supplementation (F *v.* FG + FGM) and the supplement used (FG *v.* FGM). Data are reported as means ± s.e. (s.d. for pH and N-NH_3). The statistical significance was declared at $P < 0.05$ and a tendency was noted between 0.05 and 0.1.

Results

BW did not show significant variations during the experiment ($P = 0.48$). The coefficients of digestibility for DM, OM, CP, NDF and ADF for the three diets in both periods are shown in Table 2. No interaction between treatments and periods were observed. Compared to F diet, the DM and OM digestibility of the supplemented diets increased ($P < 0.001$), and were greater for FG than FGM diet ($P = 0.009$ and 0.005 for DM and OM, respectively). Digestibility of CP tended to be affected by treatments ($P = 0.07$). The digestibility of NDF ($P = 0.015$), but not of ADF, was reduced by supplementation without differences between supplements. When comparing periods, the mean values of the three treatments for the digestibility of DM (0.72), OM (0.76), CP (0.74) and NDF (0.69) were greater in P2 than in P1 (0.70, 0.71, 0.70 and 0.64, respectively; $P \leq 0.025$).

Urinary elimination of allantoin, microbial protein yield, EMPS based on DOMI and retained N are listed in Table 3, for the three treatments in both periods. No interaction between treatments and periods was observed. Microbial protein was not affected by treatments. The orthogonal contrasts analysis showed a tendency for a reduction in EMPS and retained N in supplemented groups, without differences between supplements. Microbial protein and EMPS were greater in P2 than in P1 ($P < 0.006$ and < 0.007, respectively).

The ruminal pH values (24-h means and ranges, measured only in P2) were 6.33 for F, 6.15 for FG and 6.51 for FGM, with significant differences ($P < 0.05$) among treatments. The highest mean pH was achieved in the FGM group. The hourly evolution of pH (Figure 1a) showed similar pattern for each

Table 2 *Coefficient of digestibility of DM, OM, CP, NDF and ADF, in the three experimental groups for late (period 1) and early (period 2) forage periods*

	Period 1			Period 2				Treatment	Period	Treatment × period	Contrast (treatment)	
Variable	F	FG	FGM	F	FG	FGM	s.e.	*P*	*P*	*P*	F *v.* (FG + FGM)	FG *v.* FGM
DM	0.67	0.72	0.71	0.71	0.75	0.71	0.011	<0.001	0.025	0.271	0.001	0.009
OM	0.68	0.74	0.72	0.75	0.79	0.75	0.011	<0.001	<0.001	0.415	<0.001	0.005
CP	0.71	0.72	0.68	0.74	0.74	0.73	0.013	0.072	0.016	0.673	0.532	0.029
NDF	0.66	0.61	0.64	0.73	0.68	0.66	0.019	0.043	0.008	0.417	0.015	0.622
ADF	0.58	0.55	0.57	0.61	0.60	0.56	0.024	0.460	0.240	0.487	0.223	0.914

DM = dry matter; OM = organic matter; F = forage; FG = forage + barley grain; FGM = forage + barley grain + molasses-based product experimental groups.

Table 3 *Urinary elimination of allantoin (Uall), estimated production of MP, DOMI, EMPS and retained N in the three experimental groups for late (period 1) and early (period 2) forage periods*

	Period 1			Period 2				Treatment	Period	Treatment × period	Contrast (treatment)	
Variable	F	FG	FGM	F	FG	FGM	s.e.	*P*	*P*	*P*	F *v.* (FG + FGM)	FG *v.* FGM
Uall (mmol/kg $BW^{0.75}$)	0.49	0.44	0.46	0.63	0.59	0.60	0.056	0.817	<0.001	0.985	0.55	0.85
MP (g/day)	6.29	6.09	5.93	8.30	8.09	7.55	0.912	0.892	0.006	0.915	0.71	0.77
DOMI (g/day)	382	459	442	409	482	463	23.501	0.096	0.002	0.833	0.04	0.67
Ingested N (g/day)	57.2	56.9	61.2	79.4	65.4	70.5	5.806	0.498	0.001	0.704	0.37	0.45
Retained N (g/day)	2.55	1.38	1.98	2.28	1.23	1.10	0.494	0.127	0.268	0.705	0.05	0.67
EMPS (g N/kg DOMI)	16.4	13.9	13.4	20.8	16.7	16.2	1.812	0.173	0.007	0.836	0.07	0.89

MP = microbial protein; DOMI = digestible organic matter intake; EMPS = efficiency of microbial protein synthesis; F = forage; FG = forage + barley grain; FGM = forage + barley grain + molasses-based product experimental groups.

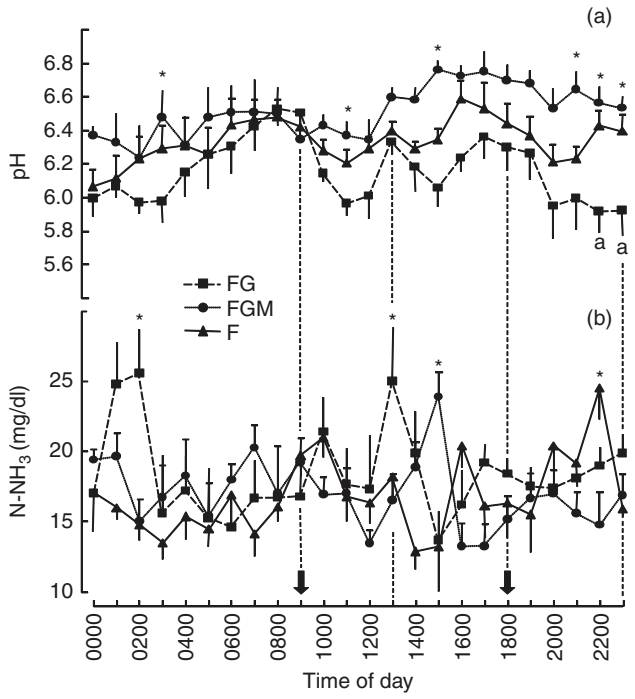

Figure 1 Mean ruminal pH (a) and N-NH_3 concentration (b) for the three experimental treatments (F = forage, FG = forage + barley grain, FGM = forage + barley grain + molasses-based product) during the daytime in period 2 ($n = 6$ sheep per group). Vertical dashed lines indicate forage distribution; arrows indicate supplement distribution for FG and FGM groups; $^{*}P < 0.05$ between supplemented groups; $^{a}P < 0.05$ between F and FG.

experimental treatment with no interaction hour × treatment ($P > 0.05$). In F and FG groups, pH dropped after meals. When pH was measured 7 to 10 h from the last meal (between 0600 h and 0900 h), mean values were high (~6.4) and without differences among the three treatments ($P = 0.73$).

The average values of the ruminal concentration of N-NH_3 (measured only in P2) were 17.4, 18.5 and 18.0 mg/dl for F, FG and FGM groups, respectively, without any significant difference among treatments or sampling hours for the same treatment. The hourly evolution of N-NH_3 for each treatment is observed on Figure 1b. Only few values were above 25 mg/dl, but at different times and not related to forage or supplement distribution.

Discussion

Digestibilities of DM and OM were greater in P2 and, although similar NDF concentrations were found in late and early forages, the NDF digestibility was also greater (11%) in P2. These effects can be attributed to the earlier phenological stage of the pasture. In both periods, NFC supplementation increased DM and OM digestibility but decreased NDF digestibility showing an interaction between NFC and forages. This concerned mainly hemicelluloses because ADF digestibility was not modified by the NFC supplementation. The observed greater digestibility for DM and OM in supplemented groups is consistent with the data found in the review by Poppi and McLennan (1995), and could be related

to the inclusion of highly digestible compounds as grain or MBP. The depressed NDF digestibility by supplements may indicate a change in the microbial population profile, prevailing the soluble-carbohydrates users. The retention time of particles in the rumen should also be considered to explain digestibility results. As the study was carried out on restricted intake (without refusals), the reduction in digestibility should not be associated with changes in total food intake. However, NFC supplementation could decrease the retention time of particles in the rumen and thereby the digestibility. The fact that DM and OM digestibility were greater with barley grain alone than with barley grain + MBP, could be attributed to redirection of the enzymatic activity of microorganisms to the rapidly fermentable substrate of added molasses, as suggested by Obara *et al*. (1991), although the MBP had only 25% of water-soluble carbohydrates. Another possible explanation is the catabolite regulatory mechanism ('catabolite repression'), well documented in rumen bacteria (Russell and Baldwin, 1978).

The mean hourly rumen pH values measured in this study for group F agree with those reported by Cajarville *et al*. (2006) on fresh forage-fed sheep, and were reasonably within the range of 6.2 to 7.0 stated by Owens and Goetsch (1988) for ruminants fed on forage-based diets. Unexpected high pH values were observed in FGM treatment. One possible explanation for our result could be related to the high proportion of $Ca(OH)_2$ (6.2%) of the Kalori 3000, that would alkalinize the rumen fluid. In fact, $Ca(OH)_2$ solutions have been used for the treatment of lactic acidosis in goats (Cao *et al*., 1987). Another explanation could be that, as the content in water-soluble carbohydrates of MBP (25%) was determined by analysis and its NFC content (52.3%) was calculated, the product could not have as much fermentable carbohydrate as the calculated NFC value would suggest.

The lower pH values observed in FG in this study are probably due to the rapidly fermentable starch present in the barley grain (reviewed by Peyraud and Apper-Bossard, 2006). It is unlikely that these changes affected the cellulolytic flora, as pH was below the critical value of 6.0 for less than 4 h (Calsamiglia *et al*., 2002). Another result, consistent with this idea, is that NDF digestibility was similarly reduced in both supplemented groups, meanwhile pH was only reduced in FG group. Calsamiglia *et al*. (2008), working *in vitro* and Martin *et al*. (1999) working *in vivo*, attributed the reduction in NDF digestibility primarily to pH changes. Moreover, Huhtanen and Khalili (1992), working *in vivo*, reported that the use of supplementation of diets containing sucrose with sodium bicarbonate, resulted in higher carboxymethylcelulase and xylanase activities than sucrose diets without buffer. Martin and Michalet-Doreau (1995), working with barley-supplemented cows with or without infusions of bicarbonate salts observed that, although the density of microorganisms was similar, glycosidase activities were higher when buffer was added. In present experimental conditions, it seemed that the reduction of NDF digestion could be associated with other fermentation products related to the diets instead of pH reduction. This decrease may be probably related to a 'substrate' effect via a catabolite repression by molecules released during NFC hydrolysis (Huang and Forsberg, 1990) or as proteinaceous inhibitor produced during glucose fermentation (Piwonka and Firkins, 1996).

The average concentrations of $N\text{-}NH_3$ in rumen fluid observed for the three treatments were above the values of 5 mg/dl established by Satter and Slyter (1974) for optimal microbial protein synthesis. It has been shown that fermentable carbohydrate supplementation reduces ruminal $N\text{-}NH_3$ increasing its capture by microorganisms and its subsequent use in protein synthesis (Berzaghi *et al*., 1996). However, Wiedmeier *et al*. (1992), comparing the effect of molasses and a molasses by-product on digestibility and rumen fermentation in hay-fed cattle, obtained $N\text{-}NH_3$ concentrations similar to those reported in this study without significant differences between treatments. Moreover, in sheep fed silage fodder, the daily intraruminal continuous infusion of 150, 300 and 450 g of sucrose (~4 to 10 times the ingested sucrose in FGM group) did not change $N\text{-}NH_3$ concentrations (Kim *et al*., 2005).

Urinary excretion of allantoin was within the range of 6.4 to 10.6 mmol/day, reported by Pérez *et al*. (1997), in sheep fed different protein sources and supplemented with high and low levels of barley. Trevaskis *et al*. (2001) also reported similar values (5.5 to 9.0 mmol/day) in sheep receiving forage and supplemented with barley. The reported increase in urinary excretion of purine bases, including allantoin, after energy supplementation (Chen *et al*., 1992; Pérez *et al*., 1997), was not observed in this study. Similarly, Jetana *et al*. (2000) did not find differences in the elimination of allantoin between control and supplemented (corn flour or paper pulp) forage-fed sheep.

The daily production of microbial protein estimated from urinary allantoin were within the range of 4.5 to 9.1 g/day observed by Chen *et al*. (1992). The values of EMPS related to DOMI were consistent with the 12.8 to 15.8 g N/kg DOMI reported by Chen *et al*. (1992). In both periods, the addition of rapidly fermentable energy sources did not improve microbial protein synthesis or EMPS. This raises the question of what other factors were limiting that would reduce the response to added fermentable carbohydrate. This result was consistent with the absence of effect on $N\text{-}NH_3$ concentration and was in accordance with the fact that supplemented sheep showed a clear reduction of retained N. This reduction was not related to the amount of N ingested. Unexpectedly, the increase in the energy intake (assuming the DOMI as an index of the ingested energy) did not enhance the N retention. Although in a previous study (Tebot *et al*., 2004) microbial protein synthesis was significantly increased by energy supplementation, the base diet was not fresh forage. Amaral *et al*. (2011) observed that starch supplementation did not improve the microbial protein entering the duodenum and even reduced the EMPS in lambs fed a diet based on ryegrass. Moreover, Aguerre *et al*. (2009) reported that ruminal microbial protein synthesis decreased linearly with increased grain addition and that EMPS was not affected by energy supplementation in wethers fed on temperate fresh pasture.

In the studies aforementioned, the levels of carbohydrates supplementation and the ingested DM and OM varied greatly among treatments and, consequently, also varied the supply of fermentable substrates to ruminal microbes. In contrast, in this study, a fixed level of supplementation was used and the amount of DOMI was similar for FG and FGM treatments. This fact was reflected in ruminal pH and N-NH_3 that did not show major changes that could have promoted changes in microbial protein production. The values of N-NH_3 were not limiting for the protein synthesis, but we assume that both the energy expenditure and N were focused to the maintenance rather than microbial growth, as has also been suggested by Feng *et al.* (1993) in lactating dairy cows.

When evaluating the NFC content in the diets as a rapid source of energy, different compounds and proportions could be included in this term, due to the large variations of sucrose, starch and pectin in food. As a consequence, increased dietary NFC does not necessarily mean better EMPS. Stokes *et al.* (1991), working with corn cobs as forage, found that bacterial efficiency rose when dietary NFC was increased from 25% to 37%, but only small improvements were observed at greater dietary NFC. Nevertheless, in this study, the NFC values for supplemented groups (29% to 39%) did not lead to a higher microbial protein synthesis. Bach *et al.* (1999), working *in vitro* with grass and legumes as forages supplemented with soybean hulls, beet pulp and corn, reported that the increment in NFC resulted in a rise of glycogen content in microbial cells and a decrease in bacterial N. Similarly to this study Feng *et al.* (1993), providing high and low NFC diets combined with rapidly or slowly degraded NDF on dairy cows, found that an increase from 29% to 39% of NFC in the diet resulted in a reduction in the EMPS. Although supplementation did not increase microbial protein yield, greater values for both measures and for DOMI were observed during P2 for all treatment groups. This could be due to the earlier phenological stage of the forage during P2. Early forage had lower NFC content and microbial protein synthesis and EMPS were greater when animals consumed this forage. A possible explanation for present results is that, even though both forages had similar NDF concentrations, the NDF digestibility was greater in forage in early phenological stage, leading to a higher energy availability in rumen. In addition, water-soluble carbohydrates and CP were greater in early than in late phenological stage of forage. In fact, Brito *et al.* (2009), feeding dairy cows with silage from alfalfa cut at sundown, with greater sugar concentration than when cut at sunup, observed an increased capacity of microbes to uptake N-NH_3 and convert it to microbial protein. Therefore, it seems that the impact of the diets on microbial protein production and EMPS was mainly related to the pattern of supply of the forage fermentable carbohydrates (as also proposed by Hall and Huntington, 2008), and that the NFC supplementation and the consequent increased DOMI did not improve these parameters.

In conclusion, although supplementation with NFC increased digestibility of the diets and kept ruminal parameters within standard ranges, the digestibility of the cell walls was reduced. The microbial protein synthesis and its efficiency were not modified when supplementation increased the NFC of the diet. Instead, a clear increasing effect on both parameters was associated to the greater NDF digestibility and soluble sugars content of the early stage of the forage. Therefore, in our experimental conditions, NFC supplementation of temperate fresh forage did not lead to the expected increase in microbial protein synthesis. Present results suggest the need to redesign the strategies for efficient use of grain supplementation and environment-friendly feeding practices.

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