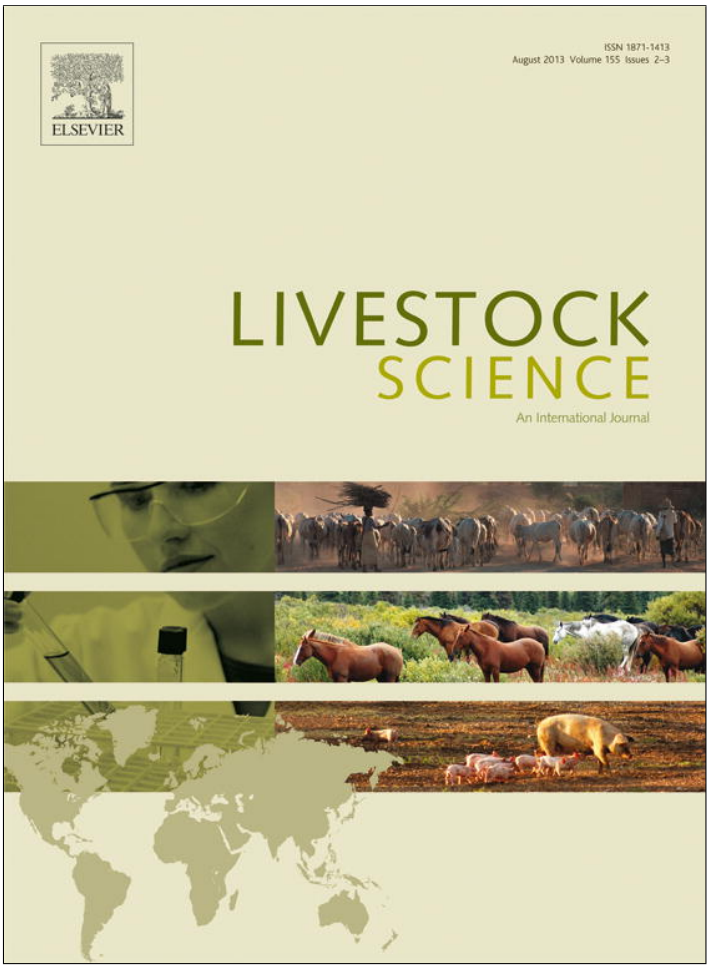

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Effects of urea supplementation on nutrient digestibility, nitrogen utilisation and rumen fermentation in sheep fed diets containing dates

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ABSTRACT

Summary: The aim of the study was to determine the influence of increasing levels of urea (i.e., 0 (U0); 10 (U10); and 15 (U15) g kg^{-1} of concentrate) in sheep fed diets containing dates (local name: Azzawi), on nutrient intake and digestibility, N utilisation and ruminal fermentation. To maintain iso-nitrogenous and iso-metabolisable energy diets, the dates were added with increasing levels of urea. Sheep were fed a 400:600 (dry matter (DM) basis) concentrate:berseem hay (*Trifolium alexandrinum*) diet. Twelve Barki sheep (53.8 ± 1.95 kg body weight) with three/diet were used in a randomised block design to determine digestibility and N balance, while four ruminally cannulated Barki sheep (56.6 ± 2.15 kg body weight) were used in a 3×3 Latin square design to determine rumen function. Experimental periods were 22 days with the first 15 days for adaptation. The calculated metabolisable energy (MJ kg^{-1} DM) and actual crude protein (CP; g kg^{-1} DM) contents were 12.17 and 156.1, 12.69 and 158.2 and 12.60 and 154.8, for the U0, U10 and U15 diets, respectively. Increased urea feeding increased ($P < 0.05$) digestibility of DM, organic matter (OM) and CP. Rumen ammonia N concentrations, allantoin in urine and the resultant microbial N supply increased linearly ($P < 0.05$), as did the total ruminal volatile fatty acid concentrations. Results suggest that urea supplementation to sheep diets containing dates improved DM, OM and CP digestibility and substantially increased rumen microbial growth as well as ruminal fermentation function.

1. Introduction

As dates produced in the Siwa Oasis of Egypt are not edible by humans, farmers in the region use date by-products as ruminant feeds, intact or after grinding, as a source of energy to replace some dietary corn grain. El-Shaarawy et al. (1989) reported that dates are rich in carbohydrates (i.e., 800 g kg^{-1} dry matter (DM)) and they are an excellent source of sugars, minerals and vitamins, while the fibre specify type of fibre content of date is only about 80 g kg^{-1} DM (Lambiote, 1982).

A high content of readily soluble sugars in ruminant diets can interfere with rumen function (Bouabidi et al., 1996) as they are water-soluble carbohydrates which are readily available in the rumen. Sugars ferment faster than starch or fibre in the rumen, and the Cornell Net Carbohydrate and Protein System assumed a fermentation rate of 300% h^{-1} for sugars (Sniffen et al., 1992).

The digestive process of ruminants is affected by a variety of factors, among them the proportion of degradable dietary N

Abbreviations: ADF, acid detergent fibre; BW, body weight; CP, crude protein; DM, dry matter; EE, ether extract; ME, metabolisable energy; NDF, neutral detergent fibre; NPN, non-protein N; OM, organic matter; NFCs, non-fibre carbohydrates; VFAs, volatile fatty acids..

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Table 1
Dietary ingredients[1] and composition (g/kg) used in formulating the concentrate mixture as well as the composition of the roughage used.

	Level of urea (g/kg concentrate dry matter)			Berseem hay (*Trifolium alexandrinum*)	Dates
	U0	U10	U15		
Ingredient composition					
Soybean meal	170	90	50		
Cottonseed meal	150	150	150		
Dates	550	600	635		
Wheat, bran	110	130	130		
Urea	0	10	15		
Salt	7	7	7		
Limestone	10	10	10		
Vitamin mineral premix[2]	3	3	3		
Feed cost/ tonnes (US$)	284	263	251	200	167
Chemical composition					
Organic matter	881	878	881	847	948
Crude protein	156	158	155	140	73
Ether extract	49	43	43	37	41
Nonfiber carbohydrates[3]	410	411	417	143	649
Neutral detergent fiber	266	265	266	526	186
Acid detergent fiber	194	198	200	388	175

[1] Diets of sheep contained 400 g of concentrate kg of the diet DM with 0 (U0, no urea), 10 (U10) and 15 (U15) g of urea per kg of the total concentrate mixture, and 600 g of berseem hay (*Trifolium alexandrinum*) as the forage/kg of diet.
[2] Composition /kg: vit. A, 4,500,00 IU; vit. D3, 1,166,666 IU; vit. E, 333 mg; Mn, 2666 mg; Zn, 20000 mg; Se, 116 mg; Co, 333 mg; Fe, 16666 mg; Cu, 1000 mg.
[3] Nonfiber carbohydrates (NFC) determined by difference (100 - (% ash+% CP+% EE+% NDF)).

needed to support ruminal microbial activity. The rumen microorganisms ferment ingested organic matter (OM) to obtain energy for maintenance and growth and produce volatile fatty acids (VFAs) and ammonia. Russell et al. (1992) reported that microbial growth is proportional to the intake and extent of fermentation of carbohydrates as long as an adequate N source is available to the bacteria. Mehrez et al. (1977) observed that the optimum microbial growth depends on the maximum rate of fermentation, which depends on the maximum ammonia concentration and the minimal ammonia concentration for maximal rate of fermentation was estimated to be 23.5 mg dl^{-1} rumen fluid.

Urea is widely used as a dietary supplement for ruminants because it is an inexpensive nitrogenous compound and has long been accepted as a replacement for some of the degradable true protein in diets (Van Horn et al., 1969; Pinos-Rodríguez et al., 2010). Urea is rapidly and extensively degraded in the rumen yielding maximum ammonia concentrations within the first few hours of ingestion (Broderick and Wallace, 1988; Puga et al., 2001). Wallace et al. (1979) observed that addition of urea to the diet of sheep fed barley grain altered the rate of ruminal fermentation, quantities of some ruminal bacterial populations and activity of some enzymes. Leibholz (1980) evaluated variables of ruminal and total tract digestion in calves fed diets with different urea concentrations but, besides urea concentration, the experimental diets also varied in N content and the forage to concentrate ratio.

The aims of this study were to determine if there was a benefit to supplementation of urea to diets containing dates on nutrient digestibility, N balance and rumen function of sheep.

2. Materials and Methods

2.1. Feeds and diets

Three experimental diets were formulated to contain 400 g of concentrate kg^{-1} of diet DM with 0 (U0, no urea), 10 (U10) or 15 (U15) g of urea kg^{-1} of the total concentrate and 600 g of berseem hay (*Trifolium alexandrinum*) as the forage kg^{-1} of diet DM (Table 1). In the concentrate mixture, urea replacement was approximately iso-nitrogenous, and the resultant loss of volume was made up by adding dates to the concentrate. Thus, the concentrate mixture was iso-nitrogenous and iso-metabolisable energy to the control rations (U0). Sheep were fed restricted amounts of the diets to meet their requirements (NRC, 1985) twice daily in equal portions at 0800 and 1600 h and had continuous access to fresh water and a vitamin/mineral block.

2.2. Animals, samples and experimental design

2.2.1. Digestibility and nitrogen balance experiments

Digestibility and N balance experiments were conducted using twelve Barki sheep (53.8 ± 1.95 kg of body weight (BW)) with four sheep fed each diet. The sheep were housed individually in metabolism crates which allowed separate collection of urine and faeces. Sheep were allowed 15 days to adapt to the diets before a 7-days collection period. An aliquot (100 g kg^{-1}) of total faecal output was collected each day for digestibility determination and dried at 60 °C for 72 h to constant weight before analysis. Urine was collected into buckets containing 100 ml of 100 ml l^{-1} (v/v) sulphuric acid to keep the urine pH below 3.0 and prevent bacterial activity

in urine. The volume of urine at each sampling was determined, and a 100 ml l^{-1} sub-sample was collected from each sheep and frozen until analysis for total N. An additional subsample was diluted by a factor of five with tap water and frozen for analyses of allantoin. Blood samples were collected after 0.5, 2, 4 and 6 h post feeding from the jugular vein and heparin (10–15 μl ml^{-1} blood) was used as the anticoagulant (Korte and Prohaska, 1987) prior to centrifugation at 2500 × *g* for 20 min at 4 °C and then stored at −20 °C for later analysis of urea.

2.2.2. Rumen function

Four ruminally cannulated Barki sheep (56.6 ± 2.15 kg BW) were used in a 3 × 3 Latin square design experiment. After a 15-days period of diets adaptation, ~100 ml of ruminal contents were collected through the cannula of each sheep 0, 2, 4, 6 and 8 h after the morning feeding, strained through four layers of cheesecloth and the pH of the resultant fluid recorded using a digital pH meter. Three drops of HCl were added to stop microbial activity, and samples were stored at −20 °C until further analyses of ammonia and total and individual VFAS.

2.2.3. Laboratory analyses

Feeds and faces were analysed in duplicate for DM (#934.01), ash (#942.05), N (#954.01) and ether extract (EE; #920.39) according to Association of Official Analytical Chemists (AOAC, 1997). Neutral detergent fibre (NDF, Van Soest et al., 1991) and acid detergent fibre (ADF) (AOAC, 1997; #973.18) were also determined. NDF was assayed without use of an alpha amylase but with sodium sulphite in the ND. Both NDF and ADF are expressed without residual ash. Ammonia N concentrations were determined by Kjeldahl distillation according to AOAC (1997; #954.01). Total and individual VFAs were analysed according to Erwin et al. (1961) using a gas chromatograph (Agilent 6890 N; Agilent Technologies, New York, NY, USA). Allantoin was measured colourimetrically by the method of Young and Conway (1942). The principle of this method is that allantoin is the first to be hydrolysed under weak alkaline condition at 100 °C to allantoic acid, which was hydrolysed to urea and glyoxylic acid in a weak acid solution. Glyoxylic acid was then reacted with phenylhydrazine hydrochloride to produce a phenylhydrazone derivative of the acid, which formed an unstable chromophore with potassium ferricyanide, and colour was read on an ultraviolet (UV) spectrophotometer at 522 nm (Jenway, 6105). Plasma urea concentration was determined according to Tietz (1986).

2.3. In vitro gas production

To measure gas production (Menke and Steingass, 1988), rumen liquor and particulate matter (~50:50) were collected from four rumen fistulated Barki sheep fed berseem hay and a commercial concentrate mixture (~60:40) before the morning meal into pre-warmed thermos flasks, then streamed through a cheesecloth. Triplicate 200 mg samples were weighed into 100-ml calibrated glass syringes. Buffered rumen fluid, 30 ml, was placed in each syringe, which was immediately placed into the water bath at 39 °C. Three syringes containing only 30 ml buffered rumen fluid were the blanks. Another three syringes, each containing 200 mg of corn grain reference standard and 30 ml buffered rumen fluid, were also incubated together with the other test and blank syringes. Gas production was recorded after 3, 6, 9, 12, 24 and 48 h of incubation. Total gas values were corrected for the blank incubation, and reported gas values were expressed as ml per 200 mg of DM. Two runs were completed for each sample and, in each run, three replicates/sample and three blanks were included. The metabolisable energy (ME, MJ kg^{-1} of DM) in feeds was calculated as:

$$ME = 1.06 + 0.157*GP + 0.084*CP + 0.22*EE - 0.081*ash$$

where GP is 24 h net gas production (ml/ 200 mg DM), and crude protein (CP), EE and ash are all expressed as % DM. The GP characteristics were estimated by fitting the mean gas volumes to the exponential equation of Ørskov and McDonald (1979) as: GP (ml/200 mg DM) = a+b $(1 - e^{-ct})$ where GP is the gas production at time '*t*', 'a' is the soluble gas fraction, 'b' is the insoluble gas fraction and 'c' is the gas production rate per hour.

2.4. In situ procedure

Two polyester bags (100% Dacron polyester) 7 × 15 cm with a pore size of 45 μm were used at each incubation time. About 3 g of the ingredients were placed in each bag, which was incubated in the rumen of each sheep and removed after 3, 6, 12, 24 and 48 h, rinsed in cold water until the water became clear and then gently squeezed prior to storage at −20 °C. Frozen bags were thawed and washed again in running water as described by Kamel et al. (1995) to eliminate microorganisms attached to the residue, after which the bags were drained, dried for 72 h at 65 °C, cooled in a desiccator and weighed. The DM and CP contents were then estimated.

Two bags were washed in running water for 15 min to determine initial losses (i.e., fraction 'a'). The kinetics of DM and CP disappearance were fitted to the equation of Ørskov and McDonald (1979): P = a+b $(1 - e^{-ct})$, where P represents disappearance at time '*t*' and 'a', 'b' and 'c' are the estimates of the soluble fraction, the degradable fraction and the rate of degradation of the degradable fraction, respectively.

2.5. Calculations and statistical analysis

Allantoin, uric acid, hypoxanthine and xanthine (Y, mmol day^{-1}) were used to calculate microbial purines absorbed (X, mmol day^{-1}) by the equation

$$Y = 0.84X + (0.15*(BW^{0.75}e^{-0.25X}))$$

where BW is in kg and microbial N supply (g day^{-1}) was calculated from the relationship

$$70X/(0.83 \times 0.116 \times 1000)$$

where 70 is the content of purines in rumen content (mg N $mmol^{-1}$), X is as defined above, 0.83 is the assumed

digestibility of microbial purines, 0.116 is the ratio of purine N/total N in mixed rumen microbes and 1000 converts milligrams to grams (Chen and Gomes, 1992).

Data on nutrient intake, digestibility, N utilisation, allantoin and microbial N supply were analysed as a completely randomised design and subjected to the GLM procedure of SAS (2002) with animal, period and treatment as effects. Data of rumen activities (i.e., pH, ammonia N and total and individual VFAs) were analysed as a 3 (treatments) × 3 (experimental periods) Latin square design but without block as an effect. The model sensitivity was improved by using an extra animal, to the treatments effects, which was allotted randomly to the treatments across periods. A linear polynomial contrast was used to examine responses of diets to increasing addition of urea.

3. Results

The levels of all major nutrients were similar among concentrates (Table 1). There was no effect of urea supplementation on nutrient intake, but increasing urea levels of the diets linearly increased ($P<0.05$) digestibility of DM, OM, CP and non-fibre carbohydrate (Table 2). There was no effect of urea supplementation on digestible energy or ME.

Urea supplementation did not affect N intake or N excreted in urine or the N balance, although there was a trend ($P=0.06$) to a lower N excretion in faeces (Table 3). However, allantoin ($P<0.01$), uric acid ($P<0.04$), hypoxanthine ($P<0.02$) and xanthine ($P<0.01$) and total purine derivative excretion levels in urine ($P<0.01$) as well as microbial N production ($P<0.01$) and plasma urea concentrations ($P=0.04$) all increased linearly with urea supplementation.

Average pH was similar among diets (Table 4), but the ammonia N and total VFAs concentrations increased linearly ($P<0.01$) on increasing the urea levels in diets. While the molar proportions of acetate ($P=0.01$) increased and propionate ($P<0.01$) decreased, the acetate:propionate ratio increased ($P<0.01$). Branched-chain fatty acids also linearly deceased with increasing the urea in diets. Supplementation of urea did not affect *in vitro* gas production parameters (Table 5) or *in situ* parameters (Table 6).

4. Discussion

The lack of a DM intake effect due to substitution of urea for soybean meal is consistent with previous research indicating that forage intake was not affected by increased substitution of non-protein N (NPN) for true protein in the diet (Kropp et al., 1977; Köster et al., 2002). However, others have reported numerical depressions in forage intake due to increased substitution of NPN for true protein in the diet, which have varied from slight (Kropp et al., 1977) to as much as 14% (Forero et al., 1980). That there was a linear increase in digestion of DM, OM, CP and non-fibre carbohydrates with increasing urea levels in the diets was not entirely consistent with the similar DM intakes among diets, while it was likely due to the increased rate of growth of rumen microorganisms due to more available N in the rumen as ammonia from the

Table 2
Effects of urea supplementation on nutrient intake and digestibility (g/kg) and nutritive value of sheep fed diets containing dates[1].

	Level of urea (g/kg concentrate DM)			SEM	*P* Linear
	U0	U10	U15		
Dry matter					
Intake	1077	1100	1055	31.0	0.81
Digestibility	671	682	719	11.0	0.02
Organic matter					
Intake	927	945	909	11.5	0.30
Digestibility	693	694	722	5.7	0.01
Crude protein					
Intake	158	162	154	20.0	0.90
Digestibility	723	744	745	5.6	0.03
Non fiber carbohydrates					
Intake	428	452	440	7.4	0.53
Digestibility	774	817	843	11.8	< 0.01
Neutral detergent fiber					
Intake	452	463	439	12.4	0.47
Digestibility	509	524	520	5.0	0.19
Acid detergent fiber					
Intake	333	342	325	8.1	0.55
Digestibility	469	478	481	6.0	0.21
Nutritive value					
DE[2], Mcal/kg	2.85	2.91	2.93	0.02	0.08
ME[3], Mcal/kg	2.34	2.38	2.41	0.02	0.08

[1] Diets of sheep contained 400 g of concentrate kg of the diet DM with 0 (U0, no urea), 10 (U10) and 15 (U15) g of urea per kg of the total concentrate mixture, and 600 g of berseem hay (*Trifolium alexandrinum*) as the forage/kg of diet.

[2] DE, Digestible energy (Mcal/kg) = 0.04409 × Total digestible nutrients (%)

[3] ME, Metabolic energy (Mcal/kg) = DE × 0.82

Table 3
Effects of urea supplementation on N balance Urinary purine derivatives (PD) and microbial N supply of sheep fed diets containing dates[1].

	Level of urea (g/kg concentrate DM)			SEM	*P* Linear
	U0	U10	U15		
Nitrogen balance (g/d)					
Intake	25.3	24.8	24.7	1.96	0.70
Fecal	8.4	7.3	6.0	0.71	0.06
Urine	12.9	14.0	14.4	0.93	0.30
Net	4.1	3.5	4.4	0.91	0.80
Urinary PD (mg/kg $BW^{0.75}$)					
Allantoin	30.8	40.2	44.2	1.77	< 0.01
Uric acid	6.1	7.9	9	0.46	0.04
Hypoxanthine	2.7	3.4	3.5	0.16	0.02
xanthine	0.6	0.8	0.9	0.06	< 0.01
Total PD	40.2	52.2	57.6	2.81	< 0.01
Microbial N, g/d	3.48	4.52	4.99	0.243	< 0.01
Plasma urea, mg/l	3.8	4.0	4.4	0.18	0.04

[1] Diets of sheep contained 400 g of concentrate kg of the diet DM with 0 (U0, no urea), 10 (U10) and 15 (U15) g of urea per kg of the total concentrate mixture, and 600 g of berseem hay (*Trifolium alexandrinum*) as the forage/kg of diet.

Table 4
Effect of urea supplementation on rumen pH, ammonia N, and total volatile fatty acids (VFA) of sheep fed diets containing dates.[1]

	Level of urea (g/kg concentrate DM)			SE M	*P* Linear
	U0	U10	U15		
pH	6.57	6.56	6.44	0.071	0.32
Ammonia N, mg/dl	24.99	28.06	30.60	0.799	< 0.01
Total VFA, mmol/l	116.3	123.2	138.4	1.81	< 0.01
Individual VFA, mol/100 mol					
Acetate (A)	46.31	48.33	50.10	0.665	0.01
Propionate (P)	27.63	25.37	24.73	0.561	< 0.01
Butyrate	18.62	19.77	18.92	0.249	0.58
Isobutyrate	1.95	1.70	1.66	0.087	0.21
Isovalerate	2.45	2.04	1.97	0.083	< 0.01
valerate	3.08	2.65	2.48	0.102	0.004
Total branched chain-VFA	4.40	3.74	3.62	0.152	0.03
A/P ratio	1.68	1.91	2.03	0.063	0.03

[1] Diets of sheep contained 400 g of concentrate kg of the diet DM with 0 (U0, no urea), 10 (U10) and 15 (U15) g of urea per kg of the total concentrate mixture, and 600 g of berseem hay (*Trifolium alexandrinum*) as the forage/kg of diet.

Table 5
Observed cumulative *in vitro* gas production after 24 h incubation and metaboliasble energy of feeds and their characteristics obtained by fitting data to the equation: $GP = a + b\ (1 - e^{-ct})$.

	Level of urea (g/kg concentrate DM)			SEM	*P* Linear
	U0	U10	U15		
Gas produced at 24 h (ml/200 mg DM)[1]	69.7	73.2	72.6	2.06	0.24
a	25.0	27.2	27.7	1.03	0.14
b	39.1	38.7	36.7	1.41	0.17
c	0.0533	0.0437	0.0407	0.005	0.16
ME (MJ/kg DM)	12.2	12.7	12.6	0.32	0.23

a; soluble fraction (ml/g OM), b; insoluble fraction (ml/g OM), c; production rate (h^{-1}).

Table 6
Effect of urea supplementation on *in situ* degradability of dates in sheep feed diets containing dates.

	Level of urea (g/kg concentrate DM)			SEM	*P* Linear
	U0	U10	U15		
DM					
a	22.3	23.3	23.2	1.38	0.60
b	44.5	43.8	45.9	2.05	0.59
c % /h	0.082	0.091	0.094	0.002	0.02
CP					
a	23.8	24.6	26.2	1.33	0.23
b	44.1	45.5	46.1	2.17	0.38
c % /h	0.109	0.105	0.110	0.003	0.92

[1]a: soluble fraction (%); b: potentially degradable fraction (%); c: rate of degradation (% h^{-1});

hydrolysis of urea (Boucher et al., 2007). Thus, the similar digestibilities of NDF and ADF among diets are not surprising. Indeed, increasing the proportion of supplemental NPN supplied to forage fed ruminants has had variable effects on fibre digestion (Kropp et al., 1977; Lee et al., 1987; Köster et al., 2002) with some studies reporting a slight depression in fibre digestion, whereas others reported small increases or no effect on fibre digestion (Lee et al., 1987; Robinson et al., 1998; Köster et al., 2002) when NPN replaced soluble and highly degradable true protein in the diet.

Microbial N synthesis (g day^{-1}) increased linearly with increasing urea supplementation, which in reflected in increased purine derivatives (PD) in the urine. The results are similar to Boucher et al. (2007), who reported the optimum ruminal ammonia N concentration required to support maximum synthesis of microbial and maximum efficiency of microbial protein synthesis when a corn silage-based diet was fed to lactating cows.

It is known that the level of NH_3-N in rumen fluid is important since microbial growth is highly dependent on it. Mehrez et al. (1977) stated that the ammonia N concentration in the rumen needs to be 23.5 (or more) mg dl^{-1} rumen fluid since this concentration is necessary for maximal rate of fermentation. Ørskov et al. (1972) showed with a barley-based diet that microbial protein produced was not altered as a result of urea supplementation while the extent of rumen fermentation and digestibility increased. Thus, the concentrations of NH_3-N in the rumen and digestibility were likely to be limiting microbial growth, at least based upon these previous findings, although the large linear increase in microbial N production, and ruminal VFA concentrations, suggests otherwise for the control diet. Harmeyer and Martens (1980) noted that the amount of N excreted in urine is mainly

influenced by the plasma concentration or urea; but this was not the case in our study where plasma urea N concentrations increased while urinary N outputs were not impacted, thereby suggesting efficient use of N in the rumen to support microbial protein synthesis on all diets. This may be supported by Russell et al. (1992) who found that only excessive ruminal production of ammonia N, and its consequent rumen absorption, increases urinary excretion of N.

Higher ruminal VFA concentrations suggest increased fermentation of carbohydrates in the rumen, although the extent of the increase in VFA concentrations (relatively large) is not entirely consistent with the extent of the increase in digestibility (relatively small). The two major dietary components required by ruminal microorganisms for growth are fermentable carbohydrates and rumen-solubilised protein and/or N. Because the carbohydrate composition of the diets was similar among diets, we assume that carbohydrate availability to the ruminal microorganisms was similar among diets. Thus, the positive effect of urea supplementation on microbial growth was likely additive to the higher levels of rumen ammonia N and concurrent release of readily soluble carbohydrates from dates, and ammonia from urea in U10 and U15 diets apparently produced better conditions for microbial growth in the rumen than did the control (U0) diet. Slyter et al. (1979) and Griswold et al. (2003) showed that urea addition results in an increase in total VFA. Hume at al. (1970) did not find differences in ruminal concentrations of total VFA when ruminal ammonia N concentrations were increased in sheep.

The ruminal fluid branched-chain fatty acids isobutyrate and isovalerate were linearly lowered in sheep with increasing urea levels, probably due to reduced microbial deamination of branched-chain amino acids in the rumen with increased NPN content of the diet (Bergman, 1990; Nguyen et al., 2005).

5. Conclusions

Supplementation of urea in a diet containing dates improved digestibility of DM, OM and CP, with increasing rumen microbial N production as well as ammonia N and VFA concentrations. Consequently, it may be useful to use NPN sources such as urea in diets containing high levels of soluble carbohydrates to reduce diet costs. Further experiments are required to study this impact on performance of sheep.

5. Conflict of interest

None.

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