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Research Article

INFLUENCE OF UREA ON *IN VITRO* METHANOGENESIS AND FERMENTATION OF FEED WITH BUFFALO RUMEN LIQUOR

P. N. Chatterjee[1]*, D. N. Kamra[2], N. Agarwal[2] and Aruna Pal[1]

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ABSTRACT: Four wheat straw-based iso-nitrogenous and iso-caloric complete feeds were formulated from commonly used feed ingredients containing graded levels of urea so as to replace 0 (U0), 15 (U15), 30 (U30) and 45 (U45) per cent protein. There was no effect on *in vitro* gas production, but methane production was decreased (P<0.05) by 10.6% at the highest level of urea inclusion in the ration (U45). The loss of GE in the form of methane was 9.25, 14.95 and 18.74 per cent less in U15, U30 and U45 rations, respectively, as compared to control (U0). The production of VFA was not affected but acetate: propionate ratio decreased with increasing level of urea in the diet. No difference was observed in protozoa population except total holotrichs, which was significantly higher (P<0.05) in the U0 ration. There was no effect of the urea levels on *in vitro* true digestibility of feed and the activities of different enzymes like Carboxymethyl cellulose, xylanase and protease. It can be concluded that upto 30 percent of replacement of feed nitrogen by urea, methanogenesis can be reduced leaving any adverse effect in rumen fermentation.

Keywords: Buffalo, Feed fermentation, Methane, Protozoa, Urea

INTRODUCTION

Rumen is a unique fermentation vat in the gastro-intestinal tract of ruminants in which a consortium of microbes acts upon the lignocellulosic feed and bioconverts it into volatile fatty acids, microbial protein, carbon dioxide and methane. In the rumen, methanogenesis is an essential metabolic process for the utilization of molecular hydrogen generated during fermentation of feed; otherwise, the accumulation of hydrogen would result in limiting the rate of feed fermentation [1]. The synthesis of methane in the rumen accounts for 2-12% of the gross energy of feed taken by the animal [2]. Further, methanogenesis by the livestock is also of great concern due to its global warming potential, which is 23 times higher than that of CO_2 [3]. Ruminants are responsible for the production of 20% of the global methane emission [4]. Therefore, methane mitigation will improve the feed efficiency of the animals as well as reduce the environmental pollution thus can make livestock production more economical and eco-friendlier.

Diet and feeding schedules have a considerable impact on rumen fermentation pattern and methane generation [5] as molar proportions of different VFA influence the amount of metabolic hydrogen generation [6]. Inclusion of feed additives based on plant secondary metabolites and inorganic compounds also affect production of methane and fermentation of feed [7, 8, 9. 10]. Feeding of urea is commonly recommended as non-protein nitrogen source and has been reported to be economically feasible and has no adverse effect when fed below the toxic levels [11, 12]. The influence of dietary urea on ruminal methanogenesis depends on the level of supplementation, diet composition, and synchronization of nitrogen with energy sources available in the rumen [13]. In recent days attempts are in vogue to devise different slow-release urea compounds that can regulate ruminal fibrolytic bacteria and redirect hydrogen utilization away from methanogenesis [14]. The level of supplementation of urea in practical diet need to be determined to chalk out a strategic feeding regime to boost the rumen

[1] *West Bengal University of Animal and Fishery Sciences, Belgachia, Kolkata-700037, India*
[2] *Animal Nutrition Division, ICAR-Indian Veterinary Research Institute, Izatnagar, UP-243122, India*
**Corresponding author. e-mail: chatterjeenutrition@gmail.com*

fermentation. In the present study, we planned to investigate the effect of urea inclusion in feed on *in vitro* methanogenesis and fermentation of feed in the rumen with buffalo rumen liquor as inoculum.

MATERIALS AND METHODS

Preparation of rations

Four iso-nitrogenous and iso-caloric complete feeds, namely U0, U15, U30 and U45 were prepared wherein 0, 15, 30 and 45 per cent of feed nitrogen was replaced with urea (Table 1). Urea was sprinkled over the respective feeds carefully and mixed well for homogeneity. Ingredients of feeds were properly ground, screened through 1 mm sieve and mixed thoroughly and used for the *in vitro* evaluation. Proximate constituents of ration and NDF and ADF contents were estimated as per AOAC [15]. Gross energy content of feeds was estimated by Gallenkamp adiabatic bomb calorimeter (Fischer Scientific Company, USA).

Feeding of animals and sampling of rumen liquor

Rumen liquor was collected from two adult fistulated buffaloes maintained at Animal Nutrition Shed of the institute, and fed on a diet of concentrate mixture (maize, 320 g; solvent extracted soybean meal, 200 g; wheat bran, 450 g; mineral mixture, 20 g and salt, 10 g per kg) and wheat straw (1:1). The wheat straw contained 909 g organic matter (OM); 31 g crude protein (CP); 15 g ether extract (EE); 835 g neutral detergent fibre (NDF) and 535 g acid detergent fibre (ADF) per kg of DM whereas, the concentrate mixture contained 895 g OM; 195 g CP; 40 g EE; 385 g NDF and 164 g ADF per kg of DM. The animals were fed at the rate of 2.5 kg DM/100 kg BW. Rumen liquor sample was collected just before feeding and was transported immediately to the laboratory under anaerobic conditions in insulated flasks, pooled in equal proportion and used as a source of inoculum.

In vitro gas production test

The *in vitro* gas production test was carried out as per Menke and Steingass [16]. The air-dried substrate samples (200±10 mg) from the four rations were weighed and placed in the graduated glass syringes (100 mL), and 30 mL of the incubation medium was dispensed anaerobically into each syringe followed by incubation at 39°C for 24 h. A similar set of syringes was run separately for estimation of *in vitro* true digestibility (IVTD) after termination of incubation, samples were collected for various analyses.

After 24 h of incubation, the gas production was recorded. For methane estimation, 100 µL of headspace gas from the syringe was injected in gas chromatograph (NUCON 5765, AIMIL, New Delhi, India) equipped with stainless steel Porapak-Q column and flame ionization detector (FID). Gas mixture (Spancan, England) consisted of 50% methane and 50% CO_2 was used as standard. Flow rates of nitrogen, hydrogen and air were kept as 20, 30 and 320 mL/min and the oven, injector and detector temperatures were 40, 50 and 50°C, respectively.

Estimation of enzyme activity and metabolites

The contents of the syringes were treated with carbon tetrachloride and lysozyme solution (0.4%) each at the rate of 5 mL per 30 mL of medium and incubated for 3 h at 39°C. The samples were sonicated for 6 min with 30 sec pulse rate in ice bath followed by centrifugation at 27000 × g for 30 min at 4°C. The clear supernatant was used as enzyme source. The activities of carboxymethyl cellulase (CMCase) and xylanase were estimated using Carboxymethyl cellulose and xylan as substrate as described elsewhere [17]. The reducing sugars produced due to enzyme activity were estimated as described by Miller [18]. A unit of enzyme activity was defined as the enzyme which produced one nanomole of reducing sugar per min under assay conditions. Protease activity was measured by using azocaesin as substrate [19]. The enzyme activity was expressed as microgram of azocaesin hydrolyzed per hour.

The ammonia nitrogen in the fermented medium was estimated as per the method of Weatherburn [20]. For estimation of volatile fatty acids, 1ml of the fermented medium was mixed with 0.20 ml metaphosphoric acid (25 ml/100 ml). The mixture was allowed to stand for 2 h at room temperature and centrifuged at 5000×*g* for 20 min. The 1μl supernatant was injected in a chromosorb 101 glass column (4 ft length and 1.8 mm diameter) fitted in gas chromatograph (Nucon-5765) equipped with FID [21]. Microscopic counting of protozoa was done as described earlier [22].

In vitro digestibility

Another parallel set of syringes was run to estimate *in vitro* true digestibility (IVTD) of feed. After 24 h incubation, contents of the syringe were transferred to a spoutless beaker by repeated washings with 100 mL neutral detergent solution. The flask contents were refluxed for 1 h and filtered through pre-weighed Gooch crucibles (Grade G1). The DM content of the residue was weighed and IVTD of the feed was calculated as follows [23].

Statistical analysis

The data were analyzed using Generalized Linear Model ANOVA procedures and differences between the means were compared by Duncan's Multiple Range Test [24].

RESULTS AND DISCUSSION

The chemical composition of different feeds is presented in Table 1, all of which were iso-caloric and iso-nitrogenous having 11.8-12.1% CP and 58.9-60.0% TDN. The NDF was reduced and acid ADF increased with increasing level of urea.

Gas and methane production per gram DM of feed are presented in Table 2. Gas production was not affected (P≥0.05) with inclusion of urea in the feed. Methane production decreased with increasing level of urea in the feed and was significantly (P< 0.05) lower with the U45 feed where 45% of the nitrogen was replaced with urea. There was 1.6, 7.1 and 10.6% less methane production in U15, U30 and U45 feeds, respectively. A distinct significant correlation (r=0.99) was observed between hemicellulose content of feeds and methane production. In the present study, methane production was decreased with increasing levels of maize in the feeds. The results of the *in vivo* experiments also indicated that less methane was generated with increasing level of concentrate in the diet and on replacing fibrous diet with starchy diet [25] [26]. A significant correlation (r=0.95) was observed between total gas and methane production in the present experiment. Chatterjee *et al.* [6] reported a relationship between TDN level in the feed and methane production. The *in vitro* methane production (ml/g DM) was similar in 80 and 100% TDN diets but methane production (ml/g DDM) was lower in 100% TDN feed. In the present study though, the rations were iso-caloric, maize grain was of variable levels.

Gross energy content of diet was higher in high urea containing rations. The per cent loss of GE as methane was in the range of 8.43-6.85% (Table 2) as described by earlier reports [27]. Loss of GE was 18.74, 14.95 and 9.25% in rations U45, U30 and U15, respectively, where 45, 30 and 15% nitrogen was replaced with urea. No difference (P>0.05) was observed in IVTD of rations on urea inclusion in the rations (Table 2). Toppo *et al*. [28] also did not find any difference in digestibility of urea supplemented and the control feeds in adult crossbred cattle.

There was no difference in microbial enzyme activities (Table 3) among the feeds. However, better

Table 1. Ingredients and chemical composition of the rations.

Ingredients	Rations†			
	U0	U15	U30	U45
Ingredients (% as fed basis)				
Maize	8.28	23.77	39.28	50.0
Wheat straw	3.41	15.53	27.66	41.65
Wheat Bran	85.32	57.70	30.06	5.35
Min. mix	2	2	2	2
Salt	1	1	1	1
Urea (g)	0	0.625	1.250	1.875
Chemical composition (% on DM basis)				
Dry matter	96.39	96.64	96.09	95.19
Crude protein	11.89	12.05	11.79	11.99
Total ash	8.25	8.20	8.08	8.46
Organic matter	91.75	91.80	91.92	91.54
Neutral detergent fibre	61.58	60.76	56.93	55.96
Acid detergent fibre	22.81	26.09	29.49	31.75
Hemicellulose	38.77	34.67	27.44	24.21

†Compete feeds containing graded levels of urea replacing 0 (U0), 15 (U15), 30 (U30) and 45 (U45) per cent of feed nitrogen

Table 2. Gas and methane (mL/g DM) production and *in vitro* true digestibility (%) of feed at 24h.

	Ration†			
	U0	U15	U30	U45
Total gas, mL/g DM	214.4±5.80	209.2±4.62	203.9±3.13	202.5±6.22
Methane, mL/g DM	37.44±0.99[a]	36.83±1.33[ab]	34.79±1.05 [ab]	33.47±1.10[b]
IVTD, %	66.49±0.64	66.42±0.57	66.31±0.40	64.42±0.86
NDF digestibility, %	47.55±0.99 [a]	46.59±0.91[ab]	43.14±0.67[bc]	41.45±1.42[c]
GE (kcal/g DM)	4.1983	4.5524	4.5867	4.6194
Energy loss as methane† (kcal/g DM)	0.3538	0.3480	0.3287	0.3163
GE loss as methane (%)	8.43	7.65	7.17	6.85

†Compete feeds containing graded levels of urea replacing 0 (U0), 15 (U15), 30 (U30) and 45 (U45) per cent of feed nitrogen.
[abc]Means with different superscripts in a column differ significantly (P<0.05)
†GE value of methane was considered as 9.45 kcal/L

Table 3. Effect of urea on microbial enzymes activity and rumen metabolites.

	Rations†			
	R1	R2	R3	R4
CMCase, nmoL/min/mL	46.42±1.39	47.41±3.26	46.45±1.73	47.56±2.08
Xylanase, nmoL/min/mL	100.8±2.75	104.1±3.25	105.7±5.91	106.1±4.14
Protease, µg/h/mL	47.21±5.41	60.36±7.65	56.27±7.15	53.34±7.55
NH_3-N, mg/L	260.2± 4.64	264.9±4.44	270.1± 4.38	272.6±6.63
TVFA,mmoL/dL	4.06± 0.38	4.02±0.14	4.41±0.08	4.20±0.14
Acetate,%	71.71±1.52	70.89±0.50	70.08±0.44	70.41±0.42
Propionate,%	19.54±1.58	20.04±0.48	20.60±0.49	20.91±0.34
Butyrate,%	8.75±0.22	9.07±0.30	9.32±0.23	8.67±0.24
Acetate: Propionate ratio	4.07±0.71	3.55±0.10	3.41±0.10	3.37±0.07

†Compete feeds containing graded levels of urea replacing 0 (U0), 15 (U15), 30 (U30) and 45 (U45) per cent of feed nitrogen.

protease activity was observed in urea containing rations. Ammonia nitrogen content increased with increasing level of urea in the rations, but the differences were not statistically significant (P>0.05). Although hydrolysis of urea by rumen microbes is very rapid but its immediate utilization for synthesis of microbial protein might have occurred due to availability of easily utilizable energy source [29]. In the present study the level of maize grain (source of starch) also increased with the increasing level of urea in the rations.

There was no difference in total VFA production among different rations tested (Table 3). The propionate content was considerably higher at two higher urea levels. The unutilized hydrogen due to reduced methanogenesis upon urea inclusion in the diet might have been diverted for propionate synthesis. This is one of the reasons responsible for 12.78, 16.22 and 17.20% lowering of acetate/propionate ratio with increasing level of urea in the rations, respectively.

The values for total and differential protozoa count as influenced by urea are presented in Table 4. There was no variation (P>0.05) in total protozoa and spirotrich counts. A positive correlation (r = 0.43) was observed between protozoa count and methane production. Finlay *et al*. [30] reported that 37% of methanogenesis in the rumen is due to symbiosis between rumen protozoa and methanogens, as some of the archaea remain attached with the exo-skeleton of protozoa to make interspecies hydrogen transfer

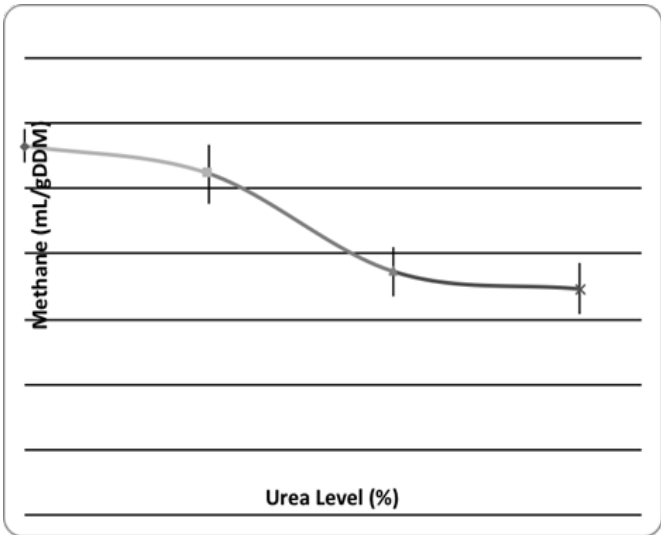

Fig.1. Methane production in buffalo rumen upon inclusion of graded level of urea.

effective. The rations producing more methane also supported higher numbers of protozoa in the present experiment. Our earlier studies have also established that the availability and type of protozoa trigger the fermentation pattern and in turn ruminal methanogenesis [31, 7]. Strategic inhibition of rumen protozoa is often associated with decreased methane output [32, 9].

The results of the present experiment indicated that inclusion of urea in the diet had some impact on the process of methanogenesis without affecting rumen fermentation pattern or digestibility of feed in *in vitro* conditions. Therefore, inclusion of urea in diet can be explored as a feasible nutritional intervention for methane mitigation in ruminants seems to have potential to reduce methane production in the ruminants.

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