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Influence of Level of Feed Intake on Concentration of Purine Derivatives in Urinary Spot Samples and Microbial Nitrogen Supply in Crossbred Bulls

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ABSTRACT : The potential of the spot urine sampling technique as an alternative to performing a total urine collection to predict the microbial nitrogen supply was evaluated in crossbred bulls. In a completely randomized design, 20 growing crossbred bulls were assigned four levels of feed intake (120, 100, 80 and 60% of voluntary dry matter intake) on diets comprised of wheat straw and concentrate mixture (50:50). After three months of experimental feeding, a metabolism trial was conducted for ten days, during which spot urine collections were performed every 6 h post feeding on days 9 and 10. The daily urinary excretion of allantoin (A) and purine derivatives (PD) decreased with the reduction in feed intake while creatinine (C) excretion remained similar in animals fed at different levels. The microbial nitrogen (MN) supply calculated from the PD excreted in total urine (35.08 to 72.08 g/d) was higher at increased levels of feed intake. PD concentration in spot urine samples had poor correlation with feed intake except at 12 h post feeding. A/C ratio and PD/C ratio in spot urine samples remained similar irrespective of sampling time and significantly ($p<0.01$) correlated with daily urinary PD excretion, digestible organic matter intake and dry matter (DM) intake. However, no significant differences were evident in these ratios among animals fed at levels 120, 100 and 80% of voluntary dry matter intake (VDMI) at different times post feeding. These results suggests that the spot urine sampling technique to predict the microbial protein supply is not suitable for detecting small differences in MN supply and hence, estimation of PD excreted in total urine (mmol/d) is necessary to assess precisely the MN supply in crossbred bulls. (**Key Words :** Crossbred Bulls, Different Levels of Feed Intake, Purine Derivatives, Microbial Nitrogen, Spot Urine, PD/C Ratio)

INTRODUCTION

In the tropics ruminants are fed on low quality roughages, crop residues and agricultural by-products, which generally show a large variability in quality. Therefore, measurement and prediction of feeding value and nutritive value of forages are essential for high levels of production (Dynes et al., 2003; Chanjula et al., 2004). The rumen microbes are able to convert fibrous feeds and low quality protein into essential nutrients for the host animal. The synthesis of microbial protein is dependent on ruminal ammonia nitrogen supply (Wanapat and Pimpa, 1999) and digestible organic matter intake (DOMI). The methods generally used for determining microbial protein production depend on the use of natural microbial markers such as RNA (ribonucleic acid) and DAPA (diamino-pimelic acid) or isotopes of ^{35}S, ^{15}N, and ^{32}P. The idea of using purine derivatives (PD) as a specific marker for rumen microbial biomass was suggested by Topps and Elliot (1965). Purines in dietary materials are degraded rapidly by microbial enzymes in the rumen (Smith and McAllan, 1970; McAllan and Smith, 1973) and they are therefore, likely to be present in negligible concentration in digesta leaving the rumen. The microbial purines on the other hand remain intact in living microbial cells and pass via the abomasum to the small intestine where they are degraded enzymatically to nucleotides and purine bases which in turn get absorbed. Therefore, the amount of PD (allantoin, uric acid, xanthine and hypoxanthine) in the urine can be used to predict the flow of microbial purines into the intestines, thus quantifying the intestinal flow of microbial protein to the animal (Verbic et al., 1990; Djouvinov and Todorov, 1994; Susmel et al., 1994). The prediction of microbial protein using urinary PD has special interest since it overcomes the disadvantages of the conventional methods. This technique is non-invasive and simple,avoiding the need for fistulated animals and those measurements related to digesta and microbial marker kinetics. However, the requirement for total urine collection limits the use of this technique under farm conditions. Therefore, a logical step forward would be

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to use the concentrations of PD in spot urine samples as an index of microbial protein supply. A number of studies in ruminant species (Daniels et al., 1994; Gonda and Lindberg, 1994; Chen et al., 1995; Dipu et al., 2006) indicated that purine derivatives: creatinine ratio (PD/C) can predict microbial nitrogen supply with reasonable accuracy as it is little affected by sampling time, while the data of Puchala and Kulasek (1992), Moorby and Dewhurst (1993) and Shingfield and Offer, (1998) indicated a diurnal variation in the PD/C ratio. There is a lack of information regarding the accuracy of the spot sampling technique to assess the daily excretion of purine derivatives under practical feeding conditions in crossbred cattle. Consequently, this study examined the influence of level of feed intake on concentration of purine derivatives in urinary spot samples and microbial nitrogen supply in crossbred bulls.

MATERIALS AND METHODS

Experimental design, diets, animals and feeding

Twenty growing crossbred (Holstein Friesian×Hariana) bulls (>1 year age and 186±6.02 kg mean body weight), were used in the study. They were housed in a well-ventilated shed with facilities for individual feeding under hygienic and uniform management conditions. Prior to initiation of the experimental feeding, the bulls were treated for endo- and ectoparasites. A concentrate mixture was formulated for feeding of animals throughout the experiment. The animals were fed *ad libitum* a mixed diet of wheat straw and concentrate (50:50) individually for one week during the preliminary feeding period. The lowest level of intake recorded during the period for all animals (4.69±0.01 kg DM/d) was set as voluntary dry matter intake (VDMI). Twenty animals were divided into four groups following a completely randomized design. The different groups were fed a mixed diet of wheat straw and concentrate mixture (50:50) at four fixed levels of VDMI. Highest level of feeding in the study was 120% of VDMI (Group 1/L-120), determined in the preliminary feeding period. The other three levels were 100% (Group 2/L-100), 80% (Group 3/L-80) and 60% (Group 4/L-60) of the VDMI. Fresh and clean drinking water was made available *ad libitum* at 10:30 h and 14.30 h. The feeding level in each group was increased fortnightly by considering the VDMI of group 2 (L-100). This procedure was followed throughout the experimental period.

Metabolism trial and collection of samples

A metabolism trial of ten days duration (excluding two days of adaptation period) was conducted after 90 days of experimental feeding. Animals were housed individually in metabolism cages, during which, at 09.00 h daily, total urine excreted (collected in plastic containers containing 500 ml 10% H_2SO_4) together with faeces were recovered, quantified and sub-sampled. Faeces was thoroughly hand-mixed, sub-sampled and collected in a plastic bag for subsequent analysis. Subsequently the animals were given their daily feed, and water was made available *ad libitum* twice daily (10:30 h and 14.30 h). Spot urine collections were performed during the last two days of the metabolism trial for which each day (24 h) was divided into four blocks at six hourly intervals, starting immediately before feeding (designated as 0 h). The first urine excreted by animals in each block was collected and a fixed sample (20 ml) was transferred into plastic vials (containing 2 ml of 10% H_2SO_4). The samples of feed, faeces and urine collected during the metabolism trial were processed and stored as per the approved protocol (IAEA-TECDOC- 945, 1997).

Measurements and chemical analysis

Measurement of apparent digestibility of dry matter (DM) and organic matter (OM) was calculated from the total collection of faeces. A sample of each daily faecal collection was homogenized, weighed and dried to estimate total faecal DM production. Feed samples were composited for the entire trial period. The DM and OM contents of feed and faeces were estimated as per AOAC, 1995. Urine samples were centrifuged, diluted (1:10), filtered (0.22 μm Millipore filter) and analyzed for PD using a Shimadzu HPLC system equipped with a UV detector (205 nm) and two C_{18} reversed-phase columns (250 mm×4.60 mm) connected in series with the mobile phase $NH_4H_2PO_4$-$NH_4H_2PO_4$-acetonitrile (80:20) gradient at a flow rate of 0.8 ml/min (Balcells et al., 1992a). Creatinine was analyzed colourimetrically based on the Jaffe alkaline picrate reaction (AOAC, 1984) using a commercial kit (Qualigens®, India).

Calculations and statistical analysis

The amount of microbial purines absorbed (X, mmol/d) corresponding to the PD excreted (Y, mmol/d) was estimated from the predictive model proposed by Verbic et al. (1990) in intragastric fed steers ($Y = 0.85X+0.385\ W^{0.75}$). The slope (0.85) in the equation represents the recovery of absorbed purines as PD in urine and the value, 0.385 represents the net endogenous contribution of PD to total excretion after correction for the utilization of microbial purine by the animal (Verbic et al., 1990).

The supply of microbial N (g/d) was estimated as follows, from the relationship derived by Chen and Gomes (1992):

$$MN\ (g/d) = 70X/(0.116\times0.83\times1,000) = 0.727X$$

The assumptions made in the above equations are i) Digestibility of microbial purines is assumed to be 0.83.

Table 1. Chemical composition of concentrate mixture and wheat straw (% DM basis)

Attributes	Concentrate mixture*	Wheat straw
Organic matter	90.15	91.74
Crude protein	22.98	3.10
Ether extract	3.37	0.94
Crude fibre	8.81	38.96
Neutral detergent fibre	40.02	88.73
Acid detergent fibre	11.10	52.99
Total ash	9.85	8.26

* Parts/100: maize 32.5, wheat bran 32, soybean meal 32, minerals and vitamins 2.5, common salt 1

This is taken as the mean digestibility value for microbial nucleic acids on observations reported in the literature. ii) The N content of purines is 70 mg N/mmol. iii) The ratio of purine N:Total N in mixed rumen microbes is taken as 11.6:100.

Statistical analysis of all experimental data was performed as per Snedecor and Cochran (1994). A completely randomized design procedure was used in the analysis of variance. The model accounts for variations caused by level of feed intake. The tables describe the mean values, SEM and the level of significance of the effects. The daily excretion of urinary PD (mmol/d) and PD/C ratio of total urine was regressed against the digestible organic matter intake (DOMI) using linear regression analysis.

Assessment of the spot sampling technique was done by calculating the correlation coefficient of the PD/C ratio (and A/C ratio) and PD concentration (mmol/L) in spot urine samples with PD concentration in daily urine sample, total PD output, DM intake and digestible OM intake during various sampling times.

RESULTS AND DISCUSSION

Intake and digestibility

Composition of the concentrate mixture and wheat straw fed to the animals, determined by chemical analysis is given in Table 1. The chemical composition of feeds did not vary throughout the experiment, so it has been concluded that the differences observed for digestive events could be attributed to the effects of intake changes. Table 2 give the values for mean body weight, feed intake (DM and OM) and the apparent digestibility of DM and OM in the whole tract. The level of DM and OM intakes was significantly different ($p<0.01$) between the treatment groups as envisaged by the experimental design. The total DM consumption (g/d) ranged from 3,298.7 in group 4 to 6,450.0 in group 1. The digestibility of DM in groups 1 and 2 was higher ($p<0.05$) compared to that in groups 3 and 4, while the digestibility of OM was significantly higher ($p<0.05$) in group 1 compared to that in groups 3 and 4. It has been suggested that the digestibility of feed is not a fixed trait but is modified by factors such as the level of feed intake (Blummel et al., 2003), age and stage of production. Restriction of feed intake often results in enhanced OM digestibility owing to higher retention time in the rumen (Schneider and Flatt, 1975; Galyean and Owens, 1991; Lechner Dol et al., 1991). However, in the present study, decrease in feed intake did not induce increased DM and OM digestibility which could be expected and this is probably due to lower availability of energy/fermentable OM which limits the growth of microorganisms (Dehority and Orpin, 1988: Tafaj et al., 2005).

Purine derivatives and creatinine excretion at different levels of feed intake

When feed is identical qualitatively, the efficiency of microbial growth will obviously depend upon the level of intake. In this experiment, four levels of feed intake were adopted to create a range of daily PD excretion and thus of microbial N supply under a variety of practical feeding conditions. Dietary treatments had a strong ($p<0.01$) influence on the urinary output of PD whereas no difference was observed in the creatinine excretion (Table 3). The mean PD excretion in urine ranged from 62.01 to 108.67 mmol/day and was similar to values reported (53.7 to 104.4 mmol/d) in crossbred heifers fed barley straw and

Table 2. Mean body weight, DM intake, digestible DM intake (DDM), OM intake, digestible OM intake (DOM) and digestibility of DM and OM in different groups

Parameters	Group I (L-120)	Group II (L-100)	Group III (L-80)	Group IV (L-60)	SEM	Significance
Body weight (kg)	252.65^{a}	212.00^{b}	217.05^{b}	207.00^{b}	16.70	*
Intake (g/d)						
DM	6,450.0^{a}	5,442.4^{b}	4,398.2^{c}	3,298.7^{d}	111.36	**
DDM	4,101.8^{a}	3,324.0^{b}	2,629.2^{c}	1,903.3^{d}	100.13	**
OM	5,865.2^{a}	4,949.5^{b}	4,000.2^{c}	3,000.2^{d}	102.11	**
DOM	3,868.8^{a}	3,136.7^{b}	2,505.3^{c}	1,812.7^{d}	91.83	**
Digestibility coefficient (%)						
DM	63.6^{a}	61.1^{a}	59.8^{b}	57.7^{b}	1.60	*
OM	66.0^{a}	63.4^{ab}	62.6^{b}	60.4^{b}	1.51	*

Means within a row with different superscripts are significantly different, * $p<0.05$, ** $p<0.01$.

Table 3. Urinary purine derivatives, creatinine excretion, PD/C and A/C ratio at different levels of feed intake

Parameters	Group I (L-120)	Group II (L-100)	Group III (L-80)	Group IV (L-60)	SEM	Significance
Allantoin (mmol/d)	102.24[a]	98.66[a]	80.05[b]	56.97[c]	3.68	**
Uric acid (mmol/d)	6.42[ab]	7.57[a]	7.17[a]	5.04[b]	0.86	*
Total PD (mmol/d)	108.67[a]	106.23[a]	87.22[b]	62.01[c]	4.24	**
Creatinine (mmol/d)	60.91	66.30	65.96	57.50	7.45	NS
PD/C ratio	1.87[a]	1.60[ab]	1.35[bc]	1.09[c]	0.151	**
A/C ratio	1.76[a]	1.49[ab]	1.25[bc]	1.00[c]	0.141	**
Microbial-N supply (calculated from total urine)						
g N/d	72.08[a]	72.61[a]	56.00[b]	35.08[c]	3.37	**
g N/kg DOMI	18.77[b]	23.18[a]	22.35[a]	19.41[b]	1.38	*

Means within a row with different superscripts are significantly different, * p<0.05, ** p<0.01. NS: non significant.

concentrate mixture (Martin Orue et al., 2000). Allantoin accounted for above 90% of the total urinary PD excreted and thus corroborated the findings of Verbic et al. (1990), Gonda and Lindberg (1994) and Shingfeild and Offer (1998). However, the proportion of allantoin was slightly lower (83.7 to 87.7%) in a study where Holstein cows were fed with silage as the basal diet (Santoso et al., 2003). No significant amounts of xanthine and hypoxanthine were detected and the level of uric acid in urine was not related to feed intake. The absence of salvageable PD (xanthine and hypoxanthine) in the urine samples confirmed the high ability of cattle to oxidise absorbed purine bases to non-re utilisable PD (Chen et al., 1990; Balcells et al., 1992b).

It has been suggested that the excretion rate of creatinine is relatively constant in healthy animals and remains independent of level of intake (Chen et al., 1992; 1995), as also observed in the present study. Moreover, the use of creatinine as an internal marker of urinary output relies on the assumption that the creatinine excretion through urine is affected neither by diet nor the physiological status of the animal, but is excreted in proportion to body weight. There was little difference in the body weight of the animals used in this study and hence they had similar excretion of urinary creatinine. In contrast, there are reports of increased excretion of creatinine when the proportion of concentrate was increased in the diet (Gonda et al., 1996). The daily mean urinary creatinine excretion observed in this study (57.50 to 66.30 mmol/d) was lower than previous reports of between 112 to 117 mmol/d (Puchala et al., 1993; Susmel et al., 1994; Gonda and Lindberg, 1994). It is conceivable that between breed/species differences in musculature and variations in body weight could account for these discrepancies. Daily mean PD/C ratio calculated from total urine was higher at increased levels of feed intake and the values ranged from 1.09 to 1.87. The relationship between digestible organic matter intake (X, g) and PD/C ratio of total urine (Y) obtained in the present study is as follows:

$$Y = 0.0004X+0.4567 \qquad (R^2 = 0.60)$$

A higher excretion of PD in groups 1 and 2 clearly indicates enhanced microbial protein synthesis in these groups, since significant relationship have already been reported between urinary PD excretion and the level of nucleic acid infused in the abomasum (Geisecke et al., 1984; Fujihara et al., 1987; Chen et al., 1990; Verbic et al., 1990) and duodenum (Antoniewicz et al., 1980). Therefore, the total daily urinary PD excreted may serve as a marker of nutritional status in cattle. However, it is important to recognize that diet of animals may vary qualitatively between farms and therefore it could be a potential source of error in measuring nutritional status based on urinary PD, if digestibility measurements are ignored. It is also conceptually sound to assume that animal performance will largely depend upon the amount of digestible OM consumed (Hall et al., 2004) and hence the use of PD excretion to predict DOMI will be the most appropriate. The relationship between digestible organic matter intake (X, g) and PD excretion (Y, mmol/d) obtained in the present study is as follows:

$$Y = 24.218X+0.399 \qquad (R^2 = 0.76)$$

Microbial nitrogen supply

Daily microbial nitrogen (N) supply (g/d) estimated from the urinary excretion of total PD and the efficiency of microbial N synthesis are summarized in Table 3. Mean values of microbial N synthesized ranged between 35.08 and 72.08 g/d. Feeding levels had significant effects ($p<0.01$) on daily production of microbial nitrogen, but the values were similar in groups 1 and 2. However, the efficiency of microbial N synthesis (gN/kgDOMI) was higher ($p<0.05$) at intermediate levels (L-100 and L-80) of feed intake when compared to the highest (L-120) and lowest (L-60) plane of nutrition. The average values of microbial efficiency (18.77 to 23.18 gN/kg DOMI) from daily urinary PD measurements are broadly within the range reported in the literature (ARC, 1984). Since, microbial protein synthesis is dependent on fermentable OM and ammonia N supply, it is anticipated that the efficiency of synthesis will decrease with the reduction in the feed intake

Table 4. Mean concentration (mmol/L) of purine derivatives and creatinine in spot urine samples, PD/C and A/C ratio at different levels of feed intake

Parameters	Group I (L-120)	Group II (L-100)	Group III (L-80)	Group IV (L-60)	SEM	Significance		
						L	P	L×P
Allantoin	10.01[a]	9.46[a]	9.26[ab]	8.49[b]	1.43	*	*	**
Uric acid	0.929	0.798	0.721	0.758	0.36	NS	**	**
PD	10.94[a]	10.25[ab]	9.99[b]	9.25[c]	1.49	*	*	**
Creatinine	5.24[b]	5.20[b]	5.12[b]	7.47[a]	0.97	**	*	**
PD/C	2.22[a]	2.12[a]	2.02[a]	1.28[b]	0.13	**	NS	**
A/C	2.02[a]	1.95[a]	1.88[a]	1.17[b]	0.12	**	NS	**

Means within a row with different superscripts are significantly different. * p<0.05, ** p<0.01. NS: Non significant.

Table 5. Correlation coefficients of purine derivatives (PD) in spot urine samples with PD concentration in total daily urine sample (PDC), total PD output (PDO), dry matter intake (DMI) and digestible organic matter intake (DOMI) during various sampling times (hours post feeding)

Sampling time (h)	PDC (mmol/L)	PDO (mmol/d)	DOMI	DMI
Concentrations of total PD in spot urine (mmol/L)				
0	0.36*	0.09	0.26	0.25
6	0.25	0.03	0.19	0.19
12	-0.60	0.47**	0.49**	0.47**
18	0.03	0.32*	0.20	0.22
Concentrations of allantoin in spot urine (mmol/L)				
0	0.37*	0.16	0.32*	0.31*
6	0.26	0.01	0.18	0.17
12	0.01	0.46**	0.45**	0.43*
18	-0.03	0.26	0.12	0.14
PD/C ratio in spot urine				
0	0.29	0.51**	0.57**	0.59**
6	0.30	0.37*	0.48**	0.47**
12	-0.21	0.53**	0.45**	0.45**
18	0.10	0.50**	0.50**	0.50**
A/C ratio in spot urine				
0	0.32*	0.53**	0.60**	0.61**
6	0.32*	0.37**	0.48**	0.47**
12	-0.17	0.54**	0.44**	0.44**
18	0.04	0.49**	0.44**	0.45**
Allantoin in total urine (mmol/L)	0.99**	0.12	0.39	0.39
Allantoin (mmol/d)	0.16	0.99**	0.90**	0.91**
DOMI (g/d)	0.38	0.87**		

Values with superscripts indicates significant correlation, * p<0.05, ** p<0.01.

as observed in the present study (from L-100 to L-60). However, animals in group 1 (fed at 120% VDMI), had the lowest efficiency of microbial N synthesis. This variation in the efficiency of microbial N synthesis may relate to the effect of level of feeding on rumen fermentation, particularly the increased chance of low rumen pH at higher levels of feed intake (Shingfeild and Offer, 1998). It has been suggested that the reduction in rumen pH below 6.0 inhibits fibre-degrading bacteria in the rumen (Mould and Orskov, 1983; Mould et al., 1983) and lowers the efficiency of microbial N synthesis due to increased energetic cost of maintaining the integrity of bacterial cells (Malestein et al., 1981).

Purine derivatives and creatinine concentration in spot urine

Mean treatment effects on urinary PD, allantoin and creatinine concentrations in spot urine samples are shown in Table 4. Allantoin accounted for proportionately 0.92 of the total PD with the remainder being uric acid. Urinary PD concentration (mmol/L) varied diurnally and ranged between 9.31 to 10.26, 7.91 to 12.00, 8.96 to 10.95 and 7.94 to 10.07 in groups 1, 2, 3 and 4 respectively. Changes in mean allantoin concentration in the various groups between sampling periods reflected those of total PD. This fluctuation in the concentration of total PD during the day is a reflection of diurnal variation in the absorption of microbial purines from the small intestine and hence, it can be reduced by increasing the feeding frequency (Chen et al., 1992). Therefore, in practical grazing conditions this diurnal variation will be minimum. The concentration of PD in spot urine samples had weak correlations (-0.06 to 0.36) with PD concentration in total urine (Table 5) and had a maximum value at 12 h post feeding (10.48) followed by 18

h (9.46), 6 h (9.32) and 0 h (7.96), but had poor correlations with intakes of DM and digestible OM except at 12 h post feeding (Table 5). In general, the concentration of PD in spot urine samples did not reflect the quantity of feed consumed for reasons related to urine volume and/or tissue catabolism (Nshalai et al., 2000).

Creatinine concentrations in the spot urine samples were higher ($p<0.01$) in L-60 compared to the other 3 groups and was significantly ($p<0.01$) influenced by sampling time and treatment interactions (Table 4). The concentration of creatinine varied from 4.67 to 5.84; 3.87 to 6.22; 4.93 to 5.96 and 6.59 to 8.04 mmol/L for groups 1, 2, 3 and 4 respectively. The concentration (mmol/L) of PD and creatinine in spot urine showed diurnal variation, but the concentrations of allantoin or total PD when expressed as molar proportions of creatinine i.e. allantoin: creatinine (A/C) and PD: Creatinine (PD/C), showed no influence of sampling time and thus confirmed the observations of previous studies (Daniels et al., 1994; Gonda and Lindberg, 1994; Chen et al., 1995). In contrast, the data of Puchala and Kulasek (1992), Moorby and Dewhurst (1993) and Shingfeild and Offer (1998) demonstrated the dependence of PD/C ratio on sampling time. In the present study, A/C ratio and PD/C ratio in spot samples remained similar irrespective of sampling time and had improved correlation with daily urinary PD excretion, digestible OM intake and DM intake, especially in the spot samples collected just before feeding (0 h) (Table 5). However, no significant differences were evident in this ratio between animals fed at levels 120, 100 and 80% of VDMI at different hours post feeding. These results indicate that the crossbred bulls have an inherent ability to maintain a constant A/C or PD/C ratio at a range of 80-120% of VDMI and therefore, the use of A/C or PD/C ratio as an index of microbial protein supply is suitable only for detecting relatively large differences in nutritional status, and more precisely when animals are at very low plane of nutrition (L-60). However the daily mean PD/C ratio (or A/C ratio) estimated by total urine collection had an improved relationship with feed intake (Table 3) and hence the PD/C (or A/C) ratio in multiple spot samples (at an interval of 2 h or less) may reflect more accurately the nutritional status of cattle.

CONCLUSION

The results revealed that the spot urine sampling technique at an interval of 6 h is not suitable for detecting small differences in microbial nitrogen supply. The estimation of PD in total urine is necessary for precise prediction of microbial nitrogen supply and nutritional status in crossbred bulls.

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