Effect of tannins on *in vitro* ruminal protein degradability of various tree forages

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Abstract

The present studies were undertaken to evaluate the effect of hydrolysable (HT) and condensed tannins (CT) (at the rate of 1 mg per 26 mg crude protein) on ruminal degradability of protein in locally available tree forages using *in vitro* gas and ammonia (NH_3) production method.

The NH_3-N content after 24 h incubation decreased with added starch. Gas production (24h) decreased with added HT and CT for all forages except in *Albizia stipulata*. *In vitro* ruminal disappearance of nitrogen (IVRDN) calculated by linear regression in untreated forages was observed to be highest (63.2 %) for *Grewia optiva* and lowest 0 %) for *Quercus incana*. With added tannins, IVRDN values varied from 4.8% (*Acacia lakoocha*) to 33.2% (*Grewia optiva)*. Addition of HT resulted in increase in IVRDN values of *Acacia catechu, Albizia stipulata, Bauhinia variegata, Dendrocalamus hamiltonii, Ficus roxburghii, Leucaena leucocephala, Morus alba* and *Quercus incana*. Comparatively, addition of HT was more effective in increasing IVRDN than CT. However, the IVRDN values when calculated with blank for NH_3-N without added HT decreased with addition of HT in all the forage samples. Tree forages with added CT exhibited an inconsistent response for IVRDN values. The values were maximum (34.4 %) for *Morus alba* and minimum (6.5 %) for *Quercus incana*. The correlation between IVRDN and fiber bound tannins was non-significant but positive and significant ($P<0.02$) between IVRDN and CP content of the forages. A higher slope in the regression equations observed in presence of tannins indicated higher uptake of ammonia by ruminal microbes and possibly to increased microbial protein synthesis.

Key words: Protein, rumen degradation, tannins, tree forages

Introduction

Ruminants fed low quality forages require supplementation with critically deficient nutrients to optimize productivity. A large increase in animal production can be achieved by alterations to the feed base and improving the ratio of protein absorbed relative to energy nutrients is the primary factor that affects its efficiency. Ensuring a nutrient non-limited microbial digestion in the rumen by supplementation automatically improves protein to energy ratio. Small amount of

escape protein stimulate both productivity and efficiency of feed utilization (Preston and Leng 1987). It is important, therefore, to identify protein sources and economic mechanisms for producing diets that contain a high proportion of escape protein.

Trees and shrubs have been introduced into cropping and grazing systems to provide green fodder high in protein to supplement the available low protein forage. Tree forages form an integral part of ruminant feed in high altitude states of India (Himachal Pradesh., Jammu & Kashmir, Uttaranchal, Uttar Pradesh and North-Eastern Regions). Recent studies, particularly of tree forages, show that anti-nutritional factors (ANF) like tannins, can affect animal nutrition in rather diverse ways (Singh et al 2003; Singh and Sahoo 2004). Formation of complexes of tannins with nutrients, especially proteins, has both negative and positive effects on their utilization (Reed 1995). Hydrolysable tannins are potentially toxic and cause poisoning in animals if sufficiently large amounts of tannin-containing plant material are consumed (Garg et al 1992). Condensed tannin are not toxic to ruminants, and when the concentration is below 4% of DM, they improve the nutritive value of herbage by binding to plant proteins and protecting them from excessive degradation in the rumen, besides preventing the establishment of parasitic nematodes (Barry and McNabb 1999). Tannins released from one species may have a role in binding proteins in other tannin free species (used as supplement) in a diet (Yu Feng 1991). In this scenario, the present investigation was aimed at evaluating the effect of HT and CT on nitrogen degradability of locally available tree forages using *in vitro* gas and ammonia production method.

Materials and methods

Tree forages

Ten locally available tree forages, *viz. Acacia catechu, Albizia stipulata, Artocarpus lakoocha, Bauhinia variegate*, *Dendrocalamus hamiltonii*, *Ficus roxburghii, Grewia optiva, Leucaena leucocephala*, *Morus alba* and *Quercus incana* were selected for the present studies. Leaves with very young stems and buds plucked, dried in a forced draft air oven at 50-55 °C, ground to pass 1 mm screen and stored in screw capped polycarbonate bottles until required.

Experimental design

The effect of hydrolysable (HT) and condensed tannins (CT) added at the rate of 1 mg per 26 mg protein equivalent of respective tree forages was assessed for *in vitro* ruminal disappearance of nitrogen (IVRDN) in an *in vitro* rumen system having glass syringes to measure gas production (Menke et al 1979). Thus the treatments were substrate (control), substrate + HT and substrate + CT.

In vitro rumen system

The CP degradation (i.e., IVRDN) was determined (Raab et al 1983) by incubating a known weight of sample corresponding to 26 mg CP in buffered rumen

liquor (BRL) taken in a 100 ml calibrated syringe (Haberle Lonsee-EttlenschieB, Germany) representing as the *in vitro* rumen system. The forage sample weight representing 26 mg CP was put in the syringes. Hundred milligram of starch also weighed in to the syringes where ever it is required. For each sample 30 syringes (3 each for blank, blank + HT, blank + CT, sample, sample + starch, sample + HT, sample + starch + HT, sample + CT, sample + starch + CT and standard hay sample) were incubated. Greased plungers were then put in to the syringes making it airtight. Two different solutions containing 1 mg per ml of CT and HT were prepared and put in to the syringes through the nozzle and clipped. Tannic acid (analytical grade) was from Qualigens, Mumbai and condensed tannins (Wattle) sample was a kind gift from CLRI, Chennai. The internal hay standard for *in vitro* gas method was obtained from University of Hohenheim, Stuttgart, Germany. The syringes were placed in incubator at 39°C and all these procedures were done the day before the run.

Distilled water 400 ml, micromineral solution ($CaCl_2.2H_2O$ 13.2 g + $MnCl_2.4H_2O$ 10.0 g + $CoC1_2.6H_2O$ 1.0 g + $FeC1_2.6H_2O$ 0.8 g per 1 L H_2O) 0.1 ml, rumen buffer solution ($NaHCO_3$ 35 g + $(NH_4)HCO_3$ 4 g per 1L H_2O) 200 ml, macromineral solution (Na_2HPO_4 5.7 g + KH_2PO_4 6.2 g + $MgSO_4$ 0.6 g per 1 L H_20) 200 ml, resazurine solution (1 mg/ml H20) 1.0 ml and reduction solution ($Na_2S.H_2O$ 280 mg + 1M NaOH 1.6 ml + 38.4 ml H_2O) 40 ml added in order in to a flat-bottomed conical flask. This mixture was prepared immediately before collection of rumen liquor, kept under CO_2 at 39 °C and stirred by a magnetic stirrer. Prior to adding rumen liquor the temperature of the medium was ascertained at 39°C with the colour of the medium changing from blue over pink to colourless, indicating anaerobic environment. Rumen contents were obtained from three adult male cattle via their rumen cannula prior to the morning feeding. The donor animals were fed a maintenance ration of wheat straw and concentrate (linseed cake, wheat bran, maize, mineral mixture and salt in a ratio of 32:32:33:2:1) at a ratio of 2:1. Rumen fluid was pooled into a pre-warmed thermos flask, capped and immediately transported to the laboratory (only 50 m). Further processing and dispensing into glass syringes was under a constant flow of CO_2 and in a water bath maintained at 39±0.5^oC. The liquid fraction was obtained by filtration through two layers of cheese cloth and used as the inoculum with buffered mineral solution (1:2. v/v*)*. Buffered rumen fluid (30 ml) was pipetted into each syringe, which was placed immediately into a thermostatically controlled water bath (39±0.5^oC) with arrangements for holding the syringes. The incubation was terminated after recording the gas volume at 24h. If gas production exceeded 80 ml, the piston was pushed back to its initial position releasing only the accumulated gas. All incubations were in triplicate.

Estimation of in vitro ruminal disappearance of nitrogen (IVRDN)

After 24h of incubation the contents of the syringes were centrifuged at 5000×*g* and the supernatant was analysed for ammonia N (NH_3-N) by Kjeltek Auto Analyser (Tecator, Sweden). Briefly, a 10 ml of aliquot was transferred to the Kjeldahl distillation tube for determination of NH_3-N by distillation with 2 ml of 1N NaOH. The liberated NH_3 was trapped in 25-30 ml of boric acid and titrated with 0.01N H_2SO_4. The N disappearance was calculated from linear regressions of NH_3-N concentration (y; mg/volume of digest/24h) versus gas production (x; ml/24h). The Y intercept (b0) is the amount of NH_3-N released when no energy is available. The difference between b0 and the NH_3-N content in the blank indicates the amount of NH_3-N liberated from degradation of protein, and other N containing compounds, in the feed incubated. The IVRDN (%) at 24 h was calculated as a proportion of total N incubated by the equation:

$$IVRDN = (NH_3\text{-N at zero gas production} - NH_3\text{-N of blank})/(\text{Total N in feedstuff incubated})$$

Further, IVRDN* (%) was calculated from simple equation (no extrapolation) involving extreme measurements of gas and NH_3-N production with and without added starch as below.

$$IVRDN^* = [A-\{(A - B)/(C - D)\}C - NH_3\text{-N of blank}]/[\text{Total N in feedstuff incubated}]$$

Where, A = mg NH_3 -N after 24 h incubation, when no starch is added

B = mg NH_3 -N after 24 h incubation, when starch is added

C = ml gas production after 24 h incubation, when no starch is added

D = ml gas production after 24 h incubation, when starch is added

Chemical analysis

Nitrogen content of the samples was determined by the standard Kjeldahl method (AOAC 1984) using Kjeltek Auto Analyser (Tecator, Sweden). Neutral detergent and acid detergent fiber in the feed samples were determined as per the procedure of Van Soest et al (1991). Neutral detergent insoluble N (NDIN) and acid detergent insoluble N (ADIN) were determined by estimating nitrogen in the residual fiber fractions and expressed as percent in NDF, DM and N. The N fraction B3 (prolamins, extensin proteins and denatured proteins) was measured from the difference between NDIN and ADIN.

Results and discussion

Grewia optiva had highest CP (24.4% of DM) followed by *Albizia stipulata*, *Leucaena leucocephala*, *Acacia catechu*, *Bauhinia variegata*, *Dendrocalamus hamiltonii* and *Acacia lakoocha*, having around 20% CP and the lowest was 12.9% in *Quercus incana* (Table 1).

Table 1. Crude protein and fiber-bound nitrogen in different tree forages

Tree forages	CP	NDIN	ADIN	% in DM			% of total N		
	% in DM	% in NDF	% in ADF	NDIN	ADIN	B3	NDIN	ADIN	B3
Acacia catechu	20.9	4.13	3.46	2.39	1.09	1.30	71.5	32.5	39.0
Albizia stipulata	22.3	3.99	3.29	2.72	1.55	1.17	76.1	43.4	32.7
Artocarpus lakoocha	19.4	3.57	1.70	1.78	0.63	1.15	57.3	20.3	37.0
Bauhinia variegata	20.6	2.22	1.29	1.39	0.56	0.83	42.2	17.1	25.0
Dendrocalamus hamiltonii	19.8	2.72	1.72	2.17	0.82	1.35	68.3	25.9	42.5
Ficus roxburghii	13.8	2.63	1.64	1.39	0.60	0.79	63.0	27.3	35.7
Grewia optiva	24.4	3.42	1.86	1.66	0.48	1.18	42.5	12.4	30.1

Leucaena leucocephala	21.1	4.38	2.68	2.02	0.55	1.47	59.8	16.3	43.5
Morus alba	17.8	3.72	2.07	1.61	0.58	1.03	56.6	20.4	36.2
Quercus incana	12.9	2.00	1.27	1.24	0.57	0.67	60.3	27.6	32.7

NDIN - neutral detergent insoluble nitrogen, ADIN - acid detergent insoluble nitrogen, B3, N fraction (represented as the difference between NDIN and ADIN)

The NDIN and ADIN (also termed as N fraction C) were closely related (R^2 = 0.87), and most of the tree forages had >20% ADIN fraction which was considered relatively indigestible (Van Soest and Mason 1991; Waters et al 1992; AFRC 1992). In Metabolizable Protein system true digestibilty of undegraded N is estimated from the concentration of ADIN on the assumption that it is completely undegradable and indigestible. The N fraction B3 was highest in *Leucaena leucocephala* (43.5%) and lowest in *Bauhinia variegata* (25.1%). Grewia optiva with highest CP had also lower B3 fraction (30.1) indicating more concentration of degradable N, which was also evident from IVRDN values (Table 2).

Table 2. *In vitro* degradable nitrogen of various tree forages with and without added tannins and regression equations (±SE of regression coefficients in parentheses) showing the relationship between gas production* (x, ml) and ammonia N after 24 h incubation (y, mg).

Tree forages	Treat-ments	Gas production (24h)		Regression equation	R^2	IVRDN, %	IVRDN*, %
		ml/substrate equivalent to 26mg N*	ml/g substrate				
Acacia catechu	UT	16.0	129	y = 6.43 - 0.0452x	0.99	10.3	9.78
	+ HT	14.5	117	y = 5.82 - 0.0513x	0.98	12.9	13.0
	+ CT	15.8	127	y = 5.98 - 0.0537x	0.99	10.6	10.7
Albizia stipulata	UT	12.8	110	y = 5.93 - 0.0470x	0.99	7.69	7.80
	+ HT	13.3	114	y = 5.24 - 0.0490x	0.99	9.38	9.42
	+ CT	12.8	110	y = 5.06 - 0.0484x	0.99	6.49	6.57
Acacia lakoocha	UT	30.3	227	y = 5.69 - 0.0459x	0.99	12.7	12.8
	+ HT	21.8	163	y = 4.32 - 0.0382x	0.99	4.81	5.10
	+ CT	27.7	207	y = 4.91 - 0.0468x	0.98	8.89	9.18
Bauhinia variegata	UT	13.0	103	y = 6.29 - 0.0568x	0.99	12.3	12.3
	+ HT	9.33	74.0	y = 5.58 - 0.0509x	0.98	18.0	18.2
	+ CT	10.0	79.4	y = 5.51 - 0.0545x	0.96	18.0	18.4
Dendrocalamus hamiltonii	UT	15.7	119	y = 6.28 - 0.0385x	0.99	13.0	13.7
	+ HT	11.7	88.9	y = 5.64 - 0.0503x	0.99	20.2	20.2
	+ CT	11.7	88.9	y = 5.63 - 0.0465x	0.99	18.3	18.9
Ficus roxburghii	UT	30.2	161	y = 6.30 - 0.0405x	0.99	0.48	0.26
	+ HT	15.0	79.8	y = 5.84 - 0.0485x	0.95	15.9	15.6

	+ CT	23.3	124	y = 5.85 - 0.0515x	0.99	8.65	8.60
Grewia optiva	UT	30.5	285	y = 7.67- 0.0561x	0.99	63.2	63.2
	+ HT	19.7	184	y = 5.63 - 0.0568x	0.99	33.2	32.3
	+ CT	26.3	246	y = 5.62 - 0.0558x	0.99	31.2	30.6
Leucaena leucocephala	UT	27.2	220	y = 6.73 - 0.0420x	0.95	11.7	12.6
	+ HT	27.2	220	y = 5.82 - 0.0392x	0.98	15.7	16.2
	+ CT	25.2	204	y = 6.02 - 0.0563x	0.98	16.8	15.4
Morus alba	UT	28.0	192	y = 7.05 - 0.0427x	0.99	18.4	18.7
	+ HT	23.0	158	y = 6.39 - 0.0669x	0.99	32.0	32.2
	+ CT	21.0	144	y = 6.21 - 0.0561x	0.98	34.4	35.0
Quercus incana	UT	15.67	78.4	y = 5.39 - 0.0431x	0.99	0.00	0.00
	+ HT	14.33	71.7	y = 5.38 - 0.0492x	0.99	14.7	13.5
	+ CT	13.00	65.0	y = 5.39 - 0.0548x	0.99	6.49	6.32

UT, untreated; HT, hydrolysable tannins, CT, condensed tannins

R^2-coefficient of determination, IVRDN-in vitro degradable nitrogen calculated by linear regression, IVRDN-in vitro degradable nitrogen calculated from the equation (Raab et al 1983)*

According to Sniffen et al (1992) increase in fractions B3 and C increases undegradability but increase in fraction C decreases intestinal digestibility. The tree forages, viz. *Grewia optiva, Leucaena leucocephala* and *Bauhinia variegata* with <20% ADIN may thus be considered useful N supplements to poor quality crop residues. Thus, it has been suggested that partitioning herbage N into neutral detergent and acid detergent soluble and insoluble portions may be used to estimate ruminally degradable and undegradable N.

Twenty-four hour gas production (ml), regression equations showing the relationship between gas production and NH_3-N (mg/volume of digest/24h) content and the IVRDN (%) values of various forages with or without added tannins are presented in table 2. The gas production was nearly double in *Grewia optiva* (30.5), *Acacia lakoocha* (30.3), *Ficus roxburghii* (30.2), *Morus alba* (28.0) and *Leucaena leucocephala* (27.2) compared to that in other tree forages. The CP content and the N fractions were observed to have had no bearing on gas production. The tree forages *Ficus roxburghii* and *Quercus incana* with around 13% CP required incubation of nearly 200 mg substrate DM compared to 107-146 mg from other forages. However, the gas production was higher in the former and lower in the later as calculated for g of substrate, which confounded the reasons attributed to higher gas production from higher OM degradation. *Grewia optiva* produced maximum gas from a minimum of substrate compared to other tree forages indicating higher *in vitro* ruminal degradability of substrate, and was also supported with higher IVRDN values (63.2%). Nsahlai et al (1994) attributed lower gas production to NDF characteristics (lignin content) and concentration of polyphenolics. Addition of HT showed more reduction in gas volume than CT except in *Albizia stipulata, Leucaena leucocephala, Morus alba* and *Quercus incana*. This variability may also be due to the reasons cited above. Further, the toxic effects of intrinsic and extrinsic HT cannot also be ruled out (Bhat et al 1998: Makkar 2003)

The NH_3-N content measured after 24 h incubation decreased with added starch. This phenomenon is quite obvious to the fact that NH_3 liberated during incubation is in part used for microbial protein synthesis and is related to the efficiency of energy utilization for microbial protein synthesis. Further, the estimation of protein synthesis from NH_3-N disappearance and gas production has had following assumptions: (1) all preformed monomers are deaminated, when no gas production occurs in 24 h incubation; (2) there exists a linear relationship between non-deaminated amino acids and gas production. The intercepts were different between the feedstuffs incubated indicating differences in the degradability of the proteins. The IVRDN values calculated by linear regression and by extreme measurements revealed a similar trend for the treatments with different forage substrates. The values ranged from 0 to 63 per cent which is similar to the range for IVRDN observed for West Aftican browses by Rittner and Reed (1992). The values in the present studies are comparatively lower than those of Raab et al (1983). Direct comparisons are not possible for want of literature. There was a single observation of negative IVRDN value for *Quercus incana* (assumed as zero), which however increased and become positive with added HT (14.7%) and CT (6.5%). When calculated from extreme values of gas and NH_3-N production, similar negative value in control (assumed as zero) that increased with added tannins was also seen. Leaves from *Quercus incana* having 2.06% N with 60.3% NDIN and 27.6% ADIN may have provided little degradable N for the inoculums and thus potentiate decreased microbial degradation. In the same line, *Ficus roxburghii* also had 2.2% N, 63.0% NDIN and 27.3% ADIN resulting in 0.5% IVRDN. A positive and significant ($P<0.02$) correlation was observed between IVRDN and CP content of the forages. According to Hedqvist and Uden (2006) soluble proteins from different feeds were degraded at substantially different rates and they advocated degradability studies of feeds high in soluble proteins in vitro over in sacco, since the latter method assumes solubility to be an equivalent to degradability.

The IVRDN with added tannins (calculated by linear regression) varied from 4.8% (*Acacia lakoocha*) to 33.2% (Grewia optiva). Surprisingly, addition of HT resulted in increase in IVRDN values of *Acacia catechu, Albizia stipulata, Bauhinia variegata, Dendrocalamus hamiltonii, Leucaena leucocephala,* and *Morus alba*. The IVRDN values were estimated taking into account the blank for NH3-N with added HT. However, when blank for NH3-N without added HT was taken for the calculation, the IVRDN values reduced with HT in all the forage samples. The choice of blank, therefore, needs to be scrutinized for estimation of IVRDN by the *in vitro* gas and NH_3-N production method. The values for IVRDN based on extreme measurements exhibited a similar trend. The IVRDN with added CT exhibited an inconsistent response increase in some and decrease for other samples. The values obtained by linear regression equation was maximum (34.4 %) for *Morus alba* and minimum (6.49 %) for *Quercus incana* and when calculated by the formula based on extreme measurements, the values ranged from 6.32 to 35.0 % for the above forages. The addition of HT was more effective in increasing IVRDN as compared to the addition of CT. In all the cases, the coefficient of determination was high and over 0.91 for all the samples, thus representing good fit of data. The increase in IVRDN values for some tree forages is in contrast to negative relationship between tannins and ruminal protein degradation (Reed 1995). Rittner and Reed (1992) showed that protein degradability was negatively correlated with soluble phenolics and soluble proanthocyaniins. Contrary to this, Messman et al (1996) studied *in situ* degradability of nitrogen in *Medicago sativa, Trifolium pratense, Lotus corniculatus* and *Lespedeza cuneata* and concluded that specific forage proteins degraded at different rates that were independent of tannin concentration. Makkar et al (1997) found a strong negative correlation ($r = -0.92$, $P<0.001$) between tannins and *in vitro* rumen protein degradability. In the same line, *Acacia catechu*, *Acacia stipulata*, *Acacia lakoocha* and *Grewia optiva* showed a decline/no change in IVRDN values with added tannins. A dose dependent slowing down in degradation of protein meal with increasing concentration of added tannins has also been observed (Hervas et al 2000; Martinez et al 2004). Tannins lower the rate of protein degradation and

deamination in the rumen and therefore lower ruminal NH_3-N (Woodward 1989). The decrease in NH_3-N could be partly due to decrease in deamination of amino acids or low availability of amino acids resulted from decrease in protein degradation, which may be attributed to formation of CT protein complexes or selective inhibition/inactivation of microbes or their enzymes involved in catabolism of protein or increased uptake of NH_3 by microbial cells. On the other hand, the increase in IVRDN values in other tree forages may have a complex set of reasons. Amongst them, *Ficus roxburghii* and *Quercus incana* contained lower CP (Table 1) but higher concentration of intrinsic tannins (both HT and CT) (Singh et al 2005), which additively resulted in decreased substrate degradation since gas production was reduced or to the toxic effect of tannins leading to microbial lysis/death or both. In the same line, although *Bauhinia variegata* and *Dendrocalamus hamiltonii* had about 20% CP the substrate degradation was minimal and thus less microbial uptake and increased unutilized NH_3-N in the supernatant after 24h incubation. However, the consequence of response was altogether different for *Leucaena leucocephala,* and *Morus alba*, which showed moderate fermentation of substrates. But, presence of other incriminating factors such as mimosine in *Leucaena leucocephala* (Hammond 1995) cannot be ruled out which might have altered this steady-state *in vitro* rumen fermentation process. From the results, it was quite obvious that a complex relationship does exist between protein characteristics and intrinsic and extrinsic tannins. Drobonick (1995) stated that the inhibitory properties of tannins were highly dependent on the total concentration of proteins in the test solution. Further, the tree forages that were evaluated in this study must be having variable concentration of tannins, which might also be contributing to N degradation parameters. Thadei et al (2001) observed an inverse relationship between tannin concentration and CP degradability, but the result was not consistent for all plants. Some plants with low tannin concentration were found to have low CP degradability than those with high tannin content. Further, the difficulties in fixing a time for estimation of protein degradability from the release of NH_3-N arise from the fact that protein degradation and microbial protein synthesis occur simultaneously. In the face of lower substrate degradation leading to lower energy availability for microbial synthesis the amount of added starch may be presumed to be insufficient. Therefore, some possible reasons for this variability may include changing degradability pattern for different protein fractions, inhibitory effects of intrinsic and extrinsic incriminating factors on substrate fermentation, interactions of these tannins with different protein fractions, protein quality and its contribution to gas production and presence of incriminating factors other than tannins which influence substrate degradation and gas production.

Positive effects of tannins on IVRDN, although non-significant, observed in the present studies are difficult to explain. However, in most of the reported studies the effects of tannins have been evaluated by the addition of tannin rich plants or purified tannins from the plants using other in vitro or in sacco methods. The role of extrinsic and intrinsic tannins in different feedstuffs needs to be further evaluated prior to any suggestions of recommended level for protection feed protein degradation in the rumen. The in vitro gas and ammonia production method is considered to be better than many other methods available for estimation of ruminal protein degradation and very few reports are available on ruminal protein degradability values by gas production method. Furthermore, the choice of blank while determining the additive effect of tannins needs to be scrutinized for estimation of IVRDN by the in vitro gas and NH_3-N production method. Additional studies are warranted on this aspect.

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