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The Effect of Protecting Lysine and Methionine with Condensed Tannin Added Into Total Mixed Ration on Efficiency of Microbial Protein Synthesis by Using an *in Vitro* Gas Production Approach

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Abstract

An *in vitro* study investigating the effects of adding CT-protected Lysine (Lys) and Methionine (Met) on gas production and efficiency of microbial protein synthesis (EMPS) of total mixed ration (TMR) was conducted at Animal Nutrition Laboratory. TMR were composed of concentrate and maize stover at 50:50 ratios in DM basis. The experiment used 4x4 factorial randomized design and 3 replications. The first factor was level of Lys and Met (3 g Lys- 1 g Met; 5 g Lys- 1.67 g Met; 7 g Lys- 2.33 g Met; 9 g Lys- 3 g Met) and the second factor was level or percentage of CT (0%, 6%, 8% and 10%) of Lys and Met.

Parameters measured were cumulative gas production recorded at 0, 2, 4, 8, 12, 16, 24, 36 and 48 h, DMD and OMD of residues, concentrations of NH_3 and VFAs, and EMPS. The results showed that there was interaction effect between levels of Lys-Met and CT on cumulative gas production and b value, with treatment A1B3 (3g Lys + 1g Met) + 8% CT) being the lowest (106.2 ml 121.9 ml) and A4B1 (9g Lys + 3g Met) being the highest (121.9 ml and 136.8 ml). Increasing level of Lys-Met alone significantly increased cumulative gas production, DMD, and ammonia-N, but did not increase c value, OMD , concentrations of acetic, propionic and butyric acids, total VFA, ratio of C2/C3, and EMPS. Increasing level of CT reduced cumulative gas production; b value, ammonia-N, C2, C3, C4, and total VFA, with 0% CT and 10% CT being associated with the highest and the lowest value of those parameters. The highest value of EMPS was found on treatment A1B4 (3g Lys + 1g Met+10% CT; 50.3 g N/kg DOM). It can be concluded that CT is able to protect Lys-Met from being fermented in the rumen, hence improving EMPS value.

Keywords: lysine, methionine, CT, *in vitro* gas production

1. Introduction

Low productivity of high-producing ruminants, especially dairy types is mainly related to low quality and quantity of local forage in Indonesia. Native grass and agricultural byproducts which are commonly available as the main sources of forage have low protein and digestibility, but contain high fibre. Even though~~,~~ attempts have been made to improve productivity by feeding commercial concentrates as energy and protein supplements, ~~but~~ the productivity remains low. A speculation exists that there has been a case of imbalance supplies of protein and energy from both forage and concentrate. Wanapat and Wachirapagoin (1990) reported that tropical forages have low value of undegraded dietary protein (UDP; 10% - 29%), as well as concentrate diet (30% - 49%), hence amino acids supply to the small intestine for absorption was low. Among the ten essential amino acids required for maximum milk yield and milk protein production of ruminant, Lysine and Methionine are the limiting factors of amino acids (Schwab et al 1992). Both of these limiting amino acids have relative low efficiency to be used for synthesizing tissue because of extensive ruminal degradation (Schwab, 1995). Therefore, it is needed an effort to protect both of these amino acids from being degraded by microbes in the rumen. Tannins, particularly Condensed Tannin (CTs) added to the diet are advantageous as they protect dietary protein from degradation in the rumen (Barry, 1989). CT is a phenol-compound which is capable of forming a strong bond with the protein in the rumen (Reid et al, 1994), so that by-pass protein as well as amino acids is available for absorption in the small intestine.

In this study, CT is expected to serve as a protector of the amino acid from ruminal degradation. Based on the overview, this study was aimed to investigate effects of protecting Lysine and Methionine using CT added into total mixed ration (TMR) on efficiency of microbial protein synthesis by using an *in vitro* gas production approach.

2. Materials and Methods

2.1 Feed preparation

Total Mixed Ration (TMR) used in this study consisted of Indonesia local feeds and they were composed of concentrate as protein and energy sources and maize stover as fibre source in the ratio of 50:50 on dry matter basis. The concentrate was formulated in such a way as shown in Table 1 to attain CP content (*CP*=15.40%) which was assumed to adequately support protein requirement of the of dairy goats after being mixed with maize stover.

Table 1. Proportion of feedstuffs

Ingredients	Proportion (%)
Wheat pollard	24.9
Maize bran	20.0
Rice bran	22.5
Coconut cake meal	11.3
Soybean meal	5.1
Catton seed meal	8.0
Molasses	5.7
Premix	2.3
Urea	0.2

2.2 Procedure of protecting Lys-Met with CT

Lysine and Methionine used were L-Lysine and DL-Methionine (A), whilst mimosa bark extract (B) as source of CT was purchased from Leather Company. The mixture of Lysine-Methionine-CT was made as follow: each of them was weighed according to the treatment (Table 2), and then every combination was dissolved in 50 ml of distilled water and allowed to stand for 24 hours, then added to the TMR and mixed well manually. The proportion of Lysine and Methionine was 3: 1 (NRC 2001) and level of CT ranged from 0% to 10% (Barry and Forss 1983).

2.3 Laboratory analysis

Samples of feeds were analyzed for dry matter (DM), organic matter (OM), crude protein (CP), and crude fibre (CF) according to procedure of AOAC (1984). Ammonia concentration was analyzed using the micro diffusion Conway Method, whilst determination of VFA proportions followed the Method described by Preston (1995). Condensed tannin (CT) content of mimosa bark extract was done following the Butanol-HCl Method of Porter et al (1986).

2.4 Experimental Design

Fermentation experiment was done in a 4 x 4 factorial design with 4 levels of Lysine-Methionine (3 g Lys- 1 g Met; 5 g Lys- 1.67 g Met; 7 g Lys- 2.33 g Met; 9 g Lys- 3 g Met) and 4 levels of CT (0%, 6%, 8% and 10%) from Lysine-Methionine according to Steel and Torrie (1980). This resulted in sixteen treatment combinations, each of which was replicated three times:

A1B1 : 3g Lys + 1g Met
A1B2 : A1B1 + 6% CT
A1B3 : A1B1 + 8% CT
A1B4 : A1B1 + 10% CT
A2B1 : 5g Lys + 1.67 Met
A2B2 : A2B1 + 6% CT
A2B3 : A2B1 + 8% CT
A2B4 : A2B1 + 10% CT
A3B1 : 7g Lys + 2.33g Met
A3B2 : A3B1 + 6% CT
A3B3 : A3B1 + 8% CT
A3B4 : A3B1 + 10% CT
A4B1 : 9g Lys + 3g Met
A4B2 : A4B1 + 6% CT
A4B3 : A4B1 + 8% CT
A4B4 : A4B1 + 10% CT

Measurement of *in vitro* gas production:

In vitro gas production was conducted according to the procedure described by Makkar et al (1997). Strained rumen fluid for *in vitro* gas production was aspirated from cannulated steer maintained at daily ration of 3 kg concentrate feed (a mixture of wheat pollard, coconut cake meal, maize brand, premix and mineral) and elephant grass *ad libitum*. The filtered rumen fluid was put into erlemeyer and purged with CO_2 and was added to the anaerobic mineral buffered solution (1:3 v/v). 100 mL calibrated glass syringes were pre-warmed at 39 °C before the injection of rumen fluid-buffer mixture. The mixture was kept stirred, under CO2 flushing at 39°C using a magnetic stirrer fitted on a hot plate. About 500 mg experimental sample (1.0 mm screen) incubated with 50 mL buffered rumen fluid under continuous CO_2 reflux in 100 mL calibrated glass syringes for 48 h in a water bath maintained at 39°C. The syringes were gently shaken every hour for 48 h incubation. Gas production was recorded at 2, 4, 8, 12, 16, 24, 36 and 48 h after incubation. Cumulative gas production data were fitted to the exponential equation (Orskov and Mc.Donald 1979; Makkar et al 1997 and Blummel et al 1997):

$$Y = b\,(1-e^{-ct})$$

Where:
Y = gas volume at time (ml),
b = gas production from the fermentable fraction (ml),
c = rate of gas production (ml/hour),
t = incubation time (hour)

After 48 h of incubation the residue of each syringe was separated from supernatant and used for determination of dry matter digestibility (DMD) and organic matter digestibility (OMD), whilst the supernatant was used for determination of volatile fatty acids (VFA) concentrations. Another two syringes containing 500 mg samples and buffered rumen fluid were incubated for 24 h to determine ammonia-N and microbial biomass (Blummel et al 1997)

Parameters measured were: gas production, dry matter digestibility (DMD), organic matter digestibility (OMD) of residue, NH_3 and VFA concentrations, microbial mass production and efficiency of microbial protein synthesis (EMPS). All data obtained were analyzed using the General Linear Model (GenStat15^{ed} PL23.1) and the treatment means were tested for significance by Duncan's multiple range tests (1955).

3. Results and Discussion

Chemical composition of concentrate, maize stover and mimosa bark extract is presented in Table 2.

Table 2. Chemical composition of concentrate, maize stover and mimosa bark extract

	DM (%)	Ash (% DM)	EE (% DM)	CF (% DM)	CP (% DM)	CT (% DM)
Concentrate	84.6	10.2	5.2	17.6	15.4	ND
Maize stover	92.4	8.4	2.5	26.6	9.7	ND
TMR	88.5	9.3	3.9	22.1	12.5	ND
Mimosa bark extract	94.9	ND	ND	ND	2.0	53.2

ND = Not Determined

Table 2 shows that CP content of TMR (12.5%) was considered adequate for supplying nitrogen for rumen microbial to grow and digest fibre part of maize stover. The addition of protected Lysine-Methionine into TMR was aimed to improve the efficiency of N utilization of the ration. In this particular study, EMPS is used as the main indicator of the extent of N utilization efficiency.

3.1 In vitro Gas production

The results of the research showed that there is an interaction between Lys-Met and CT and the highest value of 48 h gas production and b value founded at the highest level Lys-Met with the lowest level of CT. This may be due to Lys-Met as organic matter source are easily fermented and the presence of CT scan prohibit the fermentation process. Meanwhile protected Lys-Met supplementation on TMR did not affect the c value.

Table 3. The mean value of gas production, b and c value among all treatments of TMR supplemented by CT- treated Lys-Met at 48-hour incubation period using $Y = b\,(1-e^{-ct})$ model

ParaMeters measured	Lys/Met (A)	CT (B) B1	B2	B3	B4	Means	SEM (*p* value)
Gas production ml	A1	113.6bcd	112.5bcd	110.5b	106.2a	110.7a	
	A2	116.0de	114.5cde	111.5bc	106.0a	112.0a	0.406
	A3	118.3ef	119.9fg	113.3bcd	111.4bc	115.7b	(<0.001)
	A4	121.9g	120.6fg	117.7ef	117.2ef	119.4c	
	Means	117.4a	116.9a	113.2b	110.2c		
	SEM (*p* value)		0.406 (<0.001)				
	Interaction :SEM (*p* value)					0.812 (<0.015)	
b (ml/500mgDM)	A1	127.2cd	126.8cd	125.7c	121.9b	125.4a	
	A2	129.1cde	129.8def	125.5c	118.3a	125.7a	0.433
	A3	132.4efg	133.1fg	126.6cd	125.8c	129.5b	(<0.001)
	A4	136.8h	135.1gh	131.8efg	133.2fgh	134.2c	
	Means	131.4a	131.2a	127.4b	124.8c		
	SEM (*p* value)		0.433 (<0.001)				
	Interaction :SEM (*p* value)					0.806 (<0.001)	
c (ml/hour)	A1	0.047	0.046	0.044	0.043	0.045	
	A2	0.048	0.045	0.046	0.047	0.046	0.0006
	A3	0.047	0.048	0.047	0.041	0.047	(0.117)
	A4	0.046	0.047	0.047	0.044	0.046	
	Means	0.047	0.046	0.046	0.045		
	SEM (*p* value)		0.0006 (0.128)				
	Interaction :SEM (*p* value)					0.001(0.224)	

(a-c) Different superscript letters indicate significant different for CT level (P <0.01)
(a-c) Different superscript letters indicate significant different for Lys/Met level (P <0.01)
(a-g) Different superscript letters indicate significant different for CT and Lys/Met levels (P<0.05)

The absence of CT added on TMR resulted in highest value of cumulative gas production and b, and this suggests that the presence of CT in Lys-Met have been shown to protect Lys-Met from being degraded in the rumen and could be used as a strategy to increase bypass Lys-Met. Results of this study are in line with the previous study reported by El-Waziry et al (2005) where b value of soy bean meal (SBM) decreased significantly (P<0.05) when tannic acid (TA) was added and maximum addition of TA (3%) resulted in the lowest b value. Figure 1 showed the graphic of all treatments where the higher Lys-Met supplementation the higher the gas production however there was an inverse effect of CT in which the higher CT protector, the lower the gas production.

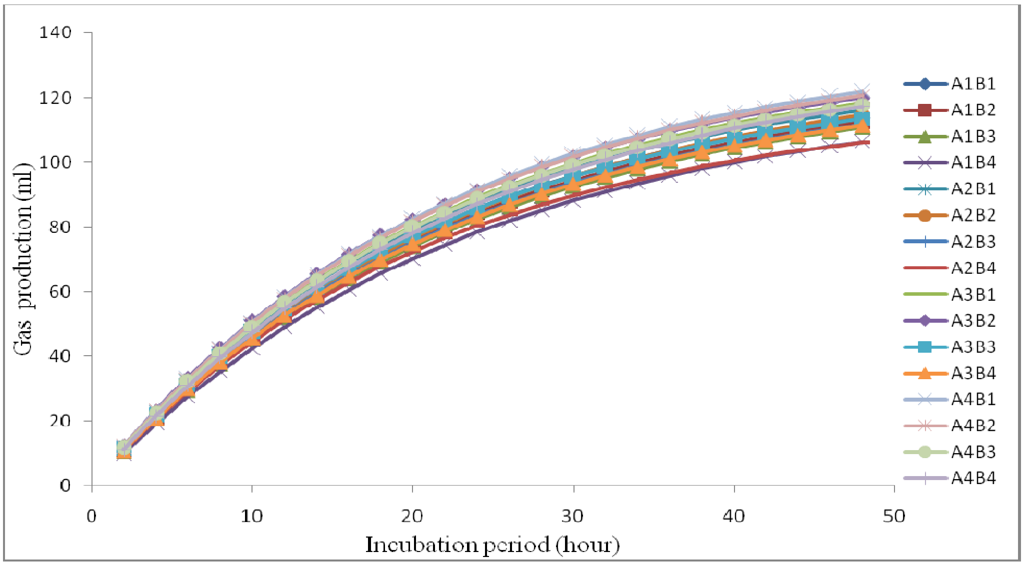

Figure 1. Graphic relationship between mean value of gas production of TMR feed supplemented by CT- treated (protected) Lys/Met at various incubation periods up to 48 hours on $Y = b(1-e^{-ct})$ model among all treatments

3.2 Digestibility

Protected Lys-Met supplementation on TMR did not affect DMD and OMD value, but the increased in DMD value was observed when Lys-Met was added. Although supplementing protected Lys-Met did not increase DMD and OMD values, there was a tendency that the DMD value was lower (P<0.060) as CT level was higher, and OMD value was higher (P<0.074) as level of Lys-Met was higher.

Table 4. DMD and OMD of residual gas production of all treatments of TMR supplemented with CT-protected Lys-Met

ParaMeters Measured	Lys/Met (A)	CT (B) B1	B2	B3	B4	Means	SEM (*p* value)
DMD	A1	58.5	54.8	52.8	54.6	55.2a	
(%)	A2	56.5	55.0	55.4	54.2	55.3a	0.725
	A3	56.8	54.9	56.2	54.0	55.5a	(0.031)
	A4	58.9	58.2	58.0	56.7	58.0b	
	Means	57.7a	55.7a	55.6a	54.9a		
	SEM (*p* value)		0.725(0.060)				
	Interaction :SEM (*p* value)					1.449 (0.841)	
OMD	A1	62.2	60.1	59.6	59.8	60.4a	
(%)	A2	60.8	60.6	59.5	59.7	60.1a	0.590
	A3	62.6	60.6	60.7	59.0	60.7a	(0.074)
	A4	61.8	62.9	62.8	61.4	62.2a	
	Means	61.8a	61.0a	60.6a	60.0a		
	SEM (*p* value)		0.590 (0.178)				
	Interaction :SEM (*p* value)					1.179 (0.865)	

(a-a) Same superscript letters indicate no significant different for CT level (P >0.05)
(a-b) Different superscript letters indicate significant different for Lys/Met level (P <0.05):DMD
(a-b) Different superscript letters indicate significant different for Lys/Met level (P >0.05):OMD
(a-a) Same superscript letters indicate no significant different for CT and Lys/Met levels (P>0.05)

That higher level of Lys-Met in TMR resulting in higher values of DMD (P <0.05) implies that Lysine and Methionine can easily be degraded in the rumen which led to the increase of digestibility value of residue.

3.3 Ammonia and VFAs concentrations

This study demonstrated that the Lys-Met could linearly increase ammonia production, but the inverse effect was observed when a higher CT was added. The presence of CT seems to prove that this polyphenolic compound is bound to Lys-Met, as Makkar (2003) stated that CT protects dietary protein from ruminal degradation and could be used advantageously to increase bypass protein to improve ruminant performance. Mohammadabadi *et al* (2010) reported that processing of sunflower meal with Tannic Acids (TA) decreased NH_3-N and 3% TA in DM had the lowest NH_3-N concentration. Furthermore Sliwinski et al (2002) found that adding chestnut wood extract containing tannins to a basal diet for dairy cows reduced the level of rumen ammonia in comparison to the without tannin diet

Table 5. Ammonia, volatile fatty acids concentrations and C2/C3 ratio values of all treatments of TMR supplemented with CT-protected Lys-Met

ParaMeters measured	Lys/Met (A)	CT level (B) B1	B2	B3	B4	Means	SEM (*p* value)
NH_3	A1	223.2	211.5	203.5	213.7	213.0a	
(mg/l)	A2	224.9	221.1	213.5	205.6	216.3a	2.93
	A3	220.1	229.9	224.1	208.0	220.5ab	(0.028)
	A4	234.2	224.8	222.3	220.8	225.5b	
	Means	225.6c	221.8bc	215.9ab	212.0a		
	SEM (*p* value)		2.93 (0.012)				
	Interaction :SEM (*p* value)					5.85 (0.382)	
VFA							
(C_2)	A1	29.2	26.9	25.3	22.9	26.1a	
(mMol/l)	A2	30.2	26.9	26.7	24.3	27.0a	0.769
	A3	28.1	28.4	27.3	26.8	27.7a	(0.195)
	A4	27.3	30.2	29.6	26.6	28.4a	
	Means	28.7b	28.1b	27.2ab	25.1a		
	SEM (*p* value)		0.769 (0.014)			1.537 (0.490)	
	Interaction :SEM (*p* value)						
C_3	A1	10.9	8.8	8.7	6.8	8.8a	
(mMol/l)	A2	11.1	8.9	8.1	8.6	9.2a	0.507
	A3	10.1	9.9	9.9	7.6	9.4a	(0.663)
	A4	10.3	9.6	9.5	9.4	9.7a	
	Means	10.6b	9.3ab	9.1a	8.1a		
	SEM (*p* value)		0.507 (0.016)				
	Interaction :SEM (*p* value)					1.015 (0.766)	
C4	A1	6.2	5.7	5.5	5.2	5.7a	
(mMol/l)	A2	7.3	5.9	5.6	5.3	6.0a	0.348
	A3	7.6	6.3	6.3	5.1	6.3a	(0.105)
	A4	7.4	7.3	6.2	6.5	6.9a	
	Means	7.1b	6.3ab	5.9a	5.5a		
	SEM (*p* value)		0.348 (0.020)				
	Interaction :SEM (*p* value)					0.697 (0.963)	
Total VFA	A1	46.3	41.45	39.45	34.93	40.52a	
(mMol/l)	A2	48.6	41.80	40.36	38.13	42.22a	1.110
	A3	45.7	44.61	43.56	39.46	43.33a	(0.053)
	A4	45.0	47.09	45.32	42.48	44.97a	
	Means	46.4b	43.74b	42.17ab	38.75a		
	SEM (*p* value)		1.110 (<0.001)				
	Interaction :SEM (*p* value)					2.221 (0.572)	
C_2/C_3 ratio	A1	2.78	3.04	2.94	3.41	3.04a	
	A2	2.79	3.18	3.39	2.87	3.06a	0.188
	A3	2.87	2.93	2.87	3.64	3.08a	(0.991)
	A4	2.70	3.32	3.12	2.84	2.99a	
	Means	2.78a	3.12a	3.08a	3.19a		
	SEM (*p* value)		0.188 (0.448)				
	Interaction :SEM (*p* value)					0.3756 (0.813)	

(a-c) Different superscript letters indicate significant different for CT level ($P<0.05$) and ($P<0.01$)
(a-a) Same superscript letters indicate no significant different for CT level ($P >0.05$) :C2/C3
(a-b) Different superscript letters indicate significant different for Lys/Met level ($P <0.05$)
(a-a) Same superscript letters indicate no significant different for Lys/Met level ($P >0.05$):NH3
(a-a) Same superscript letters indicate no significant different for CT and Lys/Met levels ($P>0.05$)

Lys-Met factor alone and interaction between Lys-Met and CT factor did not increase production of C2, C3, C4, VFA total and C2/C3 ratio, but there was a tendency that the higher level Lys-Met resulted in a higher (P <0.053) value of total VFA. Furthermore, CT factor alone could linearly decreased C2, C3, C4 and total VFA. This is evident that CT could protect organic matter fermentation in the rumen, hence lowering VFA. EL-Waziry et al (2005) reported that total VFAs concentrations were significantly decreased when soybean meal treated by TA. This implies that CT could protect Lys-Met amino acids from rumen microbial fermentation process and hence VFA production decreased. The mean value of total VFA was below the optimal range of VFA concentration for microbial growth (70-150 mmol; Mc. Donald et al 2002). The low concentration of VFA in this study may have been due to supernatant from rumen fluid was measured after incubation for 48 hours, therefore a lot of VFA has been used as a source of carbon skeleton to synthesize microbial protein (Sutardi 1980).

3.4 Efficiency of Microbial Protein Synthesis

Interaction between Lys-Met and CT did not influence EMPS value; however CT significantly influenced EMPS value. The highest value of EMPS was observed in at A1B4 treatment (Table 6).

The EMPS values (44.00 to 50.30 g microbial N/kg of OMD) were above the optimal value for EMPS (30 to 40 g N/kg of OM) as reported by Karsli and Russel (2001) indicating a good match of N supply in relation to energy availability in the form of ATP (Preston and Leng 1987).

Table 6. The mean value of microbial biomass production and EMPS of supernatant of *in vitro* gas production for 48 hours on all treatment that TMR supplemented with CT- protected Lysine-Methionine

ParaMeters measured	Lys/Met (A)	CT (B) B1	B2	B3	B4	Means	SEM (p value)
Microbial Biomass (mg/500 mg DM)	A1	158.63	162.16	159.99	172.59	163.34a	
	A2	156.81	159.76	168.38	168.12	163.27a	1.723
	A3	163.74	159.68	165.18	165.42	163.50a	(0.250)
	A4	157.75	158.26	161.32	159.64	159.24a	
	Means	159.23a	159.97ab	163.72b	166.44b		
	SEM (*p* value)		1.723 (0.019)				
	Interaction : SEM (*p* value)					3.446 (0.395)	
EMPS (g microb N/kg OMD)	A1	44.87	47.19	45.74	50.30	47.03a	
	A2	45.23	46.16	50.19	50.04	47.90a	0.718
	A3	46.70	44.86	46.21	49.27	46.76a	(0.128)
	A4	44.42	44.00	44.54	48.75	45.43a	
	Means	45.30a	45.55a	46.67a	49.59b		
	SEM (*p* value)		0.718 (<0.001)				
	Interaction : SEM (*p* value)					1.435 (0.578)	

[(a-b)]Different superscript letters indicate significant different for CT (P <0.05):MB and EMPS
[(a-a)]Same superscript letters indicate no significant different (P >0.05)

4. Conclusions

a. The higher level of Lys-Met, the higher value of all parameters such as *in vitro* gas production in 48 hours incubation, the value of b, and c, DMD, OMD, NH_3, acetate (C_2), propionate (C_3) and butyric (C_4) and total VFA.
b. The highest value of EMPS can be achieved at the lowest level of Lys-Met (3 g 1 g Lys-Met) and the highest level of CT (10% of amino acids)

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References

AOAC 1990 *Official Methods of analysis*, 15th Edition. Association of Official Analytical Chemists, Arlington, USA

Barry T N and Forss D A 1983 *The condensed tannin content of vegetative Lotus pedunculatus, its regulation by fertilizer application, and effect upon protein solubility*. J. Sci. Food Agric. 34:1047. http://onlinelibrary.wiley.com/doi/10.1002/jsfa.2740341004/abstract

Blümmel M and Becker K 1997 *The degradability characteristics of fifty-four roughages and roughage neutral-detergent fibres as described by in vitro gas production and their relationship to voluntary feed intake*. British Jornal Nutrition 77: 757-768. http://dx.doi.org/10.1079/BJN19970073

Duncan D B 1955 *Multiple range and multiple F tests*, Biometrics. 11:1-42
El-Waziry A M, Nasser M E A and Sallam S M A 2005 *Processing Methods of Soybean Meal:1-Effect of Roasting and Tannic Acid Treated-soybean Meal on Gas Production and rumen Fermentation In Vitro*. Journal of Applied Sciences Research 1(3): 313-320. http://www.aensiweb.com/jasr/jasr/313-320.pdf
Ørskov E R and McDonald I 1979 *The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage*. Journal of Agricultural Science. (Camb.) 92:499–503. http://dx.doi.org/10.1017/S0021859600063048
Karsli M and Russell J 2001 *Effects of some dietary factors on ruminal microbial protein synthesis*. Turkey Journal Veterinary Animal Science 25:681-686. http:// www.scielo.cl/scielo.php ?script= sci_nlinks&ref=997355&pid =S07185839201 200040001200024 &lng=es
Makkar H 2003 *Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds*. Small Ruminant Res. 49: 241–256. http://ciam.ucol.mx/directorios/1251/TANINOS/makkar03ei%20tannin%20review%202003.pdf
Makkar H P S, Blummel M and Becker K 1997 *Application of an in vitro gas Method to understand the effect of natural plant products on availability and partitioning of nutrients*. British Society of Animal Science. *Occasional Publication No. 22* pp. 147-150. https://euroleague.uni-hohenheim.de/publication/application-of-an-in-vitro-gas-Method-to-understand-the-effects-of-natural-plant-products-on-availability-and-partitioning-of-nutrients
McDonald P, Edwards R A, Greenhalgh J F D and Morgan C A 2002 Animal *Nutrition*. 5th Edition. Longman Scientific and Technical. New York. http://trove.nla.gov.au/ version/45747360
Mohammadabadi T, Chaji M and Tabatabaei S 2010 *The Effect of Tannic Acid on in vitro Gas Production and Rumen Fermentation of Sunflower Meal*. Journal of Animal and Veterinary Advances. Volume: 9 Issue: 2 pp: 277-280. http://medwelljournals.com/abstract/?doi=javaa.2010.277.280
National Reseaarch Council 2001 *Nutrient Requirements of Dairy Cattle*. 7th revision. Edition. Washington, DC. National Academy Press
Porter L J, Hrstich L N and Chan B G 1986 *The conversion of procyanidins and prodelphinidins to cyaniding and delphinidin. Phytochemistry*, 25:223-230. http://www.sciencedirect.com/science/arti…,
Preston T R and Leng R A 1987 *N Transaction in the rumen In : Matching ruminant production systems with available feed resources in the tropics and subtropics*. Penambul Books, Armidale, Australia.
Roger P, Darren M and Simon H 2012 *Introduction to GenStat® for Windows*TM 15th Edition. VSN International, 5 The Waterhouse, Waterhouse Street, UK. http://www.genstat.co.uk/
Schwab C G, Bozak C K, Whitehouse N L and Mesbah M M A 1992 *Amino acid limitation and flow to duodenum at four stages of lactation*. 1. Sequence of Lysine and Methionine limitation. J. Dairy Sci. 75, 3486–3502. http://www.journalofdairyscience.org/article/S0022-0302(92)78125-9/abstract
Schwab, C. G. 1995. *Protected proteins and amino acids for ruminants*. In: R. J. Wallace and A. Chesson (Eds.) *Biotechnology in Animal Feeds and Animal Feeding*. pp. 115-141. V.C.H. Press, Weinheim, Germany. Schwab, C. G. 1996. Amino acid nutrition
Schwab C G, Bozak C K, Whitehouse N L and Olson V M 1996 *Amino acid limitation and flow to duodenum at four stages of lactation*. 2. Extent of Lysine and Methionine limitation. J. Dairy Sci. 75:3503–3518. http://www.sciencedirect.com/science/article/pii/S0022030292781260
Sliwinski B J, Soliva C R, Machmuller A and Kreuzer M 2002 *Efficacy of plant extracts rich in secondary constituents to modify rumen fermentation*. Anim. Feed Sci. Technol., 101: 101-114. http://cat.inist.fr/?aModele=afficheN&cpsidt=13946949
Steel R G D and Torrie J H 1980 *Principles and Procedures of Statistics: A BioMetrical Approach*. McGraw-Hill, New York.
Sutardi T 1980 *Introduction of Animal Nutrition*. Animal Nutrition Departement. Animal Husbandary Faculty. Bogor Agriculture University.
Wanapat M and Wachirapakorn C 1990 *Techniques in Feeding of Beef and Dairy Cattle*. Funny Press. Publ., Ltd. Bangkok. 142 pp. (in Thai).