Hibiscus schizopetalus as saponin source, reduce protozoa number and increase microbial protein synthesis on in vitro sheep rumen fermentation

Asih Kurniawati and Nafiatul Umami

Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia

ABSTRACT : Effect of *Hibiscus schizopetalus* leaf as saponin source on rumen feed fermentation of sheep was studied using in-vitro gas production techniques. Grounded *Hibiscus schizopetalus* leaf equal to level of saponin 0%, 0.1%, 0.2%, 0.3% and 0.6% of feed dry matter (DM) was added to feed substrate of king grass and concentrate diet of (60:40), in the expense of king grass. Incubation was done at 39^{o}C for 48 hours. Protozoa number was significantly reduced ($P<0.05$) in line with increasing saponin level. Increasing saponin levels increased microbial protein ($P<0.01$) and gas production ($P<0.05$). The level of saponin at 0.6%DM resulted the lowest protozoa number, the highest microbial protein synthesis and the highest gas production. VFA concentration, ammonia concentration and pH of medium after 48 h fermentation on the other hand were not affected by the treatment. It could be concluded that the addition of saponin from *Hibiscus schizopetalus* up to level 0.6% of feed DM gave the positive effect on rumen feed fermentation**.**

Keys words: *hibiscus schizopetalus*, saponin, protozoa, microbial protein, rumen fermentation, in vitro

INTRODUCTION

In ruminant, volatile fatty acid (VFA), gases and microbial biomass (mainly protein) are the main end product of fermentation of feed organic matter (Alexander et al., 2008). Microbial biomass that flow to duodenum from the rumen is an importance source of protein for the host animal. To date, contribution of microbial cell biomass has been mainly considered in term of nitrogen (N) supply (David et al., 2006). Fifty to eighty persen of the N reached the small intestine is microbial origin (Demeyer 1991). The protein source for ruminant, additional to microbial protein, also come from feed protein that escaped from rumen degradation. Existence of protozoa in the rumen has an effect on flow of protein into duodenum. Protozoa represent approximately 50% of the microbial biomass in the rumen. Their presence has been shown to be important but not essential for the ruminant (Jouany 1991). Since protozoa retained in the rumen (David et al., 2006) about 70%, it is not account for microbial protein source (Weller and Pilgrim, 1974). Un like bacteria, protozoa have no ureases and can not use ammonia as a nitrogen source. They ingest bacterial and dietary proteins, and excrete as much as 50% of ingestsed nitrogen in the form of amino acids and ammonia (Jouany 1991). Their proteolitic activity and the active engulfment of bacteria is the most important on regulating the turnover of bacteria N in the rumen (Koenig at al., 2000). Several studi showed that elimination of protozoa number (defaunation) may enhanced the flow of microbial protein from the rumen, increased the efficiency of feed utilization and improved the nutrition of animal (Makkar, 1998). Removing ciliate protozoa from the rumen should prevent the recycling of N between bacteria and protozoa, and thereby increase the efficiency of N metabolism in the rumen and stimulate the flow of microbial protein into small intestine (Teferedegne, 2000). Defaunation reduced bacteria N recycle by 33% (Koenig et al., 2000). Moate (1989) cited by Hart et al., (2008) reported that defaunation is also beneficial as it increased milk yield and milk protein to fat ratio in dairy cow. Moreover protozoa also well known associated with methane emission from enteric fermentation trough interspecies commensalisms with metanogenic archaea. Seventhy percen of total methanogenesis was assosiated with the protozoa (Dore, and Gouet, 1991).

Several strategies were suggested to modify rumen fermentation to make it more efficient in fibre digestion or to have less protein degradation or less intra ruminal nitrogen turn over and hence have more outflow of protein to duodenum.

Many plants produce secondary metabolites, a group of chemicals that are not involved in the primary biochemical processes of plant growth and reproduction but are important to protect plants from insect predation or grazing by herbivores. Several thousand plant secondary metabolites have been reported. Among of them for example is saponins (Wallace et al., 1994) that have antimicrobial activity (Vincken et al., 2007).

Saponins are glycosides of aglycone linked to one or more sugar chain that are generally considered as antinutritional factors (Teferedegne, 2000). Saponin shows a toxic effect on rumen protozoa (Newbold et al., 1997). The toxicity of saponin to protozoa is obviously the result of their detergent effect on the cell membrane. The sensitivity of protozoa toward saponin is caused by their membrane sterol bind with saponin (Wina et al., 2005) which form insoluble complexes and caused cell lysis (Francis et al., 2002). Several studi of addition plants saponin have variation effect. Wina et al., (2005) reported that saponin containing a methanol extract of *Sapindus rarak* decrease protozoa number, and increase microbial protein synthesis and VFA in in vitro studi. In same way saponin extract from alfalfa on sheep decrease protozoa count, synthesis microbial N, ammonia and VFA concentration but increase feed digestibility when sheep fed concentrate (Lu and Jorgensen, 1987). Mao et al., (2010) reported that tea saponin decrease ammonia concentration and pH, protozoa number and methan production and increase VFA concentration on growing lamb. The variation of results seem to be caused by the differences of saponin source and animals. Biological activity of saponins related to their structure that have specific properties of specific sources (Francis et al., 2002). Effect of some saponin has been found to differ in different animals Teferedegne et al., (1999).

Hibiscus schizopetalus is a plant, it's leafs contain saponin that may be used as defaunating agent in ruminant. In some village area these plants commonly being used as alternative feed for ruminant, particularly on dry season, but the study of its effect on ruminant is limited. Therefore the objective of this research was to evaluate the effect of addition of *Hibiscus schizopetalus* leafs as saponin source on in vitro feed fermentation on rumen sheep.

MATERIALS AND METHODS

Plant Material, Chemical Composition and Saponin Analysis

Hibiscus schizopetalus leaf as saponin source was obtained from farmer's field. It was air dried and ground to pass a 1 mm screen. Saponin analysis was done according to Makkar et al., (2007). The data of saponin concentration was used to calculate the amount of *Hibiscus schizopetalus* leaf that was added to substrate. Dry matter (DM), organic matter (OM), crude protein (CP), extract ether (EE) and crude fiber (CF) were analyzed as per method of AOAC (1995).

Incubation

The effect of this plant addition on ruminal fermentation was examined in in-vitro gas production following procedure described by Menke et al., (1979) Substrate was a mixture of king grass (*Pennisetum hybrids*) and commercial ruminant concentrate (60:40), thoroughly homogenized and ground to pass a1mm screen. Rumen fluid as inoculums was collected from rumen sheep slaughtered at government's slaughtered house of Yogyakarta, immediately transported to laboratory in pre-warmed vacuum flask and strained through fours layer of cheesecloth. The culture medium contained 660 ml rumen fluid, 1095 ml H_2O, 730 ml buffer (4 g NH_4HCO_3 + 35g$NaHCO_3$ diluted to 1000 ml on H_2O), 365 ml macro mineral (5.7 g Na_2HPO_4 + 6.2 g KH_2PO_4 + 0.6 g $MgSO_4$.7 H_2O, diluted to 1000 ml on H_2O) , 0.23 ml micro mineral (13.2 g $CaCl_2$.2H_2O + 10 g $MnCl_2$.4 H_2O + 1 g Co Cl_2.6 H_2O + 8 g Fe Cl_3. 6 H_2O diluted to 100 ml on H_2O) , 1 ml rezasurine 0.1 % (w/v), 60 ml reducing solution (3.7 ml NaOH 1N + 580 mg Na_2S.9 H_2O diluted to 60 ml on H_2O). Prior to the adding rumen fluid, the medium had been extensively reduced by continuous bubbling CO_2 and warmed at 39^{O}C. Thirty milliliter of buffered rumen fluid was anaerobically dispensed into syringes containing 300 g of mixed substrate. Triplicate syringes for each treatment were incubated at 39^{O}C for 48 h. *Hibiscus*

schizopetalus leaf was applied in a series doses correspond to saponin level of 0% (as control), 0.1%, 0.2%, 0.3%, and 0.6% of feed dry matter, replacing equivalent amount of king grass.

Analytic Methods

After 48 h incubation, gases were measured and syringe contents were transferred to centrifuge tubes and centrifuged at 500 x g for 20 min at 4^{O}C. The pH of medium was determined using digital pH meter. A milliliter supernatant was preserved by adding 0.8 ml of formaldehyde solution (37% formaldehyde (v/v): 0.9% (w/v) NaCl, 1:9) as sample for protozoa number calculation using haemocitometer according to Diaz *et al*., (1993). To one milliliter supernatant was added with 1 ml 20% NaCl for ammonia analysis according to Wheatherburn, (1976). Carboxymethyl cellulase (CMC-ase) activity was analysis based on the determination of reducing sugar that was liberated from incubation of supernatant on buffered carboxymethylcellulose (CMC) (Halliwell *et al.* 1985). For rumen microbial protein determination, 3 ml supernatant was recentrifuged at 10.000 x g for 20 min at 4^{O}C. Protein content of the pellet then was analyzed according to Lowry method (Alexander and Griffiths 1992).

Statistical Analysis

One way ANOVA design was used to analysis the data. Treatment means were compared using Duncan's New Multiple Range Test.

RESULTS AND DISCUSSIONS

Protozoa Number and Fermentation Product

Protozoa number was affected ($P<0.05$) by the addition of leaf of *Hibiscus schizopetalus* on feed as saponin source. Protozoa number reduced in line with increasing saponin level (Table 1). The lowest number of protozoa occured when saponin was added on level 0.6%DM, the decrease was 58.83% as much as compared to control. This effect of saponin on protozoa number was in agreement with Wina et al., (2005) that studied saponin from S*apindus rarak* on in-vitro rumen fermentation, Lu and Jorgensen (1987) that evaluated Alfalfah saponin on in-vivo fermentation, and Mao et al., (2010) that explored the effect of tea saponin on growing lamb. Hanim et al., (2007) reported that protozoa number on in-vitro fermentation on feed was decreased about 28.26% (compared to control) with addition of 0.4% DM basis of saponin from *Cassia alata. L.* At the same level of saponin, 0.6% DM basis, the decreasing of protozoa number in this research (70.27%) was higher than reported by Hanim et al., (2007). This differences effect on those two study was related to the saponin sources. Biological actifity of saponins are vary depend on the sources that influence their properties and stuctures (Francis *et al.* 2002). Type of linkage and sugar composition of saponin is directly related to their biological activity (Teferedegne et al., 1999).

Microbial protein synthesis was increased with increasing of saponin addition ($P<0.01$). The highest of total microbial protein was reached when saponin added on level 0.6% of feed DM (Table 1). The increasing value was 14.84% compared to control. Newbold et al., (1997) and Diaz et al., (1993) reported that microbial protein synthesis and feed protein efficiency were increased with saponin supplementation. Saponin from tea (Mao et al., 2010), *Sapindus rarak* (Wina et al., 2005), *Sesbania sesban* (Newbold et al., 1997), *Yucca schindigera* (Valdez et al., 1986) showed to have an effect on increasing microbial protein. The increasing microbial protein synthesis was related to reduction of protozoa number, therefore less predation on bacteria by protozoa and the growth of bacteria was increased and rumen bacteria turn over was declined (Wallace et al., 1994).

In rumen, feeds are fermented resulting volatile VFA, gases and microbial biomass as main product (Alexander et al., 2008). VFA is the main energy sources for ruminant as well as for rumen microbes. The increasing of VFA product will provide more energy for ruminant production. Total VFA, in this reseach, was not influenced by saponin addition as well as parcial component of VFA (asetat, propionat, butirat) but total VFA on saponin treatment tend to higher than control. Several

research showed inconsistency result of saponin effect on VFA concentration. *Sapindus rarak* saponin (Wina et al., 2005), tea saponin (Mao et al., 2010), were found to increase VFA whereas saponin from alfalfa (Lu and Jorgense, 1987), *Yucca schidigera* and *Quilaja saponaria* (Holthausen et al., 2009) reduced the VFA concentration, while Singer et al., (2008) showed *Yucca schidigera* saponin had no effect on VFA. This inconsistency effect of saponin on VFA may be caused by the differences of saponin sources as mentioned by Francis et al., (2002) and Teferedegne et al., (1999) who stated that biological actifity of saponins were vary. The biological effect of saponin depend on its chemical properties (Makkar, 2001)

Tabel 1. Protozoa number and fermentation product at 48 h incubation of feed with addition various level of Hibiscus Schizopetalus leaf as saponin source

	Saponin Level (% feed DM)				
Parameter	0	0.1	0.2	0.3	0.6
Protozoa number, x10^3 cell/ml*	52.45^{c}	38.25^{bc}	31.21^{ab}	34.73^{ab}	15.59^{a}
Fermentation product					
Gas Production, ml/g DM*	170.50^{a}	164.10^{a}	172.30^{a}	175.95^{ab}	192.35^{b}
N microbial product, mg**	284.66^{a}	309.53^{b}	315.99^{bc}	322.03^{bc}	334.25^{c}
Total VFA, mM/l	69.87	69.44	73.84	70.95	75.24
VFA, mM/l					
Acetat	47.23	47.10	49.61	47.68	51.79
Propionat	14.82	17.50	15.45	15.06	15.24
Butirat	7.81	7.85	8.77	8.21	8.21

* ($P<0.05$); ** ($P<0.01$)
^{a,b,c} Means values within a row with different letters (a-c) differ significantly

Gas that is produced during in-vitro fermentation is one of the others end product from feed fermentation. This gas mainly composed of CO_2, CH_4 and several trace gas compound. CO_2 that form during fermentation in adittion to CO_2 from feed fermentation come from carbonate buffer in the medium. One mol CO_2 will be released from medium every 1 mol VFA being produced (Blümmel and Ørskov, 1993). Gas volume could be used to predict DM degradation (Blummel *et.al*., 1997; Kurniawati, 2007) and OM degradation (Beuvink, and Spoelstra, 1992) since gas volume have a highly corelation with both DM and OM degradation. Gas production increased with increasing level of saponin ($P<0.05$). The higher gas volume in this research was achieved when saponin added at level 0.3% and 0.6%.

Parameter of Fermentation

Manipulation on rumen fermetation should keep fermentation proscess and main function of rumen on fibre digestion going on. Ammonia concentration and pH value are two factors among other factors that influence the rumen fermentation. The adittion of saponin in this research did not influence the ammonia concentration. The ammonia concentration tend to be similar between treatment (Tabel 2). This result is in agreement to Muetzel et al., (2003) and Hristov et al., (1999) who reported *Sapindus rarak* saponin did not inhibit protein degradation and *Yucca schidigera* saponin did not affect the proteolitic activity in the rumen, respectively. Several study demontrated decreasing of ammonia concentratin by addition of saponin on rumen fermentation (Moa et al., 2010, Holtshausen et al., 2009, Lu and Jorgensen, 1987), due to defaunation of protozoa and reduction of protozoa activity on protein degradation. Ammonia in rumen fluid was product from feed protein and microbial protein degradation (Leng and Nolan, 1984). Hart *et al*. (2008) reported that the effect of saponin on ammonia concentration was not consistent.

pH and cellulolitic activity were not influenced by the saponin addition, in fact the cellulolitic activity tend to be higher on saponin treatment group than control (Table 2.). The average pH values at all treatmant were in normal level for rumen fermentation. It mean that saponin from *Hibiscus schizopetalus* did not interup the fermentation fuction of rumen. In the contrary Francis et al., (2002) explained that *Yucca schidigera* extract tend to have negative effect on cellulolitic and amilolitic bacteria. Wang et al., (2000) reported after 14 d dosed of *Yucca schidigera* saponin, *Ruminococcous*

albus and *Ruminococcous flavefaciens* loss it ability to digest celloluse. Similar to Wang et al., (2000), Ningrat et al., (2002) described that growth of cellulolitic microbes in the rumen was decreased with the addition of *Sapindus rarak* saponin.

Tabel 2. Ammonia concentration, pH, and CMC-ase activity of medium after 48 h incubation of feed with addition various level of *hibiscus schizopetalus* leaf as saponin source

	Saponin Level (% feed DM)				
Parameter	0	0.1	0.2	0.3	0.6
Ammonia (mg/100ml)^{ns}	41.95	44.01	46.23	45.09	43.37
pH^{ns}	7.44	7.38	7.31	7.42	7.36
CMC-ase activity (u/ml)^{ns}	0.028	0.056	0.016	0.055	0.039

^{ns} = non significant

CONCLUSIONS

Addition of *Hibiscus schizopetalus* leaf on rumen feed fermentation as saponin source decreased protozoa number, increased feed digestibility that was represented by gas production, and increased microbial protein synthesis without disturbing feed fermentation. VFA, pH, ammonia concentration, and cellulolitic activity were not affected. Saponin level up to 0.6% of feed DM gave positive effect on rumen feed fermentation**.**

LITERATURE CITED

Alexander, R.R., and J.M. Griffiths. 1992. Basic Biochemistry Methods 2nd Ed. A John Wiley and Sons Inc. Publ. USA

Alexander, G., B. Singh, A. Sahoo, and T.K. Bhat. 2008. In vitro screening of plant extracts to enhance the efficiency of utilization of energy and nitrogen in ruminant diets. Anim. Feed Sci. and Technol. 145: 229-244

AOAC. 1995. Official Methods of Analysis, 16th ed. Association of Official Analytical Chemists, Arlington, VA, US.

Beuvink, J.M.W., And S.F. Spoelstra.1992. Interactions between substrate, fermentation end-products, buffering system and gas production upon fermentation of different carbohydrates by mixed rumen microorganism in vitro. Appl. Microbiol. Biotechnol. 37, 505-509

Blümmel, M., and E.R. Ørskov. 1993. Comparison of in-vitro gas production and nylon bag degradability roughages in prediction of feed intake in cattle. Anim. Feed Sci. and technol. 40: 109-229

Blümmel, M., H. Steingaβ, and K. Becker. 1997. The Relationship between in-vitro gas production, in-vitro microbial biomass yield and ^{15}N incorporation and its implications for prediction of voluntary feed intake of roughages. Bri. J. of Nutr. 77: 911-921

David, R., Y. Ruiz, Scollan, N.D., Merry, R.J., and C.J. newbold. 2006. Contribution of rumen protozoa to duodenal flow of nitrogen, conjugate linoleic acid and vaccenic acid in steers fed silages differing in their water-soluble carbohydrate content. British Journal of Nutrition. 96: 861-869

Diaz, A., Avendano, M., and A. Escobar. 1993. Evaluation of S*apindus saponaria* as a defaunating agent and its effects on different rumen digestion parameters. Livest. Res. Rural Dev. 5: 1-6

Demeyer, D.I. 1991. Quantitative aspects of microbial metabolism. In: the rumen and hindgut in Rumen Microbial Metabolism and Ruminant Digestion. (J.P. Jouany eds). INRA. Paris

Dore, J., and Ph. Gouet. 1991. Microbial interaction in the rumen. In: Rumen microbial metabolisn and ruminant digestion. Jouany ed. INRA Paris

Francis, G., Z. Kerem, H.P.S. Makkar, and K. Becker. 2002. The biological action of saponins in animal systems: a review. Bri. J. Nutr. 88: 587-605

Halliwell, G., M.N.B.A . Wahab, and A.H. Patel. 1985. The contribution of endo-1,4-ß-D-glucanase to cellulolitic in *Tricoderma coningi*. J. Appl. Biochem. 7: 43-45

Hanim, C., L.M. Yusiati, and I.M. Santo. 2007. Pengaruh Penambahan Daun Ketepeng Cina (*Cassia alata L.*) sebagai Sumber Saponin pada Pakan terhadap Rumput Raja dan Dedak Halus di dalam Rumen secara In Vitro. Prosiding Seminar Nasional. Peternakan dan Pemberdayaan Masyarakat Pedesaan. UGM.

Hart K.J., D.R. Y´a˜nez-Ruiz, S.M. Duval, N.R. McEwan, and C.J. Newbold, 2008. Plant extracts to manipulate rumen fermentation. Anim. Feed Sci. and Technol.147: 8-35

Holtshausen,L., A.V. Chaves, K.A. Beauchemin, S.M. McGinn, T.A. McAllister, P.R. Cheeke, and C. Benchaar. 2009. Feeding saponin-containing *Yucca schidigera* and *Quillaja saponaria* to decrease enteric methane production in dairy cows. J. Dairy Sci. 92: 2809-2821

Hristov, A.N., T.A. McAllister, F.H. Van Herk, K.J. Cheng, C.J. Newbold and P.R. Cheeke. 1999. Effect of *Yucca schidigera* on ruminal fermentation and nutrient digestion in heifers. J. Anim. Sci. 77: 2554–2563

Jouany, J.P., 1991. Defaunation of the Rumen. In: Rumen Microbial Metabolism and Ruminant Digestion. (J.P. Jouanyeds). INRA. Paris

Koenig, K.M., C. J. Newbold, F. M. McIntosh and L. M. Rode. 2000. Effects of protozoa on bacterial nitrogen recycling in the rumen. J Anim Sci. 78: 2431-2445

Kurniawati, A. 2007. Teknik Produksi Gas In-Vitro untuk Evaluasi Pakan Ternak : Volume Produksi Gas dan Kecernaan Bahan Pakan. Jurnal Ilmiah Aplikasi Isotop dan Radiasi 3. 1: 40-51

Leng, R.A., and J.N. Nolan. 1984. Nitrogen metabolism in the rumen. J. Dairy Sci. 67: 1072-1089

Lu, C.D. and N.A. Jorgensen. 1987. Alfalfa saponin affect site and extent of nutrient digestion in ruminant. J. Nutr. 117: 919 - 927

Makkar, H.P.S. 1998. Effect of antinutrients on the nutritional value of legume diets. Proceedings of the seventh scientific workshop. European Commision. in TromsØ 18 to 21 June 1998

Makkar, H.P.S. 2001. Role of tannins and saponins in nutrition. Institute fro Animal Production in the Tropics and Subtripics, Univ. Of Hohenheim, Stuttgart. Germany. Unpublish

Makkar, H.P.S., P. Sidduraju, and K. Becker. 2007. Plant Secondary Metabolites. Humana Press. Totowa. New Jersey.

Mao, H.L., J.K. Wang, Zhou, Y.Y., and Liu, J.X. 2010. Effect of addition of tea saponins and soybean oil on methane production, fermentation and microbial population in the rumen of growing lambs. Livestock Sci. doi: 10.1016/j.livsci.2009.12.011

Menke, K.H., L. Raab, A. Slewski, H. Steingass, D. Fritz, and W. Schneider. 1979. The estimation of the digestibility and metabolisable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor. J. Agric. Sci. 93: 217-222

Muetzel, S., E.M. Hoffmann, and K. Becker. 2003. Supplementation of barley strawwith *Sesbania pachycarpa* leaves in vitro: effects on fermentation variables and rumen microbial population structure quantified by ribosomal RNA-targeted probes. Bri. J. Nutr. 89: 445-453

Newbold, C.J., S.M. ElHassan, J. Wang, M.E. Ortega, and R. J. Wallace. 1997. Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. Br. J. Nutr. 78: 237-249

Ningrat, R.W.S., P.C. Garnsworthy, and C.J. Newbold. 2002. Saponin fractions in *Sapindus rarak*: effects on rumen microbes. Reprod. Nutr. Dev. 42 (Suppl. 1), S82.

Singer, M.D., P.H. Robinson, A.Z.M. Salem, and E.J. DePeters. 2008. Impact of rumen fluid modified by feeding *Yucca schidigera* to lactating dairy cows on in vitro gas production of 11 common dairy feedstuffs, as well as animal performance. Anim. Feed. Sci. 146: 242-258

Teferedegne, B. 2000. New perspectives on the use of tropical plants to improve ruminant nutrition. Proceeding of the Nutrition Society. 59: 209-214

Teferedegne, B., F. McIntosh, P.O. Osuji, A. Odenyo, R.J. Wallace, and C.J. Newbold. 1999. Influence of foliage from different accessions of the sub-tropical leguminous tree, *Sesbania sesban*, on ruminal protozoa in Ethiopian and Scottish sheep. Anim. Feed Sci. Technol. 78: 11–20

Valdez, F.R., L.J. Bush, A.L. Goetsch, and F.N. Owens. 1986. Effect of steroidal sapogenins on ruminat fermentation and on production of lactating dairy cows. J. Dairy Sci. 69: 1568-1575

Vincken, J.P., L. Heng, A. Groot, and H. Gruppen. 2007. Saponins, classification and occurence in the plant kingdom. Phytochemistry 68: 275-297.

Wallace, R.J., L. Arthauo, and C.J. Newbold. 1994. Influence of *Yucca shidigera* extract on ruminal ammonia concentration and ruminal microorganisms. Appl. Environ. Microbiol. 60: 1762-1767

Wang, Y.X., T.A. McAllister, L.J. Yanke, Z.J. Xu, P.R. Cheeke, and K.J. Cheng. 2000. In vitro effects of steroidal saponins from *Yucca schidigera* extract on rumen microbial protein synthesis and ruminal fermentation. J. Sci. Food Agric. 80: 2114–2122.

Weatherburn, M.W. 1976. Phenol Hyphochloride Reaction for Determination of ammonia. Analysis of Chemis. 39: 971-974

Weller, R. A., and A. F. Pilgrim. 1974. Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system. Bri.J. of Nutr. 32: 341-351.

Wina, E., S. Muetzel, E. Hoffmann, H.P.S. Makkar and K. Becker. 2005. Saponin containing methanol extract of Sapindus rarak affect microbial fermentation, microbial activity and microbial community structure in vitro. Anim. Sci. and Technol. 121: 159-174.