WARU LEAF (*Hibiscus tiliaceus*) AS SAPONIN SOURCE ON *In vitro* RUMINAL FERMENTATION CHARACTERISTIC

L. Istiqomah[1], H. Herdian[1], A. Febrisantosa[1] and D. Putra[2]
[1]Research Unit for Development of Chemical Engineering Processes,
Indonesian Institute of Sciences (LIPI),
Jl. Jogja - Wonosari Km. 31, Gading, Playen, Gunungkidul, Yogyakarta 55861- Indonesia
[2]Faculty of Mathematics and Natural Science, Sebelas Maret University
Jl. Ir. Sutami 36 A Surakarta 57126 - Indonesia
Corresponding E-mail: ps_uty@yahoo.com

Received December 10, 2010; Accepted February 16, 2011

ABSTRACT

The effect of waru leaf (*Hibiscus tiliaceus*) supplementation as saponin source on ruminal fermentation characteristics were studied using in vitro gas production techniques. Rumen fluid was taken from fistulated Ongole crossbreed cattle. The treatments consisted of control treatments (Napier grass with monensin and Napier grass without waru leaf (0% saponin level) and waru leaf supplementation treatments as much as 11, 22, 33, and 44 mg of feed (in dry matter basis) or equal to 5, 10, 15, and 20% saponin level added to feed substrate of Napier grass (*Pennisetum purpureum*). The result showed that protozoa numbers and total gas production were significantly reduced ($P<0.05$) in line with the increasing of saponin level compared to 0% saponin level, while NH_3, VFA concentration and pH after 48 h fermentation were not affected by the treatment. VFA concentration increased by waru leaf up to 10% saponin level then decreased at level 15 and 20%. Ratio of acetate to propionate (A/P) and non glucogenic ratio (NGR) decreased at 5, 10, and 15% saponin level, but increased at level 20%. It could be concluded that waru leaf supplementation at 10% saponin level of feed was the optimum level which gave positive effect on rumen feed fermentation.

Keywords: In vitro, ruminal fermentation, saponin, waru leaf (H. tiliaceus)

INTRODUCTION

The main products of fermentation of feed organic matter in ruminant were volatile fatty acid (VFA), gases and microbial biomass (mainly protein) (Alexander *et al*., 2008). The protein source for ruminant, additional to microbial protein also came from feed protein escaped from rumen degradation. Existence of protozoa in the rumen has an effect on flow of protein into duodenum. Unlike bacteria, protozoa has no urea and could not use ammonia as a nitrogen source. They ingested bacterial and dietary proteins, and excreted as much as 50% of ingested nitrogen in the form of amino acids and ammonia (Jouany, 1991). Several studies showed free fatty acids reduced protozoa numbers, as well, and low pH may cause defaunation (Owens *et al*., 1998). Removing ciliate protozoa from rumen may prevent recycling of N between bacteria and protozoa, and thereby increased the efficiency of N metabolism in rumen and stimulated flow of microbial protein into small intestine (Teferedegne, 2000). Moreover protozoa also well known associated with methane emission from enteric fermentation trough interspecies commensalisms with methanogenic archaea. Seventy percent of total methanogenesis was associated with the protozoa (Dore and Gouet, 1991).

Several strategies were suggested to modify rumen fermentation to make it more efficient in fiber 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 produced secondary metabolites, a group of chemicals that were not involved in the primary biochemical processes of plant growth and reproduction but were important to protect plants from insect predation or grazing by herbivores. Several thousand plant secondary metabolites had been reported such as saponins that had antimicrobial activity (Vincken *et al*., 2007). Saponins were glycosides of aglycone linked to

one or more sugar chain that were generally considered as anti nutritional factors (Teferedegne, 2000). Saponin showed a toxic effect on rumen protozoa (Newbold *et al*., 1997). The toxicity of saponin to protozoa was obviously the result of their detergent effect on the cell membrane. The sensitivity of protozoa toward saponin was caused by their membrane sterol bind with saponin (Wina *et al*., 2005) which formed insoluble complexes and caused cell lysis (Francis *et al*., 2002).

Hibiscus tiliaceus leaf was a native tropical plant contains saponin that may be used as defaunating agent in ruminant. In some area these plants commonly being used as the alternative feed for ruminant, particularly on dry season, but the study of its effect was limited. Therefore the objective of this study was to evaluate the effect of *Hibiscus tiliaceus* leaf supplementation as saponin source on in vitro feed fermentation on rumen.

MATERIALS AND METHODS

Plant Material, Chemical Composition, and Saponin Analysis

Hibiscus tiliaceus leaf was grounded to pass a 1 mm screen and dried in oven at 55ºC for 72 h. Saponin analysis was done according to Makkar *et al*. (2007). The data of saponin concentration was used to calculate the amount of *H. tiliaceus* leaf that was added to substrate. Monensin (2 mg) was used as positive control (G.200^C Monensin Sodium Elanco Animal Health Division). Dry matter (DM), crude protein (CP), and crude fiber (CF) were analyzed by AOAC method (1995).

Incubation

The effect of this plant supplementation on ruminal fermentation was examined by in vitro gas production following procedure described by Menke *et al*. (1979). Two ruminally fistulated Ongole crossbred cattle were used as rumen liquor donor. Animals were fed twice daily with a basal diet consisting of Napier grass and concentrate (70:30, on DM basis). Rumen fluid was taken using aspirator, and immediately transported in pre warmed vacuum flask (39ºC water temperature) and filtered. *H. tiliaceus* leaf was applied in a series dosage appropriate to level of saponin 0, 5, 10, 15, and 20% of feed (in dry matter basis), replacing equivalent amount of Napier grass.

Treatments and Statistical Analysis

Experimental design of this study was Completely Randomized Design with six treatments descripted as follows:

1. *P. purpureum* (200 mg) + *H. tiliaceus* (level of saponin 0% of feed in DM basis)
2. *P. purpureum* (200 mg) + *H. tiliaceus* (level of saponin 5% of feed in DM basis)
3. *P. purpureum* (200 mg) + *H. tiliaceus* (level of saponin 10% of feed in DM basis)
4. *P. purpureum* (200 mg) + *H. tiliaceus* (level of saponin 15% of feed in DM basis)
5. *P. purpureum* (200 mg) + *H. tiliaceus* (level of saponin 20% of feed in DM basis)
6. *P. purpureum* (200 mg) + Monensin (2 mg)

Effect of treatment was analyzed using the test of variance (ANOVA) and differences between treatments were analyzed using Duncan's Multiple Range Test (DMRT) (Gomez and Gomez, 1984).

Measurement of Gas Production

Gas production was measured by Menke and Steingass procedure (1988). Fermentation was conducted in glass size 100 mL syringe (Fortuna model, Poulten and Graft Gmbh Germany). A total of 200 mg of Napier grass substrate and *H. tiliaceus* leaf were entered into the syringe and incubated for one night in an incubator at 39ºC. Then, along with CO_2 flow, a mixture of rumen fluid was added by a medium mixture of 30 mL with a ratio of 1:2 into the syringe. All of the measurements were repeated three times. Incubation was then continued for 48 h at 39ºC. Gas production measurements were performed at 0, 3, 6, 12, 24, 48 h. After 48 h incubation gas was released, whereas rumen fluid contained in syringe samples taken for analysis of protozoa population, VFA, and NH_3.

Observed Variables

Variables measured were protozoa numbers, gas production, VFA concentration, ammonia concentration, and pH rumen fluid. After 0, 3, 6, 12, 24, and 48 h of incubation, gases were measured and syringe contents were transferred to centrifuge tubes and were centrifuged at 500 x g for 20 min at 4^{o}C. The pH of medium was determined using digital pH meter. The calculation of protozoa number was done using MFS solution composed of 20 mL 35% formaldehyde solution, 0.12 g of methyl green, and 1.6 g of NaCl and hemocytometer according

to Diaz *et al*. (1993). VFA measurements were carried out according to method of Santoso and Hariadi (2007) using Gas Chromatography (Shimadzu GC8). Measurement of microbial rumen fluid was done using the Lowry *et al*. (1951) method modificated by Waterborg (2002).

RESULTS AND DISCUSSION

Nutrient composition of Napier grass (*Pennisetum purpureum*) and waru leaf (*Hibiscus tiliaceus*) are presented in Table 1. Crude protein content in *P. purpureum* and *H. tiliaceus* were higher than the minimum concentration of CP (7%) required for microbial activity (Crowder and Chheda, 1982). High protein content was good enough for cattle to protein needs. Crude fiber of *H. tiliaceus* leaf was lower than the Napier grass that was good to reduce methane gas production, while carbohydrate content of *H. tiliaceus* was high (45.91%) that may available for livestock as source of energy. Saponin content of *H. tiliaceus* leaf in this study (8.93 mg/g DM) was lower than the saponin content of *Acacia mangium Willd* (16.7 mg/g DM) (Santoso and Hariadi, 2007).

Protozoa Number and Fermentation Product

In this study the protozoa number in rumen fluid decreased significantly ($P<0.05$) by the *H. tiliaceus* leaf supplementation in line with the increasing of saponin level (Table 2). *H. tiliaceus* leaf supplementation at level 10% decreased the protozoa number as much as 43.08% (9.25 $x10^3$/mL) than that of control ($16.25x10^3$/mL). Supplementation of plant extracts containing saponins reduced the population of protozoa linearly with the increasing of saponin dose (Teferedegne *et al*., 1999). Wina *et al*. (2005) reported that saponin of methanol extract of *Sapindus rarak* decreased protozoa number and increased microbial protein synthesis as well as VFA in *in vitro* study. Saponin of tea was also reported decreased protozoa number on growing lamb (Mao *et al*., 2010).

Saponin could disrupt the development of protozoa by the bond between the saponin with sterols in protozoa cell membrane surface that may affect cell membrane permeability (Patra *et al*., 2006) and further may cause membrane rupture, cell lysis and death. The presence of cholesterol content in eukaryotic (including protozoa) cell membranes, but not in prokaryotic bacterial cell (Hussain and Cheeke, 1995), suggested a possible selective susceptibility of ruminal ciliate protozoa to saponins due to an affinity of saponin to cholesterol.

According to gas production data in Table 2, it was known that monensin and waru leaf (*H. tiliaceus*) depress gas production significantly ($P<0.05$) compared to controls. *H. tiliaceus* leaf supplementation as much as 5, 10, 15, and 20% and the monensin addition on fermentation of Napier grass by rumen microbes in vitro decreased gas production ($P<0.05$) compared to control (47.17 mL). *H. tiliaceus* leaf supplementation at level 10% decreased the gas production as much as 11.02% (41.97 mL) than control (47.17 mL), but there were no difference ($P>0.05$) among level 10 and 15% of *H. tiliaceus* leaf supplementation. Monensin treatment as positive control produced the lowest gas production (18.48 mL), followed by level 20% of *H. tiliaceus* leaf supplementation (38.47 mL).

The reduction of gas production in this study was associated with the reducing protozoa number; when the protozoa number was less, then the gas production decreased. The gas production contained CO_2 and CH_4, which was caused by the fermentation of carbohydrates in the rumen and consisted of 32% CH_4, 56% CO_2 and 3.5% O_2

Table 1. Nutrient Composition of *P. purpureum* and *H. tiliaceus* (% Dy Matter)

	P. purpureum	*H. H. tiliaceus*
Crude protein (%)	11.50	17.08
Ether extract (%)	3.20	3.45
Crude fiber (%)	29.30	22.77
Ash (%)	15.90	10.79
Carbohydrate (%)	40.10	45.91
Tannin (%)	7.55	8.93
Saponin (mg/g)	8.01	12.90

Table 2. Protozoal Number and Fermentation Product at 48 h Incubation of Feed with Various Level of Supplementation of *Hibiscus tiliaceus L*eaf as Saponin Source and Monensin

Observed Variables	Level of *H. tiliaceus* Leaf Supplementation					Monensin
	0%	5%	10%	15%	20%	
Protozoal number, x 10^3/mL	16.25^{d}	4.50^{c}	9.25^{b}	9.00^{b}	6.75^{a}	7.25^{a}
Fermentation product						
Gas production, mL	47.17^{a}	44.29^{b}	41.97^{c}	40.43^{c}	38.47^{d}	18.48^{e}
Total VFA, mM/L	137.39^{abc}	152.93^{ab}	165.81^{a}	127.15^{bc}	129.54^{bc}	106.67^{c}
VFA, mM/L						
Acetic acid	99.17^{abc}	109.38^{ab}	115.90^{a}	89.51^{bc}	90.37^{bc}	70.78^{c}
Propionic acid	25.89^{abc}	31.35^{ab}	35.01^{a}	27.35^{bc}	23.29^{bc}	25.68^{c}
Butyric acid	12.33^{abc}	12.20^{ab}	14.90^{a}	10.28^{bc}	15.87^{bc}	10.20^{c}
A/P Ratio	3.83	3.49	3.31	3.27	3.88	2.76
NGR	4.83	4.34	4.16	4.05	5.24	-

Different superscript in the same row indicates significantly different ($p<0.05$)

(Arora, 1989). The study results of Santoso and Hariadi (2007) using *Acacia mangium* in *P. purpureum* at levels 15, 30 and 45% in 100% of the substrate indicated the volume of gas after 48 h incubation decreased linearly with the increasing concentration of *Acacia mangium*, being 57, 48.2, and 37.5 mL, respectively, that was lower than that of control treatment (64.7 mL). Hu *et al*. (2005) reported in his study that tea saponin at 0, 0.2, 0.4 mg/mL has a defaunating effect on protozoa and gas production (93.0, 90.5 and 92.0 mL).

The very high differences in gas production effect between the *H. tiliaceus* leaf with monensin supplementation was occurred because the *H. tiliaceus* leaf gave an effect on the decreasing of protozoa number, but it did not gave a significant difference on bacterial activity in rumen. Meanwhile, monensin as manipulator of rumen fermentation would reduce the population of protozoa, suppressed the populations of bacteria and methane production.

Total VFA production in this study ranged from 106.67 to 165.81 mM. VFA production was a sufficient condition for optimal rumen microbial protein synthesis, because VFA range required for rumen microbial growth was 80-160 mM (Van Soest, 1994). Other studies stated that the range of VFA in the rumen was 60 mM acetic acid, 20 mM propionate and 10 mM butyrate (Madigan *et al*., 2003). The increased VFA concentration in response to the increasing of saponin could be due to the reducing outflow from the reticulorumen, inhibition of VFA absorption by the ruminal ephitelium, or increasing of microbial VFA production. Supplementation of *Sapindus saponaria* fruits contained 120 g of saponin improved the ruminal VFA profile, microbial efficiency, and duodenal flow of microbial protein in sheep fed tropical grass-alone or grass-legume diet (Abreu *et al*., 2004).

In Table 3, *H. tiliaceus* leaf supplementation resulted the ratio of acetic to propionate (A/P) lower than the control. A/P ratio of control was 3.83 while the A/P ratio of *H. tiliaceus* supplementation 5, 10, 15, and 20% and monensin were 3.49, 3.31, 3.28, 3.88 and 2.76 respectively. It has been suggested that there is an increased proportion of propionate in the rumen compared to acetate. *H. tiliaceus* played a role in glucose metabolism affected production of propionate that was glucogenic. Rumen fermentation system that leads to the propionate also resulted in the value of non-glucogenic ratio (NGR) tends to decrease. Increased propionate which was glucogenic will reduce the value of NGR. NGR value in this study by *H. tiliaceus* supplementation 5, 10, and 15% (4.34, 4.16, and 4.05, respectively) were lower than the control (4.83), while at level 20% of *H. tiliaceus* supplementation resulted greater A/P ratio and NGR than control.

Table 3. Ammonia Concentration and pH after 48 h Incubation of Feed with Various Level Supplementation *H. tiliaceus* Leaf as Saponin Source and Monensin

Observed Variables	Level of *H. tiliaceus* Leaf Supplementation					Monensin
	0%	5%	10%	15%	20%	
Ammonia (mg/100 mL)	35.63	36.72	37.96	38.13	34.88	33.99
pH	7.06	7.07	7.05	7.03	7.11	7.15

Parameter of Fermentation

Manipulation on rumen fermentation should be considered to maintain fermentation process and main function of rumen on fiber digestion. Ammonia concentration and pH value were two factors among other factors that influenced the rumen fermentation. In this study, the determination of ammonia concentration in rumen fluid was done using Raneff method (Chaney and Marbach, 1962). Table 3 showed that monensin treatment or *H. tiliaceus* leaf supplementation was not significantly different (P>0.05) compared to controls. The results also showed the tendency that *H. tiliaceus* leaf supplementation at level 5, 10, and 15% increased ammonia concentration, while the supplementation of monensin and *H. tiliaceus* leaf supplementation at level 20% tend to decrease the concentration of NH_3. Mao *et al*. (2010) reported that tea saponin decreased ammonia concentration and pH on growing lamb.

According to Madigan *et al*. (2003), the ammonia concentration in the rumen was balance between the production rate of NH_3 from the food and the use of NH_3 for microbial growth and endogenous compounds. Ammonia concentration obtained in this study varied at 30-42 mg/100mL. For maximum growth of rumen microbes, the concentration of NH_3 needed is 8.5 mg/100 mL of rumen fluid (Arora, 1989). Role of N-ammonia was very important as a raw material to form cells in the process of rumen microbial protein metabolism. In this study, rumen fluid ammonia values were relatively high. The high concentration of ammonia indicated the high value of easily degradable protein in these rations.

In this study, pH of rumen fluid was not influenced by *H. tiliaceus* (P>0.05) (Table 3). It means that saponin content on *H. tiliaceus* did not interrupt the fermentation function on rumen. This study showed the pH ranged between 7.03-7.15. Deamination produced NH_3, CO_2, and VFA, while at the decarboxylation step to produce amines and CO_2 due decarboxylase activity. Low pH caused the condition become acidic and lower rumen microbial population so the process of proteolysis would be inhibited and as a result of degradation of feed will go down (Madigan *et al*., 2003).

Nagaraja and Titgemeyert (2007) reported that ruminal pH generally was higher than 5.5 and often in the range from 5.8 to 6.5 in grain adapted cattle. Therefore it could be concluded that pH obtained in this study indicated the occurrence of cellulose fermentation process optimally. Ruminal pH was critical factor in normal and stable function of the rumen because of its profound effect on microbial populations and fermentation products, and on physiological function in the rumen, mainly on its motility and absorptive function.

CONCLUSION

Waru leaf (*Hibiscus tiliaceus*) supplementation at 10% saponin level of feed (in dry matter basis) was the optimum level because its ability to modify rumen fermentation characteristics which leads to the synthesis of propionate, reduced protozoa population and gas production, and there was no effect on the NH_3 concentration, VFA concentration, and pH value. Waru leaf (*H. tiliaceus*) supplementation also increased the proportion of propionate which was the main energy source for beef cattle.

REFERENCES

Abreu, A., J.E. Carulla, C.E. Lascano, T.E. Diaz, M. Kreuzer and D.H. Hess. 2004. Effects of *Sapindus saponaria* fruits on ruminal fermentation and duodenal nitrogen flow of sheep fed a tropica grass diet with and without legume. J. Anim. Sci. 82: 1392-1400.

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. Technol. 145:229-244.
AOAC. 1995. Official Methods of Analysis. 16th ed. Association of Official Analytical Chemists, Arlington, VA, US.
Arora, S. P. 1989. Microbes Digestion in Ruminant. 2nd ed. Gadjah Mada University Press: Yogyakarta.
Chaney, A.L. and E.P. Marbach. 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8:130-132.
Crowder, L.V. and H.R. Chheda. 1982. Tropical Grassland Husbandry. Longman Group Limited, New York, USA.
Diaz, A., M. Avendano 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.
Dore, J. and Ph. Gouet. 1991. Microbial interaction in the rumen. In: Rumen Microbial Metabolism 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. Br. J. Nutr. 88:587-605.
Gomez, K.A. and A.A. Gomez. 1984. Statistical Procedures for Agricultural Research. 2nd ed. An International Rice Research Institute Book. John Willey and Sons Inc. New York. Toronto.
Hu, Wei-lian, W. Yue-ming, L. Jian-xin, G. Yan-qiu and Y. Jun-an. 2005. Tea saponins affect in vitro fermentation and methanogenesis in faunated and defaunated rumen fluid. Zhejiang Univ. SCI 6B(8):787-792.
Hussain I. and P.R. Cheeke. 1995. Effect of *Yucca schidigera* extract on rumen and blood profiles of steers fed concentrate- or roughage- based diets. Anim. Feed Sci. and Technol. 51:231–242.
Jouany, J.P. 1991. Defaunation of the rumen. In: Rumen Microbial Metabolism and Ruminant Digestion. Jouany ed. INRA Paris.
Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.
Madigan, M.T., J.M. Martinko and J. Parker. 2003. Brock Biology of Microorganisms. Southern Illinois University Carbondale. 10th ed. Pearson Education, Inc.
Makkar, H.P.S., P. Sidduraju and K. Becker. 2007. Plant Secondary Metabolites. Humana Press. Totowa. New Jersey.
Mao, H.L., J.K. Wang, Y.Y. Zhou and J.X. Liu. 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. 129: 56-62.
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.
Menke, K.H. and Steingass, H. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Dev. 28:7-55.
Nagaraja, T.G. and E.C. Titgemeyert. 2007. Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. J. Dairy. Sci. 90: 17-38.
Newbold, C.J., S.M. El Hassan, 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.
Owens, F.N., D.S. Secrits, W.J. Hill and D.R. Gill. 1998. Acidosis in cattle: a review. J. Anim. Sci. 76:275-286.
Patra, A.K., D.N. Kamra and N. Agarwal. 2006. Effect of plant extracts on in vitro methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo. Anim. Feed Sci. Technol. 128:276–291.
Santoso, B. and B.T. Hariadi. 2007. Effect of supplementation of *Acacia mangium Willd* in *Pennisetum purpureum* on fermentation characteristic and methane production in vitro. Med. Pet. 30(2):106-113.
Teferedegne, B. 2000. New perspectives on the use of tropical plants to improve ruminant nutrition. Proceedings. 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.
Van Soest, P.J. 1994. Nutritional Ecology of The Ruminant. 2nd ed. Comstock Publishing

Associates a Division of Cornell University Press. Ithaca and London.

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

Waterborg, J.H. 2002. The lowry method for protein quantitation. In: The Protein Protocols Handbook. 2nd ed. Humana Press Inc., NJ. 7-10.

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. Feed. Sci. Technol. 121: 159-174.