

Methionine Hydroxy Analog Supplementation to Increase Feed Utilization for Indigenous Sheep

Suplementasi Analog Hidroksi Methionin Untuk Meningkatkan Utilisasi Pakan pada Domba Lokal

Bambang Waluyo Hadi Eko Prasetyono^{1*}, Mulyono¹, Widiyanto¹

Faculty of Animal and Agricultural Sciences, Diponegoro University
Undip Campus, Tembalang, Semarang, Indonesia

* Email: bambangwhep@ymail.com

Naskah diterima: 27 Januari 2020, direvisi: 27 Februari 2020, disetujui: 30 Maret 2020

Abstract

In the tropical area such as in Indonesia, ruminant productivity is relatively low due to, among others, the low quality of nutrition that leads to low-efficiency metabolism at the level of ruminal fermentation, post rumen digestibility, and intermediary metabolism. This study was conducted with the objective to analyze effect of methionine hydroxyl analog (MHA) supplementation on ruminal fermentation profiles of Indigenous sheep specifically in the increase of ruminant productivity. *In vitro* utility test was conducted using rumen fluid of the indigenous sheep and sample of ration having a proportion of grass and concentrate of 30%:70%, on dry matter basis. The treatments were three levels of MHA supplementation; T0: 0%, T1: 3%, and T2: 6% of dry matter (DM) concentrate. Variables measured were dry matter digestibility (DMD), organic matter digestibility (OMD), production of VFA, NH₃, as well as total protein, and molar proportion of partial VFA of rumen fluid. Data were analyzed using analysis of variance (ANOVA) in a completely randomized design (CRD). The 6% MHA supplementation increased OMD with the highest production of total protein from 28.57 mg/g (T0) to 40.49 mg/g (T2) (P<0.05). Meanwhile, the lowest ratio of acetate : propionate was from 2.74 (T0) to 2.33 (T2) (P<0.05). It can be concluded that supplementation of MHA up to 6% in the concentrate increases the performance of Indigenous sheep ruminal fermentation and feed utility.

Key words: methionine hydroxy analog, Indigenous sheep, ruminal fermentation, *in vitro*

Abstrak

Produktivitas ternak ruminansia di daerah tropik seringkali kurang memadai, yang antara lain disebabkan kurangnya kualitas nutrisi, yang berdampak pada rendahnya efisiensi metabolik, baik pada aras fermentasi ruminal, digesti pasca rumen maupun metabolisme intermedier. Penelitian ini dilakukan untuk menguji pengaruh suplementasi analog hidroksi metionin (AHM) terhadap kinerja fermentasi ruminal domba lokal (domba ekor tipis) yang terkait dengan peningkatan produktivitas ternak ruminansia. Penelitian ini merupakan uji utilitas pakan secara *in vitro*, menggunakan cairan rumen domba lokal dan sampel ransum rasional, denganimbangan hijauan : konsentrat : 30% : 70%, bahan kering. Perlakuan yang digunakan adalah suplementasi AHM, yang terdiri atas 3 aras, yakni T0: 0%, T1: 3%, dan T2:6% dari bahan kering konsentrat. Variabel yang diukur meliputi kecernaan bahan kering (KcBK), kecernaan bahan organik (KcBO), produksi VFA, NH₃ dan protein total serta proporsi molar VFA parsial cairan rumen. Data yang terkumpul diolah dengan analisis varians (ANAVA) dalam rancangan acak lengkap (RAL). Kecernaan bahan organik meningkat dengan suplementasi AHM dengan produksi protein total tertinggi, yakni dari 285,73 pada T0 menjadi 404,97 mg/g pada T2 (P<0,05) serta nisbah asam asetat/ asam propionate terendah, yakni dari 2,74 pada T0, menjadi 2,33 pada T2 (P<0,05). Dapat disimpulkan bahwa suplementasi AHM sampai 6% dalam konsentrat meningkatkan kinerja fermentasi rumen dan utilitas pakan pada domba lokal.

Kata kunci: Analog hidroksi metionin, domba lokal, fermentasi rumen, *in vitro*

Introduction

The increased demand for beef has to be anticipated by the increased productivity of sheep both quantitatively and qualitatively. However, in Indonesia, ruminants' production has to deal with problems; such as low feed efficiency. Among factors engendering the problems, highly heat increment in the tropical climate resulting in low energy efficiency that affects the low productivity of the sheep (Lee *et al.*, 2012). The low energy efficiency is worsening by the low quality of forage which is characterized by a relatively high level of lignin and silica. As a result, the level of protein and energy, as well as a total digestible nutrients (TDN) and digestibility, is low (White *et al.*, 2013). Furthermore, the low quality of protein, in this case, a low level of limiting essential amino acids such as methionine, is caused by the low quality of feed that decrease metabolic efficiency.

Widiyanto *et al.*, (2012) proved that Indigenous sheep having average body weight of 13 kg consuming 548 dry matter per day, 71 g crude protein (CP), and 369 g TDN in average produces only 89 g daily gain. Meanwhile, in standard feeding (National Research Council, 2007), to produce 100 g daily gain, a 13 kg sheep only needs 366 g DM with 72 g CP, and 250 g TDN. These data indicated that low metabolic efficiency occurred, especially in protein biosynthesis process that reflected in daily gain. Therefore, to increase metabolic efficiency mainly protein biosynthesis, supplementation of methionine hydroxy analog (MHA) is essential that its effect can be identified from the increased body weight of the animal. Latham *et al.* (2019) stated that methionine is a limiting essential amino acid found to be frequently deficient in the growing stage ruminants, therefore, supplementation of MHA is required, since it might efficiently supplies methionine. According to Guerrero *et al.* (2018), availability of this essential amino acid significantly increases enzyme secretion and level of cellular metabolism on growing sheep, therefore; MHA supplementation increases the efficiency of the protein biosynthesis. The essential function of MHA supplementation likely increases the biosynthesis product that reflected in the increased body weight of the growing stage of sheep (Clements *et al.*, 2017). El-Tahawy *et al.*

(2015) proved that 3.63% MHA supplementation in Rahmani Lamb for 65 days increases body weight from 31.57 kg to 43.57 kg compared to those without MHA which is from 31.57 kg to 43.3 kg.

Yet, the potential of the MHA to improve the rumen fermentation and nutrient digestibility of indigenous sheep has never been documented in Indonesia. From the background and stated problems, research on evaluating the role of MHA as a supplement in ration on ruminal feed fermentation ability is necessary. Therefore, the objective of this study was to analyze the effect of MHA supplementation on metabolic efficiency and performance of indigenous sheep. The findings of this research are expected to be implemented widely in increasing the productivity of sheep that may help farmers in increasing their income and welfare.

Materials and Methods

Ethical approval

The experiment was approved by the animal ethics committee of the Faculty of Animal and Agricultural Sciences, Diponegoro University (No. 3119/UN7.5.5/KP/2019, 23 May 2019).

Location of the study

In vitro studies were conducted at the Nutrition and Feed Science Laboratory, Department of Animal Science, Faculty of Animal and Agricultural Sciences, Diponegoro University.

Experiment details

The *in vitro* experiment was conducted using rumen fluid taken from two fistulae Indigenous sheep (thin tailed sheep) fed with standard ration according to Harris (1970); ration having crude protein level of $\geq 10\%$ and dry matter digestibility of 65 - 70%. The materials used were MHA bought from PT Surya Hidup Satwa (SHS) Semarang and feed (composed from grass and concentrate) met with growing sheep requirement containing 16.4% crude protein level and 60% TDN (Jayanegara *et al.*, 2017). The nutrient composition of the experimental feed is presented in Table 1.

The formula of experimental feed is exhibited in Table 2.

Table 1. Nutrient composition of the experimental feed

Item	CP%	CF%	NFE%	EE%	Ash	TDN%
Forage (grass land)	10.25	31.66	39.21	1.97	16.91	54.21
Rice brand	13.76	12.01	46.78	14.23	13.22	63.34
Copra meal	21.34	12.98	47.91	10.03	7.74	81.57

Note: CP: crude protein, CF: crude fiber, NFE: nitrogen free extract, EE: ether extract, TDN: total digestible nutrient

Table 2. Experimental Feed Formula

Item	Proportion %	CP%	CF%	NFE%	EE%	Ash%	TDN%
Forage	30	3.07	9.48	11.76	0.59	5.07	16.26
Rice brand	21	2.89	2.52	9.82	2.99	2.77	13.30
Copra meal	49	10.6	6.36	23.47	6.97	3.79	39.97
Total	100	16.42	18.36	45.05	10.55	11.56	69.53

Note: CP: crude protein, CF: crude fiber, NFE: nitrogen free extract, EE: ether extract, TDN: total digestible nutrient

The treatments applied were three levels of MHA: 0%, 3%, and 6% based on dry matter (DM) concentrate in which every group of treatment consisted of 5 replications. All ration treatments sampled, a ruminal fermentation test was conducted in two stages of the *in vitro* digestibility test following the Tilley and Terry method (2006), 8 ml rumen liquid and 12 ml buffer liquid of McDougall, and incubated for 24 hour. One gram sample was filled in a 50 ml fermentor tube, added, followed by digestive hydrolytic. Precipitation formed was separated by centrifuge, then added with 10 ml pepsin HCL and re-incubated at 39°C for 24 hours. The DMD was collected by calculating the proportion of DM residue to DM sample; meanwhile, the OMD was calculated by the same formula after DM sample and DM residue were subtracted by ash weight. Every L of McDougall liquid consisting of 9.80 g NaHCO₃, 7.00 g Na₂HPO₄·7H₂O, 0.57 KCl, 0.47 NaCl, 0.12 MgSO₄·7H₂O contained 0.04 g CaCl₂.

Total protein consisted of un-degraded feed protein and microbial protein. Total protein was measured by stirring the content of the fermentor so that the precipitation and the supernatant were mixed. Solution of 10 ml was precipitated by adding 20 ml of the mixture of 20% TCA and 2% SSA. The precipitation then was separated by centrifuge, and an amount of the precipitation was taken to be analyzed for its protein using Kjeldahl method.

At the 3rd hour of stage 1 fermentation, rumen liquid was collected as samples for determining the level of ammonia (NH₃) using the Conway

micro diffuse method, determining the total VFA using steam distillation and partial VFA using chromatography (Galyean, 1980). Amount 5 ml of rumen fluid was filtered through cheesecloth. That was deproteinized and extracted by adding 1 ml metaphosphoric acid (20%). Stand 30 minutes and used amount of 1 – 3 µL supernatant for gas chromatography.

Variables measured were dry matter digestibility (DMD), organic matter digestibility (OMD), NH₃ concentration, total VFA, total protein, the molar proportion of acetate, propionate, butyrate, and acetate:propionate ratio. The collected data were analyzed using analysis of variance (ANOVA) in a completely randomized design (CRD) (Steel *et al.*, 1996) by using the Costat statistical program (CoHort, 2019).

Results and Discussion

Feed utility can be measured and tested by variables of *in vivo* and *in vitro*. Among *in vitro* variables of feed utility are DMD/OMD (Table 3), VFA Production (Table 3), NH₃ (Table 3), Total Protein (Table 3), and molar proportion of partial VFA (Table 4).

Dry matter and organic matter digestibility

Table 3 shows that the DMD and OMD in the treatment group of T0: supplemented with MHA 0%, T1: supplemented with MHA 3%, and T2: supplemented with MHA 6% are 64.23% and 66.16%; 65.33% and 67.53%; and 67.71% and 69.13%, respectively. The supplementation of MHA at the level of 3% tended to increase the

Table 3. DMD/OMD, VFA Production, NH₃, Total Protein

Treatment	DMD(%)	OMD(%)	VFA (mM)	NH ₃ (mM)	Total Protein (mg/g)
T0	64.23 ^b	66.16 ^b	86.52 ^b	6.70 ^a	28.57 ^c
T1	65.33 ^b	67.53 ^{ab}	88.98 ^{ab}	5.55 ^b	34.26 ^b
T2	67.71 ^a	69.13 ^a	91.75 ^a	4.21 ^c	40.49 ^a

Note: T0: supplementation of MHA 0%; T1: supplementation of MHA 3%, T2: supplementation of MHA 6% of DM concentrate; DMD: dry matter digestibility; OMD: organic matter digestibility; VFAs: volatile fatty acids.
^{a,b,c}: different superscript in the same column indicates significant different (P<0.05).

DMD and OMD while the significantly increased DMD and OMD are found in the supplementation of MHA 6% (P<0.05).

According to Agle *et al.* (2010) the increased digestibility can occur because there is an increase in ruminal fermentability as a result of the increased proliferation of rumen microbe. Gonzales *et al.* (2013) stated that the increased microbe proliferation increases both microbe concentration and microbial enzyme production, and both of which increase catalytic activity. Furthermore, the increased catalytic activity of the microbial enzyme produces an increase of fermentation capacity that can be identified from the increase of the DMD and the OMD. The increase of the rumen microbe proliferation is supported by the availability of carbon skeleton used by biosynthesis of essential amino acid for rumen microbe (Doto and Liu, 2011). The carbon skeleton is in the form of alfa keto acid that part from the MHA degraded in rumen. According to Noftsker *et al.* (2005), approximately 37% of MHA is degraded in the rumen and provides carbon skeleton for the microbe to synthesize methionine.

Production of VFAs, NH₃, and total protein

Table 3 shows that in the treatment group of T0, T1, and T2, the production of VFAs is 86.52 mM, 88.98 mM, and 91.75 mM, respectively; the production of NH₃ are 6.70 mM, 5.55 mM, and 4.21 mM, respectively; and the production of total ruminal protein is 28.57 mg/g, 34.26 mg/g, and 40.49 mg/g, respectively.

Statistical analysis showed that the decreased production of NH₃ took place in line with the increased level of MHA supplementation (up to 6%). The 6% supplementation of MHA significantly decreased the production of NH₃

(P<0.05), and significantly increased total protein production (P<0.05). The increased production of total protein was even higher as the level of MHA supplementation given was increased (up to 6%).

The degradation of the ruminal organic matter, especially carbohydrate, produces monosaccharide as an intermediate compound which is furthered fermented to be alfa keto acid that lastly formed volatile fatty acids (VFAs) (Doto and Liu, 2011). This phenomenon was found as the production of the VFAs increased which was in line with the increased digestibility of the ruminal organic matter (Table 3). The supplementation of the MHA at the level of 3% tended to increase the production of the VFAs that significantly increased at the 6% supplementation of MHA. The production pattern of the VFAs related to the level of the MHA supplementation was corresponding to that of the OMD.

Furthermore, ammonia (NH₃) is the end product of the fermentation of the ruminal crude protein. As the crude protein is part of the organic matter, the increased OMD increases the degradation of the crude protein, and the increased degradability of the crude protein increases the production of the NH₃ as the end product of the fermented crude protein (Artegoitia *et al.*, 2017).

Meanwhile, as data presented on Table 3 suggested that the increased OMD was not followed by the increased production of the NH₃; on the contrary, it decreased the ruminal level of the NH₃ (P<0.05). This phenomenon might be a part of the NH₃ formed was used for synthesizing microbial protein that was increased as the supplementation of the MHA increased. The increasing of microbial protein was reflected in the increasing (P<0.05) of the total ruminal protein (Table 3) caused by the supplementation of the MHA increased. The increased synthesis

of the microbial protein was supported by the increased energy availability that was reflected in the increased OMD producing VFA, as the level of the MHA supplementation increased. In this case, the increased OMD reflected the availability of the carbon skeleton that was alpha-keto acids that functions to synthesis amino acid, which is the monomer of the microbial protein biosynthesis.

Molar Proportion of the Partial VFA

According to Table 4, the molar proportion of acetate in the treatment group of T0, T1, and T2 is 67.54%, 65.63%, and 63.89%, respectively. The proportion of the molar acetic acid tended to decrease in the 3% supplementation of MHA and significantly decreased in the level of 6% supplementation of MHA ($P < 0.05$).

The opposite pattern appeared in the molar proportion of propionate that it's increased corresponded to the increased level of the MHA supplementation, and the significant differences occurred at the supplementation level of 6% MHA. The molar proportion of the propionate of the treatment group of T0, T1, and T2 is 24.71%, 25.89%, and 27.51%, respectively. Furthermore, according to Table 4, the molar proportion of the butyrate in the treatment group of T0, T1, and T2 is 7.75%, 8.48%, and 8.68%, respectively. There was no significant difference in the molar proportion of butyrate between T0 and T1, and the significant increase of the molar proportion of the butyrate occurred in T2.

Meanwhile, the ratio of acetic acid to propionic acid (A/P) in T0, T1, and T2 is 2.74, 2.54, and 2.33, respectively. The ratio of A/P tended to decrease at the 3% supplementation of MHA, and the lowest was found at the 6% MHA supplementation.

Volatile fatty acids (VFAs) are produced in the rumen from the carbohydrate of feed in the form of fiber component and NFE that function

as the main substrate. The biodegraded process of the carbohydrate is catalyzed by enzymes from various species and strains of microbe and pyruvate as a universal intermediary compound that is formed through anaerobe glycolysis path that needs NAD^+ as oxidation and produces $NADH_2$ as coenzyme reduction (Matthews *et al.*, 2019).

Two mechanisms to converse pyruvate acid into acetic acid are pyruvate-formatelyase and pyruvate-ferredoxin oxidoreductase system. The pyruvate-formate lyase produces format and acetyl-CoA as an intermediary compound. The format formed then converses into CO_2 and H_2 . Meanwhile, the pyruvate-ferredoxin oxidoreductase system converse pyruvate into reduced ferredoxin, CO_2 , and acetyl-CoA. The reduced ferredoxin is oxidized by releasing hydrogen, and the acetyl-CoA formed is conversed into acetic acid and ATP by phosphotransacetylase and acetokinase enzymes (Liu *et al.*, 2019).

The ruminal fermentation process is supported by the formation of methane gas as hydrogen sink through the methanogenesis process. The methane formed maintains ruminal hydrogen pressure to let $NADH_2$ re-oxidation be NAD^+ . The availability of NAD^+ support anaerobe oxidation reaction needed to produce energy to support the life and development of rumen microorganism (Greening *et al.*, 2019). The increased proportion of molar propionate can be used as an indicator of methane production obstacle; for example, the decreased ruminal pH caused by a sudden formation of VFAs in feed fermentation having high concentrate proportion. The decreased pH of rumen liquid is supported by the increased OMD (Lee, 2018; Mamuad *et al.*, 2019).

The decreased molar proportion of acetate and the increased molar proportion of propionate corresponding to the increased level of MHA supplementation were caused by the increased

Table 4. Molar proportion of volatile fatty acids and ratio of acetate / propionate

Treatment	Acetate (%)	Propionate (%)	Butyrate (%)	Ratio A/P
T0	67.54 ^a	24.71 ^b	7.75 ^b	2.74 ^a
T1	65.63 ^{ab}	25.89 ^{ab}	8.48 ^{ab}	2.54 ^b
T2	63.89 ^b	27.51 ^a	8.68 ^a	2.33 ^c

Note: T0: supplementation of MHA 0%; T1: supplementation of MHA 3%, T2: supplementation of MHA 6% of DM concentrate. ^{a,b,c}: different superscript in the same column indicates significant different ($P < 0.05$).

methanogenesis inhibition and the increased use of hydrogen to form propionate. Table 2 shows that the experimental feed contained high NFE (45.05%) because the concentrate proportion was high (70%). The NFE is an easily fermented carbohydrate. The highly containing carbohydrate which was easy to be fermented together with the increased OMD as a result of MHA supplementation (Table 3) enabled the occurrence of the increase of acetate production acceleration followed by the increased production of H₂. This condition pushes rumen microorganisms to reduce pyruvate to be propionate to maintain hydrogen balance; as a result, the production and molar proportion of propionate increase.

The acceleration of VFA production can also decrease the pH that decreases methanogenic bacteria and inhibits methane production (Morgavi *et al.*, 2010). The increased of propionate molar proportion decreases the molar proportion of acetate. The increased molar proportion of butyrate takes place as a result of the increased use of acetate to synthesize butyrate through the opposite path of beta-oxidation (Ungerfeld, 2015). This condition was found to support the decreased molar proportion of ruminal acetate in the treatment group T2.

Acetate/Propionate Ratio

The decreased ratio of acetate/propionate (Table 4) in the group treated with 6% MHA supplementation might be caused by the decrease of methanogenesis which was reflected in the increased molar proportion of ruminal propionate. The decrease in the methanogenesis showed the occurrence of the increased efficiency of ruminal energy metabolism. According to Hill *et al.* (2015), methane gas contains 209.8 kcal/mol energy. As this energy cannot be used to metabolism and is wastage, thus the decrease production of methane gas means the increased efficiency in ruminal bioenergetics.

The ruminal fermentation pattern leading to the increased production of propionate supported the increased efficiency of protein biosynthesis in the intermediary metabolism. The propionate acid was glycogenic and the main source of the main glucose provider in ruminants (Aschenbach *et al.*, 2010). The increased supply of propionate acid

decreased the use of another glycogenic compound, in this case, amino acids. As a result, the efficient use of amino acid for protein biosynthesis would be even higher as the MHA was supplemented.

Conclusions

Supplementation of MHA up to 6% in the concentrate increases DMD, OMD, production of ruminal total protein as well as the production of ruminal propionate, but decreases the ratio of acetate/propionate. The increase of the efficiency of the ruminal energy metabolism and the microbial protein synthesis is in line with the increased level of MHA supplementation which leads to the increased feed utility. For implementation widely, MHA need to be supplemented in the ration on the level 6% in the concentrate, with the readily available energy source.

Acknowledgement

The member of researchers would like to extend an appreciation to the Rector and Head of LPPM of Diponegoro University for funding and approving the study (contract number: 329-38/UN7.P4.3/PP/2019), the Dean of Faculty of Animal and Agricultural Sciences of Diponegoro University for approving the proposal and facilitating the realization of the research in the university's Laboratory. We also would like to appreciate Ir. Suranto, MS who has helped in conducting the study from preparation until completion.

References

- Agle, M., Hristov, S., Zaman, A.N., Shneider, C. (2010). Effect of dietary concentrate on rumen fermentation, digestibility, and nitrogen losses in dairy cows. *Journal of Dairy Science*. 93(9): 4211-4222. DOI: 10.3168/jds.2009-2977
- Artegoitia, V.M., Foote, A.P., Lewis, R.M., Freetly, H.C. (2017). Rumen Fluid metabolomics analysis associated with feed efficiency, on crossbred steers. *Scientific Reports*. 7(2864): 1-14. DOI:10.1038/s41598-017-02856-0
- Aschenbach, J.R., Kristensen, N.B., Donkin, S.S., Hammon, H. (2010). Gluconeogenesis in

- dairy cows: The secret of making sweet milk from sour dough. *International Union of Biochemistry and Molecular Biology Life*. 62(12):869-77. DOI: 10.1002/iub.400
- Clements, A.R. F. A. Ireland, T. Freitas, H. Tucker, and D. W. Shike. (2017). Effects of supplementing methionine hydroxy analog on beef cow performance, milk production, reproduction, and preweaning calf performance *Journal of Animal Science*, 95(12): 5597–5605, <https://doi.org/10.2527/jas2017.1828>
- CoHort. (2019). Costat Statistics Software. Brindleyplace, Birmingham.
- Doto SP, and Liu JX. (2011). Effect of direct-fed microbial and their combinations with yeast culture on in vitro rumen fermentation characteristics. *J. Anim Feed Sci*. 20(2):259-271. DOI: <https://doi.org/10.22358/jafs/66183/2011>.
- El-Tahawy, A. S., A. Ismaeil, and H.A. Ahmed. (2015). Effects of Dietary Methionine-Supplementation on the General Performance and Economic Value of Rahmani Lambs. *J. Anim. Sci. Adv*. 5(10): 1457-1466
- Galyean, M.L. (1980). *Laboratory Procedures in Animal Nutrition Research*. Department of Animal and Food Sciences, Texas Tech University, Lubbock.
- Gonzales, A.R.C., Barraza, M.B., Viveros, J.D., Martinez, A.C. (2013). Rumen microorganisms and rumen fermentation. *Archivos de Medicina MartoinezVeterinaria*. 46(3):349-361 · DOI: 10.4067/S0301-732X2014000300003
- Greening, C., Geier R., Wang, C., Woods, L.C. (2019). Diverse hydrogen production and consumption pathways influence methane production in ruminants. *ISME Journal*. 13(6): 1-13. DOI: 10.1038/s41396-019-0464-2
- Guerrero, V.R., Lizarazo, A.C.P. and Mendoza, G.D. (2018). Effect of herbal choline and rumen-protected methionine on lamb performance and blood metabolites. *South African Journal of Animal Science*. 48(3): 427- 434. DOI:10.4314/sajas.v48i3.3
- Harris, L.E. (1970). *Nutrition Research Technique for Domestic and Wild Animals*. Vol 1. Anim. Sci. Dept. Utah State Univ. Logan. Utah.
- Hill, J., Mcsweeney, C.S., Wright, A.D.G. and Hurley GJB. (2015). Measuring methane production from ruminants. *Trends in Biotechnology*. 34(1). <https://doi.org/10.1016/j.tibtech.2015.10.004>
- Jayanegara, A., Ridla, M., Astuti, D.A., Wiryawan, K.G., Laconi, E.B. and Nahrowi. (2017). Determination of Energy and Protein Requirements of Sheep in Indonesia using a Meta-analytical Approach. *Media Peternakan*. 40(2):118-127. DOI: <https://doi.org/10.5398/medpet.2017.40.2.118>.
- Latham, C.M., Wagner, A.L. and Urschel, K.L. (2019). Effects of dietary amino acid supplementation on measures of whole-body and muscle protein metabolism in aged horses. *J. Anim. Physiol. Anim. Nutr*. 103: 283–294. DOI: 10.1111/jpn.12992
- Lee, J.K.W., Nio, A.O.X., Fun, D.C.Y, Teo, Y.S, Chia, E.V. and Lim L. (2012). Effects of heat acclimatization on work tolerance and thermoregulation in trained tropical natives. *Journal of Thermal Biology*. 37 (5): 366-373. doi:10.1016/j.jtherbio.2012.01.008
- Lee, M. (2018). Changes in the ruminal fermentation and bacterial community. *AJAS*. 32(1): 92-102. DOI: <https://doi.org/10.5713/ajas.19.0323>
- Liu, H., Xu, T., Xu, S., Ma, L., Han, X., Wang, X., Zhang, X., Hu, L., Zhao, N., Chen, Y., Pi, L. and Zhao, X. (2019). Effect of dietary concentrate to forage ratio on growth performance, rumen fermentation and bacterial diversity of Tibetan sheep under barn feeding on the Qinghai-Tibetan plateau. *Peer J*. 7:e7462; 1-22. DOI 10.7717/peerj.7462
- Mamuad, L.L., Lee, S.S. and Lee, SS. (2019). Recent insight and future techniques to enhance rumen fermentation in dairy goats.

- AJAS* 2019; 32(8): 1321-1330. Special Issue. 32(8): 1321-1330. DOI: <https://doi.org/10.5713/ajas.19.0323>
- Matthews, C., Crispie, F., Lewis, E., Reid, M., O'Toole, P.W. and Cotter PD. (2019). The rumen microbiome: a crucial consideration when optimizing milk and meat production and nitrogen utilization efficiency. *Gut Microbes*. 10(2): 115-132. doi: 10.1080/19490976.2018.1505176
- Morgavi, D.P., Forano, E., Martin, C. and Newbold, C.J. (2010). Microbial ecosystem and methanogenesis in ruminants. *Animal*. 4(7): 1024-1036. DOI: 10.1017/S1751731110000546
- National Research Council. (2007). *Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids*. Washington, DC: The National Academies Press. Doi.org/10.17226/11654
- Noftsker, S., St-Pierre, N.R. and Sylvester, J.T. (2005). Determination of rumen degradability and ruminal effects of three sources of methionine in lactating cows. *J. Dairy Sci*. 88:223-237.
- Steel, R.G.D., Torrie, J.H. and Dickey, D.A. (1996). *Principles and Procedures of Statistic: A Biometrical Approach*. McGraw-Hill College.
- Tilley, J.M.A. and R.A. Terry. (2006). A Two-Stage Technique for the in vitro Digestion of Forage Crops. *Grass and Forage Science*. 18(2): 104-111. DOI: 10.1111/j.1365-2494.1963.tb00335.x
- Ungerfeld, E.M. (2015). Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. *Frontiers in Microbiology*. 6(37): 1-17. <https://doi.org/10.3389/fmicb.2015.00037>
- White, D., Peters, M.S. and Home, P.Y. (2013). Global impacts from improved tropical forages: A meta-analysis revealing overlooked benefits and cost, evolving values and new priorities. *Biology*. 1: 12-24. DOI:10.17138/tgft
- Widiyanto, Soejono, M., Bachruddin, Z., Hartadi, H. and Surahmanto. (2012). Inclusion of kapok seed oil in the diet for growing of thin-tailed sheep to reduce cholesterol and to improve omega-six fatty acid contents of lamb. *J. Indonesian Trop. Anim. Agric*. 37(3): 202-212