Article

In vitro ruminal fermentation and methane production of PUFA-containing rations as treated by flavonoid and essential oil from *Piper betle* L.

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Simple Summary: Reduction of methane gas without persuading any problems have been considered by animal science experts through antimicrobial supplementation. However, the commonly antimicrobial agents, namely from PUFA and polyphenol-containing plant that were reports separately. Using *in vitro* gas production technique inviting PUFA substrate collections, we found initially recommended dose for quercetin 0.2-3.0 mg and eugenol 0.11-1.62 mg from *Piper betle* L. as potential herb, significantly to prohibit ruminal methane production inviting nurture rumen. This study promotes an option way to improve rumen diet development corresponding to environment friendly aspect, mentioning assessment of reducing methane pollution.

Abstract: This study had the objective to evaluate the effect of *Piper betle* L. powder (PP) at 5 different doses in substrate incubated by sunflower oil as secondary function of PUFA using *in vitro* gas production technique. The treatments of this study were run as a 2X5 factorial arrangement in a completely randomised design using the PROC GLM procedure of SAS 9.4: (1) control (S1) without supplementation of PP; (2) 15 mg PP (S2); (3) 30 mg PP (S3); (4) 45 mg PP (S4); and (5) 60 mg PP (S5), while sunflower oil was supplemented in all treatments: low 15 mg/incubation and high 30 mg/incubation. A 500 mg of TMR (hay: concentrate, 50:50) was assigned to basal substrate. The PP containing 1.84 mg/g DM quercetin and 1.00 mg/g DM eugenol altered rumen fermentation without change pH ($p < 0.001$) and methane production was lesser ($p < 0.001$) about -30% and -25% for DM and OM measurement, respectively. Gas kinetic, degradability, and ammonia level was significantly affected by supplementing PP ($p < 0.01$). Overall, this study suggested quercetin and eugenol deriving from PP acted three major accelerations: assembled carbon dioxide, behaved antimicrobial role and performed the balance water molecules in the rumen kinetic. This study suggests that PP promotes changing *in vitro* rumen fermentation and diminishing methane production within recommended doses, 0.1-15 mg/incubation in DM.

Keywords: quercetin; eugenol; organic compounds; rumen; environment; HPLC-DAD; polyphenols-containing plants

1. Introduction

Regardless of fat source, poly-unsaturated fatty acid (PUFA) was been known as long chain expressing several secondary functions in modulation rumen fermentation [1,2]. Linoleic acid and linolenic acid have undertaken a widely performance, particularly sunflower oil as linolenic source had been confirmed to suppress methane number (-18% of total gas production) through alteration

of rumen fermentation [3]. Sunflower oils have behaved more a hydrophobic role to interfere the inhabitant in the rumen. Carreño et al. [4] sunflower oil interacted a stability of outer liner in bacteria physiology through a membrane semipermeable that lower resistant toward a differential water race in the rumen. This activity would be assessed as a substantial function as antimicrobial value. However, supplementing single PUFA expressed a limitation to elaborate more toxicity in oxidative reactive [5]. Thus, researchers have been looking for alternatives for combining with other natural fractions. Due to their antimicrobial functions, plant-containing polyphenols is considered owning the potential properties in diminishing methanogenesis coming along with nurturing rumen.

Piper betle L. is tropical-Asian-plant owning a host of secondary metabolites. Purba and Paengkoum [6] *Piper betle* L. had abundant of flavonoid and essential oil detected by a HPLC-DAD deriving from polar and non-polar extraction. Hitherto, *Piper betle* L. investigations on rumen fermentation and methane production have a limiting information. There is comprehensive hypothesis mentioning interaction of *Piper betle* L. compounds and PUFA's is prerequisite in modulating rumen fermentation and methane loss. Hopefully, understanding pathway of this interaction might improve the strategies in rumen diet development. Therefore, the objective was to evaluate the effect of *Piper betle* L. powder (PP) at 5 different doses in substrate incubated by sunflower oil as secondary function of PUFA using *in vitro* gas production technique.

2. Materials and Methods

All experimental procedures were approved and completed in accordance with the Rules of Animal Welfare of Suranaree University of Technology (SUT 4/2558) for animal protection used and/or applied for experimental purposes in accordance.

2.1. Animal, diet, and Piper betle L. powder (PP)

In vitro study was conducted following to previous study [7]. Four female Saanen goats (body weight= 43±1.29 Kg) were as rumen inoculum donors. All goats were obtained the TMR feedstuff (hay: concentrate, 50:50), based on pangola hay (particle size > 4 cm) and concentrate, TMR was offered in two portions (60% at 9:00h and 40% at 17:00 h) and this feed was dedicated as substrate on *in vitro* incubation. Randomly mineral block and freely clean drinking water were applied to face abundant of nutrient requirement [8]. Formulation and proximate analysis of diet is presented in Table 1.

Piper betle L. leaves were purchased from local market, Prachinburi area, east Thailand. Fresh biomass of leafy material plant was pooled, rinsed and kept overnight at 4°C. The leaves were air-dried using oven set 40 °C for 2 d, made a powder, loaded in sealed plastic until, and kept in desiccator until usage time. Quercetin and eugenol content were extracted, fragmented, and detected following earlier method by Purba and Paengkoum [6] using water, methanol, and hexane under HPLC-DAD wavelength. All content of these organic compounds is presented in Table 1.

2.2. Treatments

The treatments of this study were run as a 2X5 factorial arrangement in a completely randomised design: (1) control (S1) without supplementation of PP; (2) 15 mg PP (S2); (3) 30 mg PP (S3); (4) 45 mg PP (S4); and (5) 60 mg PP (S5), while sunflower oil was supplemented in all treatments: low 15 mg/incubation and high 30 mg/incubation, containing (g/Kg FA): 16:0 (57.67), 18:0 (30.89), cis-9 18:1 (401.38) and 18:2n-6 (476.83). Sunflower oil and PP were emulsified in 1:99 v/v ethanol 96% and aqueous solution, respectively, then added into glass syringe.

2.3. In vitro experiment

After 15 d adaptation period, 1000 ml rumen fluids were compulsory suctioned from three-based rumen position through oral lavage by suction pump (Hitachi CV-SF18, Japan) before morning feeding time. Rumen fluids were strictly pH checking, 6.6-6.8. Rumen fluids were taken in a

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pre-warmed thermal flask moved to laboratory, then strained using a nylon membrane (400µm; Fisher Scientific S.L., Madrid, Spain) while bubbled with CO_2. The artificial solution was prepared following to Menke and Steingass [7] and mixed with strained rumen fluids (2:1, ml/ml) under continuously CO_2 at 39 °C. Feed (500 mg) was filled into each a hundred scale of glass syringe and combined with early sunflower oil and *Piper betle* L. powder. Thirty-milliliters of rumen mixture was added for last preparation of incubation way. Once the glass syringes were locked by 3-way stop cocks and capped by glass plungers, the glass syringes were abruptly shaken and placed in water bath set 39 °C. The incubation was been running initially recorded at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, and 72 h including shaken per hour. In this study provided a separated group of parameter syringes: gas production, methane accumulation, and degradability. The glass syringes incubated only rumen fluid and feed mixing artificial solution were dedicated as blank 1 and blank 2 (data not shown in Table) for considering pre-judgement significantly error and significantly different, respectively. Each parameter was run at triplicate.

2.4. Sample analysis

Feed and *Piper betle* L. leaves were prepared (#950.02) and analysed for DM (#925.04), ash and organic matter (#942.05), crude protein (#984.13), and crude fat/ether extract (#920.39). Neutral detergent fibre (#2002.04) without amylase (sodium sulphite instead) and acid detergent fibres (#973.18) were determined using an Fibertec8000 fibre analyser (Auto fibre analysis system tecator line). All parameters followed in [9]. Metabolize energy was measured using Parr6200 calorimeter.

On 12 and 24 h, 20 ml of gas was transferred into a disposal syringe for injecting directly in gas chromatography machine (Agilent 7890A, USA) to measure methane level. Other groups of glass syringes were directly strained with a frilled flask and filtrates were divided into two portions of 15 ml falcon tubes. Each falcon tube was filled with 5 ml strained rumen fluid added 1 ml metaphosphoric acid (25%) for volatile fatty acid (VFA) detection by gas chromatography (Hewlet Packard hp 6890, USA) and 4 ml strained rumen fluid added 1 ml metaphosphoric acid (25%) for NH_3-N measurement using Kjeldahl method (Foss Kjeltech8100, USA). All remaining supernatant could be stored in -20 °C for further study.

After 72 h, the glass plungers were released and the contents were filtered under vacuum through glass crucibles with a sintered filter. The residue was then dried under 50 °C for 2 d. The consecutive steps in degradability parameters namely *in vitro* dry matter degradability (IVDMD), *in vitro* organic matter degradability (IVOMD), *in vitro* crude protein degradability (IVCPD), and *in vitro* neutral detergent fibre (IVNDD) followed the aforementioned method [9].Once the glass syringes unplugging, pH was immediately measured using pH meter (Oakton 700, USA) in all observations above.

2.4. Calculation and statistical analysis

Feed and Recording gas production at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, and 72 h was read and measured adapting from gas pressure technique following to Theodorou et al. [10]. To calculate a cumulative volume of gas production, the number measurement was fitted to the model of [11] as:

$$y = a + b\,(1 - e^{(-ct)}), \quad (1)$$

where a (ml/g DM) is the gas production from the soluble fraction, b is the gas production from the insoluble fraction (ml/g DM), c (/h) is the gas production rate constant for the insoluble fraction (b), t (h) is the incubation time, (a + b) (ml/g DM) the potential extent of gas production and y the gas produced at time 't' (ml/g DM).

Regarding statistical analysis, data were subjected to analysis as a 2X5 factorial arrangement in a completely randomised design using the PROC GLM procedure of SAS 9.4 [12]. Multiple comparisons among sunflower oil supplementations, *Piper betle* L. powder treatments, and interactions were performed using the Tukey HSD [13]. Orthogonal polynomial contrasts were used to test for linear, quadratic, and cubic trends in all parameters. Differences among means with $p < 0.05$ were accepted as representing statistically significant differences.

3. Results

3.1. Effect of sunflower oil and Piper betle L. powder on gas kinetic, gas accumulation and degradability

As shown in Table 1, the soluble fraction on gas kinetic increased by adjusting sunflower oil (SO) level, yet, *Piper betle* L. powder (PP) decreased a level of soluble fraction ($p < 0.001$). Similarly, insoluble fraction was affected to climb gas kinetic by altogether of presenting SO and PP ($p < 0.001$). As a result, the potential gas kinetic rose a gradually volume following to the supplementing level of SO and PP ($p < 0.001$). However, fermentation rate preferred an opposition of potential gas kinetic ($p < 0.001$).

There was quadratic trend on IVDMD degradability due to a shift of fermentation rate result ($p < 0.001$) and cubic trend on IVOMD, IVCPD, and IVNDD ($p<0.001$). Overall, the gas kinetic accumulation for 72 hours was change as supplementing SO and PP. Although, the presenting PP escalated slightly on gas kinetic accumulation with cubic trend ($p<0.001$). Unfortunately, a high level of PP supplementation, particularly at 30, 45, and 60 mg/60 mg sunflower oil plunged insoluble fraction, potential gas kinetic, gas kinetic accumulation, IVOMD, and IVCPD irregularly ($p < 0.001$).

3.1. Effect of sunflower oil and Piper betle L. powder on rumen fermentation and methane production, pH and NH_3-N

Rumen fermentation and methane production including pH and NH_3-N level were presented in Table 2. Interaction of SO and PP altered a volatile fatty acid (VFA). There was similarly trend both of supplementing SO level (low and high) enhancing higher VFA at 30-45 mg PP addition, however, slightly over 45-60 mg PP diminished the VFA ($p < 0.001$). Basically, supplementing 30 mg SO at 0 mg of PP increased the VFA ($p < 0.001$). For VFA partition, supplementing more PP level declined a number of acetates leading to more propionates ($p<0.001$). Indeed, these results promoted lesser fermentation of acetate to propionate. Other fractions of VFA were shown in Table 2.

Moreover, methane production whether in DM and OM were affected by supplementing PP. Clearly, quercetin and eugenol deriving from PP decreased methane production ($p < 0.001$). Even tough, more supplementing PP, especially 30 mg PP onwards in low or high SO addition tended to greater methane accumulation ($p < 0.001$). Here, 15 mg PP was abundant of quercetin and eugenol to provoke lesser methane production. Furthermore, adjusting level of PP decreased NH_3-N, except infusing 30-60 mg PP/60 SO producing more NH_3-N. In order to alteration of VFA and methane production, pH was not change in all studies ($p > 0.05$).

4. Discussion

4.1. Gas accumulation and degradability

As shown in It had been known in advance that gas production is a result of fermentation activity in the rumen [14]. Gas kinetic, gas accumulation, and degradability are presented in Table 2. In this study, dietary quercetin and eugenol deriving from PP increased the potential gas kinetic ($p < 0.001$). This result was similar with earlier report by Oskoueian et al. [15]. Value of potential gas kinetic interpreted abundant of energy from soluble-insoluble fraction and was dominated by increase of insoluble fraction ($p < 0.001$). According to Table 1, sum of SO and eugenol (PP) were tantamount at 20% of mixture incubation. These compounds might prefer being active to possess interaction with hydrophobic base while quercetin (PP) was at hydrophilic base. As a result, fermentation rate was affected favouring an opposition of potential gas kinetic ($p < 0.001$) in this study. Oskoueian et al. [15] and Sinz et al. [16] reported incubating feed with flavonoid on *in vitro* fermentation preferring a number of similar fermentation rate and no affection in dry matter degradability (IVDMD).

More previously, Castillejos et al. [17] eugenol had no effect on degradability of dry matter, organic matter, crude protein, and neutral detergent fibre, even eugenol eminent as antimicrobial rumen. When fermentation rate is greater enhancement, thereby promoting slighter growth of bacterial rumen to degrade and metabolize feed. In this study, supplementing single sunflower oil

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decrease IVDMD, yet, combining with PP resulted a negligible effort to rise in IVDMD, IVOMD, IVCPD, and IVNDD ($p < 0.001$). These results were similarly report in previous studies [18,19]. Halushka et al. [20] explained metabolised quercetin performed to produce more carbon dioxide. Therefore, quercetin (PP) may enhance less toxicity oxygen form in the rumen, greater anaerobe improving bacterial rumen to degrade fibre's fraction resulting higher degradability.

Furthermore, the gas kinetic accumulation for 72 hours was change as supplementing SO and PP. Gas production addresses a result fermented nutrient in the rumen. In this study, adjusting level of PP showed an impressively accumulating gas production ($p < 0.001$). Even tough, several studies claimed existing pure flavonoid in diet incubation with sunflower oil no change of gas production [21,22], even Kim et al. [23] suggested a decrease number relating other parameters. As aforementioned, even quercetin is flavonoid member, this compound had independent secondary metabolism that favoured to balance the hydrophobic section of sunflower oil and eugenol. However, adding level of PP on higher SO supplementation demonstrated a remarkably reverse ($p < 0.001$). As shown in table 2, a high level of PP supplementation, particularly at 30, 45, and 60 mg/60 mg SO plunged insoluble fraction, potential gas kinetic, gas kinetic accumulation, IVOMD, and IVCPD irregularly ($p < 0.001$). Berger et al. [21] confirmed quercetin had antimicrobial properties to inhibit microbial rumen. Consequently, overloading PP in diet tended to more antimicrobial behaviour as quercetin and eugenol whereby suggested behaviour suppressing nutrient metabolism.

4.2. Rumen fermentation and mitigating methane production

As shown in Table 3, rumen fermentation and methane production including pH and NH_3-N level were significantly affect by treatment ($p < 0.001$). Basically, dietary SO and PP enlarged volume of total VFA ($p < 0.001$) without altering pH in rumen situation. It might elaborate role of bacterial rumen undertakes major nutrient, especially fibre's group by more possessively attachment and colonization in the diet due to maintenance metabolism. These results were similar with recently studies when diet supplementing with quercetin [21] and incubating single SO [22], tough, unsimilar with dietary eugenol [24]. Lourenço et al. [25] compared quercetin and eugenol on *in vitro* incubation had a different way to produce total VFA. Quercetin offered more acetate and propionate rather than eugenol performance.

In this study, supplementing PP were considered to construct more propionate ($p < 0.001$) relatively. The PP was enthusiasm to shift lesser acetate to propionate form ($p < 0.001$). As a result, methane production was lesser about -30% and -25% for DM and OM measurement, respectively. Another, NH_3-N was slightly rough when feed incubated with PP ($p < 0.001$). These results were literally similar with recently study by Oskoueian et al. [15] and Sallam et al. [26]. However, Berger et al. [21] reported quercetin had no affection on mitigating methane production, even Kim et al. [23] suggested a shift of methane accumulation depending on quercetin sources. A lower methane production could be predicted because quercetin and eugenol intruded short chain fatty acid, gathering more propionate leading to hydrogen lower to supply methanogenesis. Clearly, an evidence was shown with lower ammonia properties dedicating a merely information about a shift microbial rumen. Even tough, adding more PP, specifically 30 mg PP onwards per 30 mg or 60 mg of SO incubation caused a bulk of methane accumulation ($p < 0.001$) and 15 mg PP was abundant of quercetin and eugenol to provoke lesser methane accumulation and ammonia production in the rumen. Mao et al. [27] suggested developing diet strategies could help friendly environment, via methane loss and declined ammonia production might decrease the overall nitrogen excretion by the animal.

Overall, in this study suggested that quercetin and eugenol deriving from PP acted three major accelerations. Firstly, quercetin might help to improve anaerobe condition through assembling carbon dioxide on their secondary metabolism. Secondly, quercetin and eugenol were altogether behaved antimicrobial role tending to rumen alteration. Thirdly, PP as big home of these duo forecast, probably performed the balance water molecules in the rumen kinetic. Thus, interaction from several functions above might promise the development on modulating ruminal diet.

5. Conclusions

This study suggests that quercetin and eugenol deriving from herb plant, *Piper betle* L. promotes changing *in vitro* rumen fermentation and diminishing methane production within recommended doses, 0.1-15 mg/incubation in DM.

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References

1. Zhang, C.M.; Guo, Y.Q.; Yuan, Z.P.; Wu, Y.M.; Wang, J.K.; Liu, J.X.; Zhu, W.Y. Effect of octadeca carbon fatty acids on microbial fermentation, methanogenesis and microbial flora *in vitro*. *Anim. Feed Sci. Tech.* **2008**, *146*, 259-269, doi:http://dx.doi.org/10.1016/j.anifeedsci.2008.01.005.
2. Huws, S.A.; Kim, E.J.; Kingston-Smith, A.H.; Lee, M.R.F.; Muetzel, S.M.; Cookson, A.R.; Newbold, C.J.; Wallace, R.J.; Scollan, N.D. Rumen protozoa are rich in polyunsaturated fatty acids due to the ingestion of chloroplasts. *FEMS Microbiol. Ecol.* **2009**, *69*, 461.
3. Rasmussen, J.; Harrison, A. The benefits of supplementary fat in feed rations for ruminants with particular focus on reducing levels of methane production. *ISRN Vet. Sci.* **2011**, *2011*, 613172-613172, doi:10.5402/2011/613172.
4. Carreño, D.; Hervás, G.; Toral, P.G.; Belenguer, A.; Frutos, P. Ability of different types and doses of tannin extracts to modulate *in vitro* ruminal biohydrogenation in sheep. *Anim. Feed Sci. Tech.* **2015**, *202*, 42-51, doi:http://dx.doi.org/10.1016/j.anifeedsci.2015.02.003.
5. Castillo, C.; Hernandez, J.; Bravo, A.; Lopez-Alonso, M.; Pereira, V.; Benedito, J.L. Oxidative status during late pregnancy and early lactation in dairy cows. *Vet. J.* **2005**, *169*, 286-292, doi:https://doi.org/10.1016/j.tvjl.2004.02.001.
6. Purba, R.A.P.; Paengkoum, P. A HPLC method of *Piper betle* L. for quantifying phenolic compound, water-soluble vitamin and essential oil in five different solvent extracts. *J. Appl. Pharm. Sci.* In press.
7. Menke, K.H.; Steingass, H. *Animal Research and Development* **1998**, *28*, 7-55.
8. NRC. *Nutrition requirements of Small Ruminants*; The National Acadimies Press: Washington DC, 2007.
9. AOAC. *Official methods of analysis*; AOAC International Suite 500: Gaitherburg, Maryland, USA, 2005.
10. Theodorou, M.K.; Williams, B.A.; Dhanoa, M.S.; McAllan, A.B.; France, J. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Anim Feed Sci. Tech.* **1994**, *48*, 185-197, doi:https://doi.org/10.1016/0377-8401(94)90171-6.
11. Orskov, E.R.; Mcdonald, I. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *J. Agric. Sci., Camb* **1970**, *92*, 499-503.

12. SAS Institute Inc. *Step-by-step programming with base SAS® edition 9.4*; SAS Institute Inc.: Cary, NC, USA, 2015.
13. Tukey, J.W. *The problem of multiple comparison*; Princeton Univ.: Princeton, NJ., 1953.
14. Menke, K.H.; Raab, L.; Salewski, A.; Steingass, H.; Fritz, D.; Schneider, W. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. *J. Agric Sci.* **1979**, *93*, 217-222, doi:10.1017/S0021859600086305.
15. Oskoueian, E.; Abdullah, N.; Oskoueian, A. Effects of Flavonoids on Rumen Fermentation Activity, Methane Production, and Microbial Population. *BioMed Res. Int.* **2013**, *2013*, 8, doi:10.1155/2013/349129.
16. Sinz, S.; Kunz, C.; Liesegang, A.; Braun, U.; Marquardt, S.; Soliva, C.R.; Kreuzer, M. *In vitro* bioactivity of various pure flavonoids in ruminal fermentation, with special reference to methane formation. *Czech J. Anim. Sci.* **2018**, *63*, 293-304.
17. Castillejos, L.; Calsamiglia, S.; Ferret, A. Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in *in vitro* systems. *J. Dairy Sci.* **2006**, *89*, 2649-2658, doi:http://dx.doi.org/10.3168/jds.S0022-0302(06)72341-4.
18. Gao, W.; Chen, A.; Zhang, B.; Kong, P.; Liu, C.; Zhao, J. Rumen degradability and post-ruminal digestion of dry matter, nitrogen and amino acids of three protein supplements. *Asian Australas. J. Anim. Sci.* **2015**, *28*, 485-493, doi:10.5713/ajas.14.0572.
19. Castillejos, L.; Calsamiglia, S.; Ferret, A.; Losa, R. Effects of a specific blend of essential oil compounds and the type of diet on rumen microbial fermentation and nutrient flow from a continuous culture system. *Anim. Feed Sci. Tech.* **2005**, *119*, 29-41, doi:http://dx.doi.org/10.1016/j.anifeedsci.2004.12.008.
20. Halushka, P.V.; Walle, U.K.; Walle, T. Carbon dioxide is the major metabolite of quercetin in humans. *J. Nut.* **2001**, *131*, 2648-2652, doi:10.1093/jn/131.10.2648.
21. Berger, L.M.; Blank, R.; Zorn, F.; Wein, S.; Metges, C.C.; Wolffram, S. Ruminal degradation of quercetin and its influence on fermentation in ruminants. *J. Dairy Sci.* **2015**, *98*, 5688-5698, doi:https://doi.org/10.3168/jds.2015-9633.
22. Vargas, J.E.; Andrés, S.; Snelling, T.J.; López-Ferreras, L.; Yáñez-Ruíz, D.R.; García-Estrada, C.; López, S. Effect of sunflower and marine oils on ruminal microbiota, *in vitro* fermentation and digesta fatty acid profile. *Front. Microbiol.* **2017**, *8*, 1124-1124, doi:10.3389/fmicb.2017.01124.
23. Kim, E.T.; Guan, L.L.; Lee, S.J.; Lee, S.M.; Lee, S.S.; Lee, I.D.; Lee, S.K.; Lee, S.S. Effects of flavonoid-rich plant extracts on *in vitro* ruminal methanogenesis, microbial populations and fermentation characteristics. *Asian-Australas J. Anim. Sci.* **2015**, *28*, 530-537, doi:10.5713/ajas.14.0692.
24. Busquet, M.; Calsamiglia, S.; Ferret, A.; Kamel, C. Plant Extracts Affect in vitro rumen microbial fermentation. *J. Dairy Sci.* **2006**, *89*, 761-771, doi:https://doi.org/10.3168/jds.S0022-0302(06)72137-3.
25. Lourenço, M.; Cardozo, P.W.; Calsamiglia, S.; Fievez, V. Effects of saponins, quercetin, eugenol, and cinnamaldehyde on fatty acid biohydrogenation of forage polyunsaturated fatty acids in dual-flow continuous culture fermenters1. *J. Anim. Sci.* **2008**, *86*, 3045-3053, doi:10.2527/jas.2007-0708.
26. Sallam, S.M.A.; Abdelgaleil, S.A.A.; Bueno, I.C.S.; Nasser, M.E.A.; Araujo, R.C.; Abdalla, A.L. Effect of some essential oils on *in vitro* methane emission. *Arch. Anim. Nutr.* **2011**, *65*, 203-214, doi:10.1080/1745039X.2011.568275.
27. Mao, H.L.; Mao, H.L.; Wang, J.K.; Liu, J.X.; Yoon, I. Effects of *Saccharomyces cerevisiae* fermentation product on in vitro fermentation and microbial communities of low-quality forages and mixed diets. *J. Anim. Sci.* **2013**, *91*, 3291-3298, doi:10.2527/jas.2012-5851.

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328 **Table 1.** Ingredient and chemical composition of feed and *Piper betle* L. powder

Item	Feed[a]	*Piper betle* L. powder
Ingredient, % dry matter		
Dehydrated Pangola hay	50	-
Cassava chip	3	-
Cassava pulp	19	-
Rice brain	5	-
Molasses	4	-
Palm meal	13	-
Soybean meal	4	-
Urea	0.9	-
Sulphur	0.1	-
Mineral[b]	0.8	-
Premix[c]	0.2	-
Chemical composition		
Dry matter, %	95.8	92.6
	----------% of dry matter----------	
Organic Matter	94.1	85.3
Crude Protein	10.8	2.26
Ether Extract	2.4	-
Acid Detergent Fibre	57.5	61.5
Neutral Detergent Fibre	66.5	73.5
Energy MJ/kg	18.96	21.8
Quercetin	-	19.9
Eugenol	-	10.8

329 [a] Contained (g/kg of total fatty acids): 16:0 (66.89), 18:0 (12.44), cis-9 18:1 (62.84), 18:2n-6 (60.96) and 18:3n-3
330 (0.63),
331 [b] Contained (g/kg): NaCl (600), P (160), Ca (240),
332 [c] Vitamin A (4,200.000 IU/Kg), Vitamin A3 (840,000 IU/Kg), Vitamin E (10,000 IU/Kg), Vitamin K3 (2 g/Kg),
333 Vitamin B1 (2.4 g/Kg), Vitamin B2 (3.5 g/Kg), Vitamin B6 (1.8 g/Kg), Vitamin B12 (0.01 g/Kg), Vitamin B5 (4.6
334 g/Kg), Vitamin C (12 g/Kg), Folic acid (0.28 g/Kg), Vitamin 7 (0.4 g/Kg), Coper (12 g/Kg), Manganese (40 g/Kg),
335 Zinc (3.2 g/Kg), Vitamin B1 (2.4 g/Kg), Iron (42 g/Kg), Iodine (0.8 g/Kg), Cobalt (0.8 g/Kg), Selenium (0.35 g/Kg).

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336 **Table 2**. Effect of Piper *betle* L. powder on gas kinetic, total gas production and degradability over 72 h[1]

Sunflower oil[2]	Treatment[3]	Gas kinetic[4]				GP 72 ml/g	Degradability[5] %			
		a	b	c	a+b	DM substrate	IVDMD	IVOMD	IVCPD	IVNDD
Low	S1	41.0^{b}	115.6^{f}	0.100^{a}	156.6^{g}	154.5^{g}	64.0^{d}	86.7^{f}	99.4^{a}	51.2^{g}
	S2	38.1^{d}	124.2^{d}	0.076^{c}	162.3^{e}	161.8^{e}	70.5^{a}	92.5^{e}	99.4^{a}	55.9^{d}
	S3	38.5^{d}	130.5^{c}	0.069^{f}	169.0^{d}	168.0^{d}	66.9^{b}	94.5^{d}	99.4^{a}	58.9^{c}
	S4	39.4^{c}	133.7^{b}	0.068^{f}	173.1^{b}	172.0^{bc}	58.0^{f}	96.6^{c}	91.7^{b}	56.4^{d}
	S5	39.0^{c}	134.1^{b}	0.073^{d}	173.2^{b}	172.5^{b}	58.0^{f}	99.9^{a}	91.3^{b}	54.3^{e}
High	S1	42.0^{a}	118.2^{e}	0.093^{b}	160.2^{h}	158.0^{f}	57.1^{g}	81.5^{g}	99.4^{a}	53.1^{f}
	S2	39.4^{c}	137.5^{a}	0.063^{g}	176.9^{a}	175.4^{a}	54.5^{h}	99.4^{ab}	99.4^{a}	58.7^{c}
	S3	39.1^{c}	132.8^{b}	0.071^{e}	171.9^{b}	171.1^{bc}	52.7^{i}	94.9^{d}	99.4^{a}	66.0^{b}
	S4	41.1^{b}	129.7^{c}	0.070^{e}	170.8^{bc}	169.8^{cd}	61.6^{e}	98.0^{bc}	89.0^{c}	53.2^{f}
	S5	37.5^{e}	130.7^{c}	0.070^{e}	168.2^{d}	167.4^{d}	65.1^{c}	98.1^{bc}	80.1^{d}	70.8^{a}
	SEM[6]	0.256	0.386	0.002	0.502	0.499	0.184	0.307	0.286	0.180
Comparison	Sunflower oil	***	***	***	***	***	***	***	***	***
	Treatment	***	***	***	***	***	***	***	***	***
	Interaction	***	***	***	***	***	***	***	***	***
Orthogonal polynomial contrast	Linear	**	***	***	***	***	ns	***	***	***
	Quadratic	***	***	***	***	***	***	*	***	***
	Cubic	**	***	***	***	***	ns	***	***	***

337 Notes: means in the same line with different superscripts are significantly different ($p < 0.05$), ns, non-significant ($p > 0.05$); *: significant different ($p < 0.05$); **: significant different ($p <$
338 0.01); ***: significant different ($p < 0.001$).

339 [1] Values are averages of three replicates obtained from independent incubation;

340 [2] sunflower oil was supplemented in all treatments: low, 15 mg/incubation and high, 30 mg/incubation;

341 [3] supplementing Piper *betle* L. powder: S1, 0 mg; S2, 15 mg; S3, 30 mg; S4, 45 mg; S5, 60 mg;

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342 [4] a, the gas production from the soluble fraction (ml/g DM); b, the gas production from the insoluble fraction (ml/g DM); c, the gas production rate constant for the insoluble fraction
343 (/h); (a + b), the potential extent of gas production (ml/g DM).;
344 [5] IVDMD, *in vitro* dry matter degradability; IVOMD, *in vitro* organic matter degradability; IVCPD, *in vitro* crude protein degradability; IVNFD, *in vitro* neutral detergent fibre;
345 [6] SEM, standard error of mean.
346

347 Table 3. Effect of Piper *betle* L. powder on rumen fermentation and methane production (CH_4)[1]

Sunflower oil[2]	Treatment[3]	pH	NH_3-N (mg/dl)	*In vitro* VFA								CH_4 ml/ g DM	CH_4 ml/g OM
				Total VFA (mmol/l)	C_2%	C_3%	Iso-C_4%	C_4%	Iso-C_5%	C_5%	C_2:C_3		
Low	S1	6.59	31.1^{b}	72.0^{i}	62.2^{c}	20.1^{e}	0.9^{a}	11.0^{e}	1.2^{a}	4.6^{b}	3.1^{c}	6.5^{c}	4.8^{b}
	S2	6.63	22.6^{d}	322.2^{a}	60.2^{de}	25.1^{abc}	0.6^{d}	12.5^{cd}	0.8^{c}	0.9^{h}	2.4^{f}	4.4^{h}	3.4^{g}
	S3	6.62	22.6^{d}	310.0^{b}	56.2^{f}	26.1^{ab}	0.8^{b}	14.5^{b}	1.1^{a}	1.2^{h}	2.2^{h}	5.7^{f}	4.0^{d}
	S4	6.63	17.0^{f}	300.2^{c}	61.0^{cd}	23.3^{a}	0.7^{c}	12.2^{d}	1.0^{b}	1.8^{d}	2.6^{e}	5.8^{ef}	3.5^{f}
	S5	6.63	19.8^{e}	233.1^{e}	59.2^{e}	24.8^{bc}	0.7^{c}	12.7^{c}	0.9^{b}	1.7^{e}	2.4^{f}	6.1^{d}	3.5^{f}
High	S1	6.61	25.4^{c}	90.0^{h}	67.2^{a}	17.6^{f}	0.7^{c}	8.6^{h}	0.8^{c}	5.0^{a}	3.8^{a}	7.5^{a}	5.2^{a}
	S2	6.65	17.0^{f}	289.3^{d}	57.5^{f}	24.9^{bc}	0.7^{c}	14.5^{b}	1.0^{b}	1.5^{g}	2.3^{g}	6.6^{bc}	3.5^{f}
	S3	6.64	19.8^{e}	290.8^{d}	65.4^{b}	21.9^{de}	0.5^{e}	10.6^{f}	0.4^{e}	1.2^{h}	3.0^{d}	6.7^{b}	3.7^{e}
	S4	6.63	19.8^{e}	170.4^{f}	53.0^{g}	27.0^{cd}	0.9^{a}	16.0^{a}	1.2^{a}	2.0^{c}	3.0^{d}	5.9^{e}	3.7^{e}
	S5	6.60	50.9^{a}	156.1^{g}	66.4^{ab}	20.7^{e}	0.5^{e}	10.1^{g}	0.7^{d}	1.6^{f}	3.2^{b}	5.1^{g}	4.5^{c}
	SEM[4]	0.009	0.130	4.417	0.819	0.534	0.025	0.402	0.043	0.254	0.091	0.152	0.112
Comparison	Sunflower oil	ns	***	***	***	***	***	***	***	***	***	***	***
	Treatment	ns	***	***	***	***	***	***	***	***	***	***	***
	Interaction	ns	***	***	***	***	***	***	***	***	***	***	***
Orthogonal polynomial contrast	Linear	ns	***	***	***	***	***	***	***	***	***	***	***
	Quadratic	ns	***	***	ns	***	***	***	***	***	***	***	***
	Cubic	ns	***	***	***	***	***	***	***	***	***	***	***

348 Notes: means in the same line with different superscripts are significantly different ($p < 0.05$), ns, non-significant ($p > 0.05$); ***: significant different ($p < 0.001$).

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[1]Values are averages of three replicates obtained from independent incubation;

[2] sunflower oil was supplemented in all treatments: low, 15 mg/incubation and high, 30 mg/incubation;

[3] supplementing Piper *betle* L. powder: S1, 0 mg; S2, 15 mg; S3, 30 mg; S4, 45 mg; S5, 60 mg;

[4] SEM, standard error of mean.