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ANIMAL PRODUCTION

Enhanced conjugated linoleic acid and biogas production after ruminal fermentation with *Piper betle* L. supplementation

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ABSTRACT: *Piper betle L. is edible plant richer in polyphenols that might improve feed utilization in rumen diet. The objective of the present study was to investigate the effect of various Piper betle L. powder (PL) doses on in vitro rumen microorganisms, ruminal biogas and fermentation end-product production, and biohydrogenation including lipolysis-isomerization. The completely randomized design used five levels of PL supplementation (0, 25, 50, 75 and 100 mg DM) incubated with 400 mg of a basal substrate of Pangola hay and concentrate (50:50). The matrix compounds (g/kg DM) of 0.27 catechin, 0.11 rutin, 3.48 quercetin, 0.41 apigenin, 0.04 myricetin, 0.27 kaempferol, 0.76 eugenol and 0.22 caryophyllene derived from PL altered the fermentation pattern, with an increase in degradable nutrients and total volatile fatty acids and acetogenesis without shifting pH during fermentation. These values promoted in vitro gas production, with higher carbon dioxide and lower methane production. Although, hydrogen recovery from lipolysis-isomerization in biohydrogenation was limited, PL successfully promoted stearic acid (C18:0) accumulation by changing the biohydrogenation pathway of fatty acids, causing more C18:1 trans-11 rather than C18:2 trans-11, cis-15. Consequently, this resulted in more conjugated linoleic acid (CLA) cis-9, trans-11, CLA trans-10, cis-12 and CLA trans-11, cis-13. Enhanced PL supply increased total bacteria and fungal zoospores due to a reduction in rumen protozoa. In conclusion, our results demonstrated that PL is a feed additive with potential for ruminants, promising improved ruminal fermentation and biohydrogenation, while reducing methane production.*
Key words: *fatty acids, feed additive, organic compounds, polyphenol compounds, rumen.*

Aumento na produção de ácido linoléico conjugado e biogás após fermentação ruminal com suplementação com *Piper betle* L.

RESUMO: *Piper betle L. é uma planta comestível rica em polifenois que podem melhorar a utilização de alimentos na dieta de ruminantes. O objetivo do presente estudo foi investigar o efeito de várias doses de Piper betle L em . pó (PL) sobre microrganismos do rúmen in vitro, biogás ruminal e produção de produtos finais de fermentação e bio-hidrogenação, incluindo lipólise e isomerização. O delineamento inteiramente casualizado utilizou cinco níveis de suplementação de PL (0, 25, 50, 75 e 100 mg de MS) incubados com 400 mg de um substrato basal do feno de Pangola e concentrado (50:50). Os compostos da matriz (g / kg MS) de 0,27 catequina, 0,11 rutina, 3,48 quercetina, 0,41 apigenina, 0,04 miricetina, 0,27 kaempferol, 0,76 eugenol e 0,22 cariofileno derivado de PL, alteraram o padrão de fermentação com o aumento de nutrientes degradáveis e voláteis totais, ácidos graxos e acetogênese sem alterar o pH durante a fermentação. Esses valores promoveram a produção de gás in vitro, com maior dióxido de carbono e menor produção de metano. Embora a recuperação de hidrogênio da lipólise-isomerização na bio-hidrogenação tenha sido limitada, o PL promoveu com sucesso o acúmulo de ácido esteárico (C18: 0) alterando a via de bio-hidrogenação dos ácidos graxos, causando mais C18: 1 trans-11 do que C18: 2 trans-11, cis -15. Consequentemente, isso resultou em mais ácido linoléico conjugado (CLA) cis-9, trans-11, CLA trans-10, cis-12 e CLA trans-11, cis-13. O suprimento aprimorado de PL aumentou o total de bactérias e zoósporos de fungos devido a uma redução no número de protozoários do rúmen. Em conclusão, nossos resultados demonstram que o PL é um aditivo alimentar com potencial para ruminantes, prometendo fermentação ruminal e bio-hidrogenação aprimoradas, enquanto reduz a produção de metano.*
Palavras-chave: *ácidos graxos, aditivo em alimentos para animais, compostos orgânicos, compostos de polifenóis, rúmen.*

INTRODUCTION

Piper betle L. is tropical, edible and affordable plant which is reported to contain a host of potent polyphenol compounds, such as flavonoids and essential oils (PURBA & PAENGKOUM, 2019). LOURENÇO et al. (2014) incubated forage with quercetin and eugenol, which did not affect C18:2 *cis*-9, *cis*-12 yield, but altered the biohydrogenation pathway of C18:3 n-3. DURMIC et al. (2008)

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reported that Australian plants selected as CLA-degrading inhibitors were successful in targeting ruminal bacteria such that biohydrogenation yielded more CLA. Unfortunately, the non-specific bioactive compounds were not reported. Since *Piper betle* L. has other flavonoids and essential oils (namely catechin, rutin, apigenin, myricetin, kaempferol and caryophyllene), it was hypothesized that *Piper betle* L. might potentially change fermentation pathways with regard to pyruvate stage and biohydrogenation. Therefore, the objective of present study was to investigate the influence of various inclusions of *Piper betle* L. powder (PL) on *in vitro* rumen microorganisms, ruminal biogas and fermentation end-product production, as well as biohydrogenation including lipolysis-isomerization.

MATERIALS AND METHODS

Animal, feed, Piper betle L. powder and experimental design

Piper betle L. leaves were planted, harvested and sampled from random spots at SUT Organic Farm, Nakhon Ratchasima, Thailand (14°52'48"N, 102°00'54"E at an elevation of 243 m above sea level). Leaves were removed from the plant and kept at 4 °C overnight to avoid any nutrient destruction (PURBA & PAENGKOUM, 2019). The leaves were mechanically ground (Retsch SM 100 mill; Retsch Gmbh, Haan, Germany) into a powder (PL) and particles were passed a 1-mm sieve, and placed into plastic, sealed pouches in the desiccator (Auto dry desiccator model OH-3S, SPC RT, Thailand) until usage time. In addition, three female Saanen goats (40 ± 1.51 kg body weight) were prepared as rumen donors, with *ad libitum* access to water and a maintenance total mixed ration (TMR) consisting of Pangola hay and concentrate (50:50) (NRC, 2007). This feed was also presented as a basal substrate in the *in vitro* experiment. Animals were allocated to the TMR for 20 d, including a 15 d adaptation period and 5 d of sampling. Formulation and chemical composition of the PL and feeds (basal substrate) are presented in table 1. The study was designed as a completely randomized design with five levels of PL at 0, 25, 50, 75 and 100 mg DM, incubated with 400 mg of the basal substrate of TMR and hay (50:50).

In vitro experiment and sampling

The *in vitro* experiment was conducted using the Hohenheim gas test method of MENKE & STEINGASS (1988), as modified by PAENGKOUM et al. (2015). On the 16th, 18th and 20th day of the feeding trials, rumen fluid was extracted from the rumen via oral lavage by suction pump (Hitachi CV-SF18, Japan) before morning feeding time, avoiding saliva collection (LODGE-IVEY et al., 2009). Rumen fluid was strained using a nylon membrane (400 µm; Fisher Scientific S.L., Madrid, Spain) into Conical flask, and mixed with salivary buffer (1:2, ml:ml) under CO_2 and kept at 39 °C for 24 h. The composition of the rumen fluid buffer mixture was as follows: 474 ml rumen fluid, 0.60 g $MgSO_4.7H_2O$, 1.32 g $CaCl_2.2H_2O$, 0.10 g $MnCl_2.4H_2O$, 0.10 g $CoCl_2.6H_2O$, 0.80 g $FeCl_3.6H_2O$, 35 g $NaHCO_3$, 4 g NH_4HCO_3, 5.70 g Na_2HPO_4, 6.20 g KH_2PO_4, 10 mg resazurin and 0.40 g NaOH, made up to 1000 ml with distilled water (MENKE & STEINGASS, 1988). The incubation was prepared using a 100 ml glass syringe filled with basal substrate, the PL dose and the rumen fluid/buffer mixture. For example, the treatment group using 100 mg of PL contained 400 mg substrate plus 100 mg PL, mixed with 30 ml rumen fluid/buffer. The sample for incubation was complete after 8 g/kg DM sunflower oil was infused into each glass syringe. Ten replicates of each treatment were prepared in three consecutive runs, with three blank controls added per run, containing only the rumen fluid/buffer mixture.

After 24 h of incubation, total gas production was read based on the model of ORSKOV & MCDONALD (1970). A 30 ml sample of gas from the glass syringe was used to measure methane and carbon dioxide levels by gas chromatography (Agilent 7890A, USA). The glass syringe was kept on ice to impede fermentation (JAYANEGARA et al., 2012a). Once the glass syringe was uncapped, the pH of the fermented content was measured directly using a pH meter (Oakton 700, USA). The fermented content was divided into four portions. The first portion was centrifuged at 6,000 × g at 4 °C for 15 min and the supernatant was stored at -20 °C. For volatile fatty acid (VFA) analysis, the supernatant was fixed with 25% metaphosphoric acid (ERWIN et al., 1961; FILÍPEK & DVOŘÁK, 2009). Rumen ammonia detection was performed in accordance with the micro-Kjeldahl method (Foss Kjeltech 8100, USA) (AOAC, 2005). The second portion was stored at -20 °C for fatty acid (FA) analysis. The third portion was treated with 10% formalin solution in a sterilized 0.9% saline solution for observation and calculation of total bacteria, protozoa and fungal zoospores using a counting chamber (Neubauer, Germany) as described by WANAPAT et al. (2013). The last portion was prepared for *in vitro* dry matter degradability (IVDMD) and *in vitro* organic matter

degradability (IVOMD) following the method of TILLEY & TERRY (1963) using pre-weighed Gooch crucibles (Isolab, Gmbh, Germany).

Laboratory analysis and calculation

The samples of substrate and PL were chemically analyzed for dry matter (DM), organic matter, ash, crude protein (total N × 6.25) and crude fat following prior protocols (AOAC, 2005). Acid detergent fiber and neutral detergent fiber were measured as described previously (VAN SOEST et al., 1991), with residual ash included. The gross energy was determined using a bomb calorimeter with an O_2 carrier (Parr 6200 bomb calorimeter, Parr Instruments Co., Moline, IL) according to the manufacturer's instructions. Concentrations of rutin, apigenin, quercetin, kaempferol, myricetin, eugenol and caryophyllene was determined by a mean peak rate of signal following the High Performance Liquid Chromatography with Diode Array Detector principle (PURBA & PAENGKOUM, 2019). All measurements were performed in triplicate and chemical standards were included in each analytical run as appropriate (Table 1).

Fatty acid (FA) measurement was conducted following the guidelines of FOLCH et al. (1957), as modified by (DE WEIRDT et al., 2013) using gas chromatography (Agilent 7890A, USA) with a CP-Sil88 column for FA methyl esters (100 m × 0.25 mm × 0.2 µm; Chrompack Inc., Middelburg, the Netherlands). Heptadecanoic acid (C17:0; Sigma-Aldrich) was applied as an internal standard (LASHKARI & JENSEN, 2017) and the C17:0 value was excluded in the calculation. The column temperature was kept at 70 °C for 4 min, then increased by 13 °C/min to 175 °C and held for 27 min, then increased by 4 °C/min to 215 °C and held for 17 min, then increased by 4 °C/min to 240 °C and held for 10 min. More detailed information about the detection and calculation methods of peak area is given by LOURENÇO et al. (2014). The FA profile of feed and PL is presented in table 1. Calculation of the efficiency of lipolysis and isomerization (C18:2 n-6 → C18:2 *cis*-9, *trans*-11), and the hydrogenation processes of C18:2 n-6 and C18:3 n-3 in incubations were calculated as determined by BOECKAERT et al. (2007), as modified by PANYAKAEW et al. (2013) (Table 4).

The supernatant of the 2 µL VFA sample was injected into the gas chromatography machine (Hewlett Packard GC system HP6890 A; Hewlett Packard, Avondale, PA, USA), in triplicate, following the previous study by ERWIN et al. (1961), with modification by FILÍPEK & DVOŘÁK (2009). Acetic acid, propionic acid, iso-butyric acid, butyric, iso-valeric acid and valeric acid (Carlo Ebra, France) were prepared with 1% formic acid and considered as standards for calibration. Hydrogen recovery (HR) was calculated as (2 Propionate + 2 Butyrate + 4 CH_4)/ (2 Acetate + Propionate + 4 Butyrate), with acetate, propionate, butyrate and CH_4 (MARTY & DEMEYER, 1973). HR measurement was performed in triplicate and expressed as net molar production (mol/mol).

Statistical analysis

Statistical analysis accounted for the completely randomized design using the PROC GLM procedure of SAS 9.4. Data were analyzed using the model:

$Y_{ST} = A + B_S + E_{ST}$

where Y_{ST} = the dependent variable, A = the overall mean, B_S = the influence of the various PL doses ($_S$ =1–5), and E_{ST} = the residual effect. Because the runs were not significantly different, results are presented as mean values with the standard error of the mean. Differences between treatment means were calculated by Tukey's test (KAPS & LAMBERSON, 2004). Orthogonal polynomial contrasts were used to estimate the linear and quadratic PL effects. All differences with $P<0.05$ were accepted as representing statistically significant differences.

RESULTS AND DISCUSSION

Ruminal biogases, fermentation end-products and microorganisms

The effect of the inclusion of *Piper betle* L. powder (PL) on fermented substrate in terms of ruminal biogas, fermentation end-products and microorganisms after 24 h, is presented in table 2. The present matrix of flavonoids and essential oils derived from PL gradually increased total volatile fatty acids (VFAs) corresponding to an increase in degradability, especially of fermented organic matter. In this study, *in vitro* dry matter degradability (IVDMD) and *in vitro* organic matter degradability (IVOMD) rose with the increase of PL. These results increased the total VFAs. However, unchanged numbers of total VFAs and degradability efficiencies were found previously, where they were compared to the control after substrate was incubated with quercetin, rutin and eugenol (CASTILLEJOS et al., 2006; OSKOUEIAN et al., 2013). Contradictions could be due to different basal substrates in proportion to the bioactive plant compound. The former studies subjected the ruminants

Table 1 - Ingredient and chemical composition of concerned treatments.

Item	Feed	*Piper betle* L. powder
Ingredient, g/kg DM		
Dehydrated Pangola hay	500	
Cassava chip	170	
Rice bran	70	
Molasses	40	
Palm meal	100	
Soybean meal	100	
Urea	9	
Sulphur	1	
Mineral[1]	8	
Premix[2]	2	
Chemical composition, g/kg DM		
Organic matter	870.7	778.8
Crude protein	129.5	25.6
Crude fat	22.5	3.4
Neutral detergent fiber	684.7	639.4
Acid detergent fiber	545.4	509.1
Gross energy, MJ/kg DM	22.9	17.7
Total polyphenols compounds, g/kg DM		
Catechin	-	0.3
Rutin	-	0.1
Quercetin	-	3.5
Apigenin	-	0.4
Myricetin	-	0.1
Kaempferol	-	0.3
Eugenol	-	0.8
Caryophyllene	-	0.2
Fatty acid proportion, g/100 g FA		
C14.0	4.8	1.9
C16.0	22.0	23.5
C18.0	4.1	5.1
C18:1n-9	20.7	13.5
C18:2n-6	20.1	18.5
C18:3n-3	0.2	-

[1]Contained (g/kg): NaCl (600), P (160), Ca (240).
[2]Vitamin A (4,200.000 IU/kg), vitamin A_3 (840,000 IU/kg), vitamin E (10,000 IU/kg), vitamin K_3 (2 g/kg), vitamin B_1 (2.4 g/kg), vitamin B_2 (3.5 g/kg), vitamin B_6 (1.8 g/kg), vitamin B_{12} (0.01 g/kg), vitamin B_5 (4.6 g/kg), vitamin C (12 g/kg), folic acid (0.28 g/kg), coper (12 g/kg), manganese (40 g/kg), zinc (3.2 g/kg), iron (42 g/kg), iodine (0.8 g/kg), cobalt (0.8 g/kg), selenium (0.35 g/kg).

to a 60:40 forage: concentrate diet (CASTILLEJOS et al., 2006; OSKOUEIAN et al., 2013), whereas in our study, a 50:50 Pangola hay: concentrate diet was applied. Also, those reports purchased a commercial secondary compound product. Notably, *in vitro* studies with supplementing active plant compound to modulate rumen performance may result in varied outcomes due to different purity rate, binding behavior and hydroxyl compound (PURBA et al., 2020). Despite PL derived from conventional procedure, PL had been reported owning abundant hydroxyl groups and as carbohydrate sources that enhanced the fermented substrate during fermentation (PURBA & PAENGKOUM 2019). Hence, this elaboration may also account for why the final results were slightly different. Higher VFAs values reflect a greater amount of fermented substrate by rumen microbes that could also be recognized in the total

Table 2 - The mean value of substrate incubated with *Piper betle* L. powder (PL) on ruminal biogases, fermentation end-products and microorganisms after 24 h incubation.

Parameter[1]	----------PL dose (mg/incubation)----------					SEM[2]	----------Contrast[3]----------	
	0	25	50	75	100		Linear	Quadratic
pH	6.9	6.8	6.8	6.9	6.9	0.013	0.438	1.000
Total gas production (ml/g OM)	30.1^{d}	36.8^{c}	38.7^{b}	42.4^{a}	42.9^{a}	0.670	<0.001	<0.001
CO_2 (ml/g OM)	14.2^{d}	18.6^{c}	20.6^{b}	26.8^{a}	27.1^{a}	0.706	<0.001	<0.001
CH_4 (ml/g OM)	11.9^{a}	10.8^{b}	9.1^{c}	8.4^{d}	7.9^{d}	0.218	<0.001	<0.001
IVDMD (%)	49.9^{c}	51.6^{b}	52.4^{a}	52.9^{a}	52.7^{a}	0.179	0.016	0.009
IVOMD (%)	50.5^{c}	65.2^{b}	66.4^{a}	65.8^{ab}	64.99^{b}	0.872	<0.001	<0.001
NH_3-N (mg/100 ml)	19.7^{b}	20.7^{a}	20.5^{a}	19.9^{b}	20.0^{b}	0.066	0.996	0.029
Total volatile fatty acid (mM)	61.1^{c}	66.4^{b}	69.4^{a}	69.1^{a}	68.8^{a}	0.460	0.004	<0.001
Acetate (C_2) (mol/100 mol)	52.1^{d}	54.3^{c}	56.5^{b}	57.9^{a}	58.2^{a}	0.342	0.001	<0.001
Propionate (C_3) (mol/100 mol)	20.1^{a}	19.5^{b}	19.2^{b}	18.8^{c}	18.0^{d}	0.105	<0.001	<0.001
Iso-butyrate (mol/100 mol)	5.6^{a}	4.9^{b}	4.2^{c}	3.6^{d}	3.6^{d}	0.109	<0.001	<0.001
Butyrate (mol/100 mol)	10.2^{d}	10.8^{cd}	11.4^{c}	12.3^{b}	12.9^{a}	0.141	<0.001	<0.001
Iso-valerate (mol/100 mol)	4.5^{a}	4.0^{b}	3.4^{c}	2.9^{d}	3.0^{d}	0.089	<0.001	<0.001
Valerate (mol/100 mol)	7.5^{d}	6.5^{d}	5.3^{c}	4.5^{b}	4.3^{a}	0.174	<0.001	<0.001
C_2:C_3	2.6^{d}	2.8^{cd}	2.9^{c}	3.1^{b}	3.2^{a}	0.032	<0.001	<0.001
HR (mol/mol)	0.66^{a}	0.61^{b}	0.55^{c}	0.52^{c}	0.50^{d}	0.008	<0.001	<0.001
Total bacteria, × 10^7 cells/ml	5.1^{c}	5.2^{b}	5.3^{a}	5.3^{ab}	5.2^{b}	0.021	<0.001	<0.001
Total protozoa, × 10^5 cells/ml	5.0^{a}	4.1^{b}	4.0^{c}	3.6^{d}	3.4^{d}	0.075	<0.001	<0.001
Total fungal zoospore, × 10^5 cells/ml	3.1^{c}	3.1^{c}	3.1^{c}	3.2^{b}	3.5^{a}	0.022	<0.001	<0.001

[abc] Within a row, different superscript represents the significant differences by Tukey P<0.05.
[1]OM= organic matter; IVDMD= *in vitro* dry matter degradability; IVOMD= *in vitro* organic matter degradability; HR= hydrogen recovery (calculated in materials and methods (Marty and Demeyer 1973).
[2]SEM=standard error of mean.
[3]Orthogonal polynomial contrast P<0.05.

production of gas during fermentation. In this study, gas production was promoted by the addition of PL, with more carbon dioxide and less methane gas being produced. A reduction in methane production due to failed methanogenesis through the use of flavonoids and essential oils has been reported in an earlier review (PATRA & SAXENA, 2010), but the role of rutin, apigenin, myricetin, kaempferol and caryophyllene was not discussed. A smaller proportion of methane gas seems to imply that rumen methanogenesis occurred sluggishly in this study, causing a shift of the profile of VFAs, particularly the acetate, propionate and butyrate fractions.

In the present study, PL during fermentation resulted in higher acetate and butyrate production, but lower propionate, valerate and branched-chain VFAs (the iso-acid fraction). As calculated, this study also showed an inhibited hydrogen supply (HR) to methanogenesis and biohydrogenation. According to prior studies, propionate produced from pyruvate increases hydrogen consumption, and results in less efficient methanogenesis (MOSS et al., 2000; TAVENDALE et al., 2005). However, a meta-analysis by JAYANEGARA et al. (2012b) reported no significant relationship between the propionate surge and methane inhibition. GREENING et al. (2019) confirmed that hydrogen metabolism is a more complex and widespread trait among rumen microorganisms, and claims that the hydrogen yield is usurped by other consumers (not only methanogens) in fumarate and nitrite reduction (*Selenomonas* spp.) and acetogenesis (*Blautia* spp.). In the present study, PL supplementation increased the acetate fraction, providing more evidence that hydrogen was consumed by acetogenesis (*Blautia* spp.) and hydrogen supply was depleted for this reason. Valeric and iso-acid fractions in a recent study (ANDRIES et al., 1987) were lower as consequence of less propionic acid. All changes in fermentation behavior above occurred with an unchanged ruminal pH across all treatments, while an alteration in pH

occurred in the deamination stage after incubation of PL and substrate (Table 2). The range of pH and ammonia concentration in the present study was 6.8-7.0 mg/100 ml and 19.9-20.1 mg/100 ml, which was acceptable for ruminal microorganisms to survive by modulating microbial growth and fermentation efficiency (WANAPAT & PIMPA 1999).

Enhanced PL supply reduced protozoa in the rumen (Table 2). A decrease in protozoa number due to the supplementation of secondary compounds has been reported before (CASTILLEJOS et al., 2006; ZHOU et al., 2011; OSKOUEIAN et al., 2013; ANDRÉS et al., 2016; SZCZECHOWIAK et al., 2016) and a primary reason is the ability of bioactive compounds to alter cell wall synthesis or nucleic acid synthesis of protozoa. This reduction in protozoa may be reason why the bacteria and fungal zoospores increased within the incubation period. DEHORITY (2003) mentioned protozoa engulfing rumen bacteria at approximately 20,000 cells per hour. Thus, increased bacteria numbers were contingent on the eliminated protozoa. NEWBOLD et al. (2015) also reported that fungal zoospores were inversely proportional to protozoal number, indicating a competitiveness between protozoa and fungal zoospores for substrate during fermentation. In another report, CHERDTHONG et al. (2019) confirmed that fungal zoospores had a defensive mechanism for survival in the presence of bioactive compounds.

Lipolysis, isomerization and biohydrogenation

The effect of the addition of *Piper betle* L. powder (PL) to fermented substrate on the FA profile of rumen fermentation after 24 h is presented in table 3.

Table 3 - The mean value of substrate incubated with *Piper betle* L. powder (PL) on ruminal fatty acid profile (g/100 g) after 24 h incubation.

Parameter[1]	----------PL dose (mg/incubation)----------					SEM[2]	----------Contrast[3]----------	
	0	25	50	75	100		Linear	Quadratic
Total SFA[4]	65.851^{a}	62.840^{b}	61.746^{c}	61.498^{c}	61.585^{c}	0.257	0.006	0.002
C14.0	0.919^{c}	0.959^{a}	0.949^{ab}	0.940^{b}	0.919^{c}	0.003	0.132	0.171
C16.0	20.570	20.621	20.591	20.480	20.611	0.035	1.000	0.741
C18.0	36.734^{a}	33.588^{b}	32.519^{c}	32.388^{c}	32.368^{c}	0.245	<0.001	<0.001
Total MUFA[5]	22.120^{c}	25.302^{b}	26.446^{a}	26.644^{a}	26.501^{a}	0.248	<0.001	<0.001
C18:1 *trans*-6–8	0.638^{c}	0.716^{a}	0.726^{a}	0.656^{b}	0.652^{b}	0.005	<0.001	<0.001
C18:1 *trans*-9	0.486^{a}	0.463^{b}	0.480^{a}	0.470^{b}	0.461^{c}	0.002	<0.001	0.529
C18:1 *trans*-10	0.414^{c}	0.468^{b}	0.491^{a}	0.490^{a}	0.485^{a}	0.004	0.001	<0.001
C18:1 *trans*-11	2.476^{e}	5.428^{d}	6.439^{c}	6.681^{b}	6.782^{a}	0.231	<0.001	<0.001
C18:1 *trans*-15	0.959^{c}	0.983^{b}	0.993^{b}	1.008^{a}	0.953^{c}	0.003	0.004	0.004
C18:1 *cis*-9	5.222	5.244	5.251	5.252	5.252	0.009	1.000	0.996
C18:1 *cis*-11	0.762^{c}	0.752^{c}	0.769^{b}	0.787^{a}	0.695^{d}	0.005	<0.001	<0.001
C18:1 *cis*-15	0.717^{c}	0.762^{b}	0.794^{a}	0.795^{a}	0.718^{c}	0.051	<0.001	0.006
Total PUFA[6]	12.028	11.858	11.808	11.858	11.914	0.022	1.000	0.660
C18:2 *trans*-11, *cis*-15	0.038^{a}	0.037^{b}	0.035^{c}	0.035^{c}	0.033^{d}	0.003	<0.001	<0.001
CLA *cis*-9, *trans*-11	0.204^{c}	0.360^{b}	0.376^{b}	0.374^{a}	0.363^{a}	0.009	<0.001	<0.001
CLA *trans*-10, *cis*-12	0.010^{d}	0.020^{c}	0.020^{c}	0.021^{b}	0.022^{a}	0.063	<0.001	<0.001
CLA *trans*-11, *cis*-13	0.505^{d}	0.528^{c}	0.537^{c}	0.659^{b}	0.735^{a}	0.013	<0.001	<0.001
C18:2n-6	5.869^{a}	5.516^{b}	5.445^{bc}	5.375^{c}	5.364^{c}	0.028	0.001	0.001
C18:3n-3	0.152^{a}	0.151^{b}	0.150^{b}	0.148^{c}	0.149^{c}	0.032	0.186	0.998

^{abc} Within a row, different superscript represents the significant differences by Tukey P<0.05.
[1]SFA= saturated fatty acids; MUFA= mono unsaturated fatty acids; PUFA= mono unsaturated fatty acids.
[2]SEM=standard error of mean.
[3]Orthogonal polynomial contrast P<0.05.
[4]Sum of all SFA: C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C18:0, C20:0, C22:0 and C23:0.
[5]Sum of all MUFA: C14:1 *cis*-9, C15:1 *cis*-9, C16:1 *trans*-9, C16:1 *cis*-9, C18:1 *trans*-6–8, C18:1 *trans*-9, C18:1 *trans*-10, C18:1 *trans*-11, C18:1 *trans*-12–14, C18:1 *trans*-15, C18:1 *cis*-9, C18:1 *cis*-11, C18:1 *cis*-12, C18:1 *cis*-13, C18:1 *cis*-14, and C18:1 *cis*-15.
[6]Sum of all PUFA: C18:2 *trans*-11, *cis*-15; C18:2 *trans*, *trans* isomers; C18:2 *cis*, *cis* isomers; C18:2 *cis, trans* isomers; CLA *cis*-9, *trans*-11; CLA *trans*-10, *cis*-12; CLA *trans*-11, *cis*-13; C18:2n-6; C18:3n-6; C18:3n-3; C20:2n-6; C20:3n-6; C20:3n-3; C20:5n3; C24:1n-9 and C22:6n-3.

Clearly, the present matrix of flavonoids and essential oils derived from PL inhibited biohydrogenation, where the biohydrogenation of C18:2n-6 and C18:3n-3 was abated, as poly unsaturated fatty acids (PUFAs) were observed. Reference to this shift in biohydrogenation, including the isomerization of FA by lipolysis, using flavonoids and essential oils of PL, is relatively limited. The application of only phenolic acid, quercetin, anthocyanin and eugenol for *in vitro* study is available in the literature (LOURENÇO et al., 2014; YANZA et al., 2018; BRYSZAK et al., 2019). In our study, PL encouraged rumen biohydrogenation to yield a more considerable PUFA accumulation, e.g. CLA *cis*-9, *trans*-11, CLA *trans*-10, *cis*-12 and CLA *trans*-11, *cis*-13, less saturated fatty acid as C18:0 and less mono-unsaturated fatty acid accumulation of C18:1 *trans*-11, rather than C18:2 *trans*-11, *cis*-15. It is reported that the biohydrogenation pathway could be shifted by the provision of flavonoids in the animal diet (YANZA et al., 2018), resulting in an increased amount of CLA *cis*-9, *trans*-11 produced in rumen fluid and milk (BRYSZAK et al., 2019).

As shown in table 4, enhanced PL supply interacted in the first biohydrogenation pathway of C18:2n-6, resulted in the accumulation of C18:2 *cis*-9 rather than C18:2 *trans*-11, *cis*-15, which was observed to be different to other studies (LOURENÇO et al., 2014). The latter study reported that quercetin and eugenol were unable to alter the proportion of C18:2 *cis*-9 through the first biohydrogenation pathway of C18:2n-6, although, it seemed successful at a slight reduction in C18:2 *trans*-11, *cis*-15. However, later biohydrogenation regarding the transformation of C18:1 *trans*-11 to C18:0 in these reports showed similar outcomes, addressing the limited supply of C18:1 *trans*-11, leading to a decrease in stearic acid (C18:0) accumulation. A possible reason for this difference concerns the FA isomer in the diet. LOURENÇO et al. (2014) fed rumen fermenters with more C18:3n-3 than C18:2n-6. In contrast, the present study provided an abundance of C18:2n-6 from sunflower oil. This assertion could be true if the FA component was a major factor in modulating biohydrogenation (ROY et al., 2013). However, biohydrogenation efficiency is more complex because this process promotes rumen biohydrogenation bacteria and should be wisely considered for other factors, such as hydrogen supply (LOURENÇO et al., 2010). In this study, stearic bacteria (that were encouraged by the presence of PL) were responsible for undertaking CLA formation, but identification of specific ruminal microorganisms to confirm this was outside the scope of the present study. It is possible that small protozoa and big bacterial populations could influence lipase activity (LOURENÇO et al., 2010). Therefore, the change in rumen microorganisms, especially bacteria and protozoa, should not be ignored.

CONCLUSION

This study demonstrated that the matrix of flavonoids and essential oils in *Piper betle* powder can stimulate conjugated linoleic acid accumulation from biohydrogenation products, without interrupting nutrient fermentation. Methane production is also reduced. Another perspective of the use of these compounds can be assessed *in vivo*, where animals are fed with these bioactive compounds. It might be an alternative strategy to improve the quality of ruminant feeds.

Table 4 - The mean value of substrate incubated with *Piper betle* L. powder (PL) on the efficiency of lipolysis + isomerization (C18:2 n-6 → C18:2 c9t11) and hydrogenation processes of C18:2 n-6 and C18:3 n-3 in incubations.

Parameter	----------PL dose (mg/incubation)----------					SEM[1]	--------Contrast[2]---------	
	0	25	50	75	100		Linear	Quadratic
C18:2 n-6→ C18:2 *cis*-9, *trans*-11	71.0^{b}	72.7^{a}	73.1^{a}	73.4^{a}	73.4^{a}	0.319	0.053	0.095
C18:3 n-3→C18:2 *trans*-11, *cis*-15	28.1	28.3	28.5	29.2	28.5	0.126	1.000	0.149
C18:2 *trans*-11, *cis*-15→C18:1 *trans*-11	35.6^{d}	37.5^{cd}	41.1^{c}	42.3^{b}	44.0^{a}	0.471	<0.001	<0.001
C18:2 *cis*-9, *trans*-11→C18:1 *trans*-11	98.6	97.6	97.5	97.5	97.6	0.396	0.981	0.956
C18:1 *trans*-11→C18:0	83.8^{a}	53.7^{b}	43.4^{c}	41.5^{d}	40.6^{d}	2.338	<0.001	<0.001

^{abc} Within a row, different superscript represents the significant differences by Tukey P<0.05.
[1]SEM=standard error of mean.
[2]Orthogonal polynomial contrast P<0.05.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

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.

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