Flavonoids and their aromatic derivatives in *Piper betle* powder promote *in vitro* methane mitigation in a variety of diets

Flavonóides e os seus derivados aromáticos no pó de *Piper betle* promovem a mitigação *in vitro* do metano em diversas dietas

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ABSTRACT

At present, there is little information regarding whether supplementation with *Piper betle* powder (PBP) and sunflower oil (SFO) has a synergistic effect on lowering methane emissions without negatively impacting ruminal fermentation. This study investigated the effects of PBP, supplemented either with or without SFO, on biogas release, fermentation end-products, and microorganisms in the rumen of lactating goats. The treatments were run in a completely randomized 3 × 5 factorial arrangement, whereby 0, 15, and 30 mg SFO were combined with 0, 15, 30, 45, and 60 mg PBP on a dry matter basis. The outcomes were assessed *in vitro*. PBP was obtained from the perennial plant *Piper betle* L., which is an abundant source of flavonoids and their aromatic derivatives. SFO, which reduces dietary methane emissions, was supplemented to confirm whether it interacted with other nutrients in the ruminant diet. SFO × PBP significantly ($p < 0.05$) decreased methane production, enhanced total volatile fatty acid concentrations, and decreased the number of rumen protozoa. We found that 15–30 mg, but not 45–60 mg, PBP combined with 0, 15, and 30 mg SFO increased ($p < 0.05$) total gas production (including CO_2) from fermentation. However, our results suggested that at least 45 mg PBP, either alone or combined with SFO, was required to reduce ammonia-N ($p < 0.05$). Not all treatments affected rumen pH. In conclusion, supplementing PBP (< 30 mg), either alone or combined with SFO, has a suppressing effect on methane production while preserving an optimum rate of rumen fermentation.

Index terms: Environment; feed utilization; fermentation; methane reduction potential; polyphenols.

RESUMO

Atualmente, há pouca informação sobre se a suplementação com *Piper betle* em pó (PBP) e óleo de girassol (SFO) tem um efeito sinérgico na redução das emissões de metano sem impacto negativo na fermentação ruminal. Este estudo investigou os efeitos da PBP, complementada com ou sem SFO, na liberação de biogás, produtos finais de fermentação e microrganismos do rúmen de cabras lactantes. Os tratamentos foram realizados em um arranjo fatorial completamente randomizado de 3 × 5, em que 0, 15 e 30 mg de SFO foram combinados com 0, 15, 30, 45 e 60 mg de PBP em base de matéria seca. Os resultados foram avaliados *in vitro*. PBP foi obtida da planta perene *Piper betle* L., que é uma fonte abundante de flavonóides e seus derivados aromáticos. SFO, que reduz as emissões de metano na dieta, foi complementado para confirmar se interagia com outros nutritivos na dieta ruminante. A SFO × PBP diminuiu significativamente ($p < 0,05$) a produção de metano, aumentou as concentrações totais voláteis de ácidos graxos e diminuiu o número de protozoários de rúmen. Observou-se que 15-30 mg, e não 45-60 mg, PBP combinado com 0, 15, e 30 mg SFO aumentou ($p < 0,05$) a produção total de gás (incluindo CO_2) a partir da fermentação. Contudo, os nossos resultados sugeriram que pelo menos 45 mg de PBP, isoladamente ou em combinação com SFO, é necessário para reduzir a amonia-N ($p < 0,05$). Nem todos os tratamentos afetaram o pH do rúmen. Em conclusão, complementar PBP (< 30 mg), sozinho ou combinado com SFO, tem um efeito de supressão na produção de metano, preservando ao mesmo tempo uma taxa ótima de fermentação do rúmen.

Termos para indexação: Ambiente; utilização dos alimentos; fermentação; potencial de redução do metano; polifenóis.

INTRODUCTION

Methane accounts for approximately 14% of greenhouse gases worldwide; this gas is emitted by ruminants under anaerobic conditions during ruminal fermentation (Sejian et al., 2011). As a result, there has been global interest in suppressing methane emissions without deleterious consequences on feed utilization (Yanza et al., 2018). Providing feed materials such as plant polyphenols and vegetable oils was expected to alter rumen inhabitants, resulting in improved rumen fermentation characteristics while reducing methane production since these supplementations have well-documented antimicrobial effects (Hook; Wright; McBride, 2010). Despite the considerable benefits of supplementing plant polyphenols and vegetable oils from a fermentation perspective, these rumen modifiers could modulate other nutrients in the ruminant diet (Lourenço et al., 2014; Elghandour et al., 2017; Purba; Paengkoum; Paengkoum, 2020). Notably, sunflower oil (SFO) has been suggested as a cost-effective way to alleviate methane production and provide concurrent nutrients such as fat inclusions (Vargas et al., 2017).

Piper betle L. is a part of the *Piperaceae* family and has been traditionally used raw as a natural antiseptic for humans, particularly for cleaning their mouths. *P. betle* leaves have protein, fat, carbohydrate, mineral, and other contents of about 3-3.5%, 0.4-1.0%, 0.5-6.10%, 2.3-3.3%, and 86.5-93.5% respectively, of total nutrients in dry matter (Guha, 2006). Further, these leaves contain a host of polyphenols, especially flavonoids and essential oils (Purba; Paengkoum, 2019). Polyphenols originating from an extracted assay or a whole plant could be considered methane inhibitors as they have been shown to decrease enteric methane production (Jayanegara; Leiber; Kreuzer, 2012; Vasta; Daghio; Cappucci, 2019; Purba; Yuangklang; Paengkoum, 2020). However, there were adverse responses to feed digestion and ruminal fermentation when plant polyphenols were dosed at exceedingly high thresholds to obtain optimal methane mitigation (Patra; Yu, 2013; Vasta; Daghio; Cappucci, 2019). n detrimental cases, animals experienced toxicity resulting in impaired ruminant productivity and health (Waghorn; McNabb, 2003). In contrast, the above disastrous effects can be avoided at a lower threshold, but the methane mitigation would be decreased as well (Patra; Yu, 2015). A combination of a low amount of methane inhibitors, such nitrate and saponin, has been reported to decrease enteric methane production while maintaining feed digestion or substrate fermentation (Patra; Yu, 2013). At present, however, it is unknown whether supplementation with *Piper betle* powder (PBP) and SFO has a synergistic effect and can boost animal performance. We postulated that PBP could alter the ruminal fermentation pathway. Therefore, the objective of this study was to investigate the effects of PBP at five different doses, either with or without SFO, on biogas production, fermentation end-products, and microbial composition in rumen fluids from lactating goats, as estimated by *in vitro* techniques.

MATERIAL AND METHODS

All experimental procedures were approved and carried out in accordance with the Rules of Animal Welfare and all research on animals was conducted according to the Institutional Committee on Animal Use (SUT 4/2558).

Animal, feed and *Piper betle* L. powder

Four multiparous Saanen goats, *Capra aegagrus hircus*, (43 ± 1.29 kg body weight) were assigned as rumen inoculum donors. All goats received a total mixed ration (TMR) consisting of a 50:50 mix of Pangola hay (particle size > 4 cm) and concentrate, and had free access to drinking water. The TMR was offered in two portions (60% at 09:00 and 40% at 17:00) and this feed was dedicated as a basal substrate in the later *in vitro* incubation. The formulation and chemical analysis of the feed (substrate) are presented in Table 1.

P. betle leaves were purchased from a local market in the Prachinburi area of east Thailand. Fresh biomass of leafy plant material was pooled, rinsed, and kept overnight at 4 °C. The leaves were air-dried using an oven set at 40 °C for 2 days, turned into a powder (hereafter referred to as *P. betle* powder, PBP), loaded in sealed plastic, and kept in a desiccator until used. Flavonoid and aromatic contents were extracted and assayed in water, methanol, and hexane. The quantification procedure was performed using a high-performance liquid chromatography machine with a diode-array detector (HPLC-DAD Agilent Technologies 1260 Infinity, USA and Canada) (Purba; Paengkoum, 2019). Briefly, 5 g of PBP were extracted using 20 mL solvents individually and then run in a Soxhlet apparatus for 3–4 hours. The extracts were finally filtered through 0.45 μm polyvinyl difluoride syringe filters and subsequently diluted with mobile phase solution (1:9, HPLC-grade acetonitrile:1% acetic acid). We injected 20 μL of diluted extract into the HPLC-DAD machine, in which the extract was set in a 10-mm flow cell with 100 loops of automatic sample injection and four solvent delivery system quaternary pumps. Separation was achieved using a reversed-phase Zorbax SB-C18 column (3.5 μm particle size, i.d. 4.6 × 250 mm) (Agilent Technologies). A standard stock solution was prepared using commercial quercetin

and eugenol (Sigma-Aldrich, St. Louis, MO). Data collection was performed using OpenLAB CDS v1.8.1 (Agilent Technologies). All measurements were performed in triplicate and chemical standards were included in each analytical run as appropriate (Table 1).

Table 1: Ingredient and chemical composition of substrate and *Piper betle* L. powder.

Item	Substrate[a]	*Piper betle* L. powder[b]
Ingredient (g kg⁻¹ DM)		
Dehydrated Pangola hay	500	-
Cassava pulp	220	-
Rice bran	50	-
Molasses	40	-
Palm meal	130	-
Soybean meal	40	-
Urea	9	-
Sulphur	1	-
Mineral[c]	8	-
Premix[d]	2	-
Chemical composition (g kg⁻¹ DM)		
Organic matter	982.62	921.73
Crude protein	112.33	24.40
Ether extract	24.71	3.57
Acid detergent fiber	600.59	664.05
Neutral detergent fiber	694.65	793.88
Catechin	0	3.06
Rutin	0	1.28
Quercetin	0	39.59
Apigenin	0	4.72
Myricetin	0	0.48
Kaempferol	0	3.11
Eugenol	0	8.65
Caryophyllene	0	2.86

[a]Contained fatty acid (g kg⁻¹ DM): 16:0 (5.12), 18:0 (0.26), 18:2n-6 (4.95) and 18:3n-3 (0.07); [b]Contained fatty acids (g kg⁻¹ DM): 16:0 (0.62), 18:0 (0.13), cis-9 18:1 (0.35), and 18:2n-6 (0.48); [c]Contained (g kg⁻¹): NaCl (600), P (160), Ca (240); [d]Vitamin A (4,200.000 IU kg⁻¹), vitamin A₃ (840,000 IU kg⁻¹), vitamin E (10,000 IU kg⁻¹), vitamin K₃ (2 g kg⁻¹), vitamin B₁ (2.4 g kg⁻¹), vitamin B₂ (3.5 g kg⁻¹), vitamin B₆ (1.8 g kg⁻¹), vitamin B₁₂ (0.01 g kg⁻¹), vitamin B₅ (4.6 g kg⁻¹), vitamin C (12 g kg⁻¹), folic acid (0.28 g kg⁻¹), vitamin 7 (0.4 g kg⁻¹), coper (12 g kg⁻¹), manganese (40 g kg⁻¹), zinc (3.2 g kg⁻¹), iron (42 g kg⁻¹), iodine (0.8 g kg⁻¹), cobalt (0.8 g kg⁻¹), selenium (0.35 g kg⁻¹).

Treatments

Treatments followed a 3×5 factorial arrangement in a completely randomized design, whereby three doses of SFO (0, 15, and 30 mg) were combined with five doses of PBP (0, 15, 30, 45, and 60 mg) on a dry matter (DM) basis. SFO composition (in g kg⁻¹ fatty acid) was as follows: 16:0 (51.07), 18:0 (27.36), *cis*-9 18:1 (355.43), 18:2n-6 (422.24), and 18:3n-3 (1.74). SFO and PBP were emulsified in a 1:99 v v⁻¹ 96% ethanol: aqueous solution, then decanted into a glass syringe. The glass syringe that contained 0 mg of PBP and 0 mg of SFO was designated as the control treatment. The PBP and SFO doses were selected based on prior studies and were considered both safe for the animals and practical for the farmers (Lourenço et al., 2014; Vargas et al., 2017; Purba; Paengkoum; Paengkoum, 2020).

In vitro experiment

After a 15-day adaptation period, rumen fluids were suctioned from the rumen using an oral lavage solution and a suction pump (CV-SF18, Hitachi, Tokyo, Japan) before morning feeding time (Tian et al., 2018). The pH of the rumen fluids were strictly checked to avoid contamination from goat saliva (Menke; Steingass, 1988). Rumen fluid was moved to the laboratory (14°52'36''N, 102°00'54''E; elevation above 200 m) in a pre-warmed thermal flask, then strained using a nylon membrane (400 µm; Fisher Scientific S.L., Madrid, Spain) while bubbled with CO_2. The artificial solution was prepared per an earlier protocol (Menke; Steingass, 1988) and mixed with strained rumen liquors (2:1, volume: volume) under continuous CO_2 at 39 °C. Every hundred glass syringes containing the earlier SFO and PBP as treatment were combined with 500 mg of basal substrate. For instance, the control treatment contained 500 mg of basal substrate, 0 mg of SFO, and 0 mg of PBP. We then added 30 mL of rumen mixture as a final preparation prior to incubation. Once the glass syringes were locked with 3-way stopcocks and capped by glass plungers, the glass syringes were subsequently shaken and placed in a water bath set at 39 °C. The incubation was run for 72 h, including shaking once per hour. To get representative results, all incubations were completed in 10 replications over three runs on separate days and gas production was corrected with blanks (three glass syringes that only contained rumen mixture were included in each run).

Sample analysis

DM was prepared (#950.02) and analyzed (#925.04) from 2.0 g of grinded sample after drying

using a forced-air oven at 105 °C for 4 h (AOAC, 2005). Organic matter (OM) content was calculated as a hundred percent minus ash percentage, which was obtained after incineration in a muffle furnace at 550 °C for 5 h (#942.05) (AOAC, 2005). Total N was measured using the Kjeldahl method and crude protein concentration was calculated as total N × 6.25 (#984.13), (AOAC, 2005). Ether extract concentration was measured by extraction with petroleum ether (#920.39) (AOAC, 2005) and fatty acid concentration was calculated from methylation using a gas chromatography machine (Agilent 7890A GC, Agilent Technologies) with external FAME standards (Supelco 37-Component FAME Mix, Supelco Inc., Bellefonte, PA) (Lourenço et al., 2014). Concentrations of acid detergent fiber and neutral detergent fiber were measured by sequential analysis without amylase (we used sodium sulfite instead) and expressed with residual ash included (Van Soest; Robertson; Lewis, 1991). 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. All measurements were performed in triplicate and chemical standards were included in each analytical run as appropriate (Table 1).

Gas production was read, recorded, and measured at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, and 72 h by adapting a prior gas pressure technique (Theodorou et al., 1994). To calculate the cumulative volume of gas production (Equaton 1), the numerical measurement was fitted to the model of Orskov and McDonald (1970) as:

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

where a (mL g^{-1} DM) is the gas production from the soluble fraction, b (mL g^{-1} DM) is the gas production from the insoluble fraction, c (h^{-1}) is the gas production rate constant for the insoluble fraction (b), t (h) is the incubation time, (a + b) (mL g^{-1} DM) is the potential extent of gas production, and y (mL g^{-1} DM) is the gas produced at time 't'.

At 24 and 72 h, 10 mL of gas was transferred into a disposal syringe for directly injecting into the gas chromatography machine (Agilent 7890A, Agilent Technologies) to measure methane and carbon dioxide levels. To obtain the acceptable separation, internal standards were applied into the peak rate for methane (FIDI A, front signal, 0.900–0.999 min) and carbon dioxide (TCD2 B, back signal, 0.924–0950 min). The methane, carbon dioxide, and cumulative gas production were expressed as mL/g of OM disappeared after 24 and 72 h of incubation (Ribeiro Junior et al., 2014).

After 24 h of incubation, rumen fluids were then filtered through four layers of cheesecloth. Once the glass syringes were unplugged, pH was immediately measured using a pH meter (Oakton 700, Cole-Parmer, Vernon Hills, IL). Samples were divided into 2 portions; the first portion was centrifuged at 6,000 × g for 15 min at 4 °C and the supernatant was stored at -20 °C before NH_3-N analysis using the micro-Kjeldahl methods (Kjeltec 8100, Hilleroed, Denmark) (AOAC, 2005) and volatile fatty acid (VFA) detection by gas chromatography (Agilent 6890 GC, Agilent Technologies) (Erwin; Marco; Emery, 1961). The second portion was fixed with 10% formalin solution in a sterilized 0.9% saline solution for calculating total bacteria, protozoa, and fungal zoospores using a Neubauer counting chamber. Subsequently, the fixed solution was diluted with autoclaved deionized water as a medium 100, 10, and 10 times and counted using 10 × 40, 10 × 40, and 10 × 10 ocular × objective for total bacteria, fungal zoospores, and protozoa, respectively (Galyean, 1989).

In vitro degradability was determined after 24 and 72 h of incubation following an earlier protocol (Tilley; Terry, 1963). The filtered contents were collected through pre-weighed Gooch crucibles and residual dry matter was calculated. The percent loss in weight was calculated and dedicated as the *in vitro* dry matter degradability. The dried feed sample and residue left from the above process was incinerated in a furnace at 550 °C for 5 h for determination of the *in vitro* organic matter degradability.

Statistical analysis

The data were analyzed in a 3 × 5 factorial arrangement in a completely randomized design using the PROC GLM procedure in SAS 9.4. Since the data from the three consecutive runs of incubations were comparable, the data were averaged and analyzed using the one-way analysis of variance. Multiple comparisons among SFO supplementations, PBP treatments, and interactions were performed using Tukey's honestly significant difference test (Kaps; Lamberson, 2004). Differences among means at p < 0.05 were accepted as representing statistically significant differences.

RESULTS AND DISCUSSION

The PBP used in this study contained roughly 52.24 g/kg DM and 11.51 g/kg DM of secondary metabolites, which accounted for flavonoids and aromatic/essential oils, respectively (Table 1). Among the present

secondary metabolites, quercetin and eugenol were major compounds. In the present study, we tested whether these two active plant metabolites interacted with SFO supplementations to modulate rumen fermentation and its biogas production. All outcomes were expected to reduce methane production and enhance the fermentation end-product. Therefore, we evaluated the effect of different levels of SFO supplementation with different doses of PBP.

Ruminal biogas and degradability

Increased PBP levels in different substrates (0, 15, 30 mg of SFO) did not show a reduction in gas production after 72 h of incubation (Figure 1). Adding PBP at 15–30 mg, either with or without SFO, increased both total gas production (including CO_2) from fermentation and organic matter degradability (Table 2). SFO did not affect those parameters. Exceedingly high doses of PBP (45–50 mg) were shown to have an opposite effect. SFO × PBP significantly ($p < 0.05$) affected methane production after 24 and 72 h of incubation. Methane production decreased when PBP was combined with 0 mg of SFO (up to 37%), 15 mg of SFO (up to 20%), and 30 mg of SFO (up to 16%).

The gas production trend of substrates treated by *Piper betle* L. powder (PBP). S1, control or no PBP content; S2, 15 mg PBP; S3, 30 mg PBP; S4, 45 mg PBP; and S5, 60 mg PBP. (a) No SFO supplementation; (b) Low SFO, 15 mg; (c) High SFO, 30 mg. Data reported as least-squares ± standard error of mean (N=30).

It has been previously reported that gases such as hydrogen (H_2), methane (CH_4), and carbon dioxide (CO_2) are produced as a result of substrate fermentation in the rumen (Wolin; 1979). Carbohydrates, proteins, and lipids of substrates are degraded by rumen microorganisms and shifted into microbial cells. In the present study, the lack of SFO effect on total gas production (including CO_2) from fermentation and degradability was consistent with a previous study by Vargas et al. (2017), who reported comparable results for estimated total gas production and degradability rate maintained in semi-continuous flow fermenters (RUSITEC) in rumen fluid from sheep. This notion was substantiated by the fact that increasing SFO supplementations in the substrate tended to maintain total gas production (including CO_2). Thus, it appears that SFO is a relatively unfermentable nutrient source. However, in the present study, increasing PBP, which contained flavonoids and their aromatic derivatives, enhanced the fermentable organic degradability in substrate fermentation, resulting in a greater total gas production (including CO_2). Notably, PBP is an abundant carbohydrate source (Table 1). It has been reported that plant polyphenols

richer in flavonoids could be degraded in nutrient fermentation, especially by carbohydrate-degrading bacteria (McSweeney et al., 2001). Given this information, the presence of PBP has been shown to increase ruminal biogases whereby the degraded PBP made a predominant contribution to additional fermentable nutrient sources, such as carbohydrates (Orskov; McDonald, 1970). The carbohydrate content of the feed substrate was identical at the onset of each treatment in the present study; thus, the surge in ruminal biogases and degradability rates were
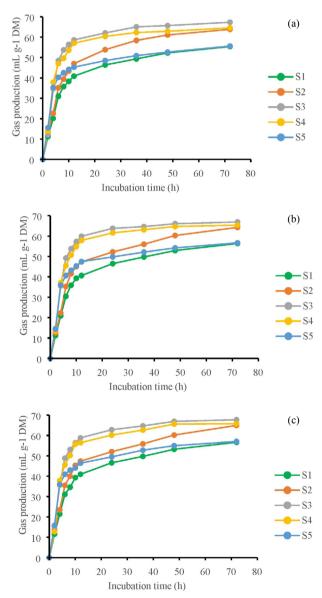

Figure 1: The trend of gas production for 72 h in a variety of diets.

Table 2: Effect of treatments on biogas production and *in vitro* degradability at 24 and 72 h after incubation.

SFO[1]	PBP[2]	Total gas production (mL g^{-1} OM)		CO$_2$ production (mL g^{-1} OM)		CH$_4$ production (mL g^{-1} OM)		*In vitro* dry matter degradability (%)		*In vitro* organic matter degradability (%)	
		24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
No	S1[3]	85.83e	177.76e	71.39d	140.67e	14.41ax	36.64ax	55.76	63.01	54.01d	60.23d
	S2	99.65c	205.64c	87.72b	181.09c	11.89bx	23.97bx	56.01	63.29	56.26c	62.61c
	S3	114.87a	226.62a	104.88a	207.31a	9.95cx	18.64cx	56.27	62.86	64.15a	67.72a
	S4	111.75b	218.89b	102.18a	198.58b	9.52cx	19.67cx	56.22	62.91	59.07b	65.46b
	S5	89.76d	182.08d	80.29c	162.42d	9.44cx	19.14cx	56.26	62.97	55.07d	60.33d
Low	S1	86.08e	179.65e	74.04d	153.29e	12.01bx	25.87bx	55.93	63.20	54.04d	60.15d
	S2	96.62c	203.35c	86.20b	180.70c	10.39by	22.07by	56.09	63.38	56.59c	62.04c
	S3	118.12a	229.20a	109.00a	210.28a	9.07dy	18.24cy	56.33	62.98	63.60a	66.52a
	S4	114.02b	222.58b	104.76a	202.63b	9.22dy	19.30cx	56.36	62.96	58.67b	64.71b
	S5	92.19d	186.30d	83.07c	166.83d	9.09dy	18.93cy	55.96	63.24	54.62d	60.51d
High	S1	86.24e	180.34e	75.89d	154.08e	10.32by	25.77bx	55.90	63.17	54.29d	60.23d
	S2	96.34c	204.20c	87.13b	182.93c	9.17dy	20.68bz	55.88	63.15	56.51c	62.18c
	S3	116.30a	228.76a	108.73a	211.82a	7.53ez	16.26dz	55.78	63.03	64.30a	66.42a
	S4	111.43b	220.63b	103.72a	203.38b	7.66ez	16.59dz	55.97	63.25	57.99b	63.23b
	S5	91.64d	186.38d	84.09c	169.56d	7.51ez	16.27dz	56.14	62.97	54.23d	60.38d
SEM[4]		0.318	0.514	0.344	0.600	0.048	0.136	0.051	0.040	0.096	0.069
P-values											
SFO		0.064	0.057	0.051	0.056	0.038	0.025	0.620	0.987	0.087	0.080
PBP		0.019	0.002	0.027	0.036	0.019	0.012	0.555	0.987	0.021	0.015
SFO × PBP		0.144	0.493	0.303	0.086	0.042	0.027	0.539	0.961	0.469	0.493

[1]SFO, sunflower oil supplementation per incubation at no (0 mg); low (15 mg); high (30 mg); [2]PBP, *Piper betle* L. powder supplementation per incubation at S1 (0 mg); S2 (15 mg); S3 (30 mg); S4 (45 mg); S5 (60 mg); [3]dedicated as control treatment (only substrate); [4]SEM, standard error of mean. Within a parameter as column, means with different superscript (a, b, c) differ at p < 0.05 for the PBP effect in substrate; with different superscripts (x, y, z) at p < 0.05 for the SFO effect in substrate.

expected from modulated carbohydrates and other glucose fermentations degraded by rumen microorganisms during substrate incubation. However, exceedingly high levels of PBP (> 10%) supplementation have shown an adverse effect that might increase toxicity for rumen inhabitants (Yanza et al., 2018).

Furthermore, the supplementation of SFO and PBP modulated ruminal fermentation and effectively reduced methane production. The extent of methane mitigation by SFO and PBP was equal to that reported by previous studies (Vargas et al., 2017; Yanza et al., 2018). This methane reduction might have occurred due to the flavonoids and essential oils of PBP. Previous references reported similar results that flavonoids and essential oils had a well-documented antimicrobial

effect in rumen methanogenesis through interfering with the outer membrane of gram-positive bacteria (Patra; Saxena, 2010; Canova et al., 2015; Purba; Yuangklang; Paengkoum, 2020). This motion caused bacteria to lose their control over ion gradients, electron mobilization, phosphorylation cascades, protein translocation, and other enzymatic reactions (Ultee; Bennik; Moezelaar, 2002). In the present study, the combination of SFO and PBP tended to cause a reduction in CH$_4$. To our knowledge, the present study is the first investigation of the effects of a combination of SFO and polyphenol on enteric methane production. However, a previous study combining SFO and microalgae could be assessed due to the polyphenolic properties ascertained in microalgae (Haoujar; Cacciola; Abrini, 2019). *Schizochytrium* microalgae and SFO

appeared to alleviate ruminal methane production in rumen fluid of steers and goats (Elghandour et al., 2017). It is worth highlighting that the varied responses to SFO and microalgae inclusions in goat rumen were expected due to the type and dose of the basal diet and lipid inclusion (Hook; Wright; McBride, 2010). Our results elaborated that the combination of SFO and PBP attenuated bacteria activity in the rumen, especially the methanogen group, in which flavonoids of PBP may compete with SFO to possess the target sites through their hydrophobic interaction. Antimicrobial properties of flavonoids and oils have been observed to understand the efficacy of their activity against varied bacteria (Mandalari et al., 2007). This notion was substantiated by the fact that increasing SFO supplementations with PBP levels led to augmented rumen methanogenesis activity.

Fermentation parameter

There were significant interactions ($p < 0.05$) between SFO and PBP for total VFA and the ratio of acetic to propionic acid and its acid fractions, except iso-acids (Table 3). Increased PBP levels in different substrates (0, 15, 30 mg of SFO) increased acetic acid without altering pH. Supplementing at least 45 mg of PBP, either alone or in combination with SFO, decreased ammonia-N ($p < 0.05$).

It has been previously reported that adding SFO (*ca.* 2% of total feed substrate) containing unsaturated fatty acids increased VFA concentration (Vargas et al., 2017). Furthermore, polyphenol utilization by adding doses of commercial quercetin and eugenol at 250–500 mg L^{-1} has shown inconsistent results (Lourenço et al., 2014). Quercetin, but not eugenol, resulted in a greater concentration of VFA. Given those reports, the increased

Table 3: Effect of treatments on pH, NH_3-N and total volatile fatty acid (TVFA).

SFO[1]	PBP[2]	pH	NH_3-N (mg (100 mL)$^{-1}$)	TVFA (mmol L^{-1})	Acetic acid (C_2) (%)	Propionic acid (C3) (%)	Iso-butyric acid (%)	Butyric acid (%)	Iso-valeric acid (%)	Valeric acid (%)	Ratio $C_2:C_3$
No	S1[3]	6.92	17.43a	68.36cx	56.27c	19.60ax	4.63	10.14c	4.95	4.41ax	2.87cx
	S2	6.83	17.69a	70.07bx	56.55c	19.63ax	4.19	11.26c	4.28	4.09bx	2.88cx
	S3	6.83	17.86a	71.82bx	58.89b	19.47ax	2.35	12.39b	3.51	3.39cx	3.02bx
	S4	6.83	18.02a	73.62by	59.72a	18.46bx	2.35	12.54a	3.53	3.40cx	3.24ax
	S5	6.83	16.24b	75.46ax	54.34c	18.44bx	3.79	12.69a	5.69	5.05az	2.95bx
Low	S1	6.83	17.46a	73.14by	54.84c	20.67ay	3.71	10.39c	5.55	4.84ay	2.65cy
	S2	6.83	17.02a	78.11ay	57.40b	20.61ay	2.45	12.22b	3.67	3.65by	2.79cx
	S3	6.83	17.19a	78.50ay	57.42b	20.34ay	2.10	14.20a	3.16	2.78cy	2.82cx
	S4	6.83	16.80b	78.60ay	57.84b	19.89by	2.37	12.90a	3.55	3.45cx	2.91by
	S5	6.83	16.55b	80.94az	54.94c	19.43by	3.52	12.44a	5.29	4.38ax	2.83by
High	S1	6.83	17.55a	78.02ay	54.87c	21.87az	3.07	10.41c	4.60	5.18az	2.51cz
	S2	6.83	17.52a	83.11az	62.70a	18.39bx	1.60	13.37a	2.41	1.53dy	3.41az
	S3	6.83	17.51a	83.60az	59.81a	17.82cz	2.29	13.41a	3.43	3.24cx	3.36az
	S4	6.83	15.89b	62.32cx	57.89b	17.75cz	3.07	11.49b	4.61	5.19az	3.29az
	S5	6.83	15.70b	59.10cx	55.00c	17.67cz	4.10	11.23c	6.17	5.83az	3.11ax
SEM[4]		0.007	0.184	0.184	0.061	0.319	0.234	0.313	0.028	0.290	0.007
P-values											
SFO		0.987	0.273	0.027	0.285	0.034	0.051	0.060	0.067	0.033	0.044
PBP		0.953	0.025	0.001	0.041	0.029	0.050	0.031	0.082	0.005	0.001
SFO × PBP		0.765	0.066	<0.001	0.217	0.022	0.056	0.022	0.112	0.012	0.004

[1]SFO, sunflower oil supplementation per incubation at no (0 mg); low (15 mg); high (30 mg); [2]PBP, *Piper betle* L. powder supplementation per incubation at S1 (0 mg); S2 (15 mg); S3 (30 mg); S4 (45 mg); S5 (60 mg); [3]dedicated as control treatment (only substrate); [4]SEM, standard error of mean. Within a parameter as column, means with different superscript (a, b, c) differ at p < 0.05 for the PBP effect in substrate; with different superscripts (x, y, z) at p < 0.05 for the SFO effect in substrate.

total VFA in the present study might be due to the content of unsaturated fatty acids and carbohydrate sources in SFO and PBP, respectively. However, a previous meta-analysis reported that additional oil in a diet did not modulate rumen NDF degradability and displayed a negative effect, e.g. reducing dry matter intake (Weld; Armentano, 2017). It is worth noting that constituent nutrients of PBP may be more easily degraded than SFO by rumen inhabitants, thereafter boosting fermentation to produce more VFA (McSweeney et al., 2001). However, taken altogether, the present results were similar to prior studies both observed *in vitro* (Elghandour et al., 2017; Vargas et al., 2017) and *in vivo* (Atikah et al., 2018). Presumably, SFO might still affect the total volatile fermentation by modulating fiber-degrading bacteria in the rumen.

In the present study, SFO increased propionic acid, which was in agreement with a prior study (Vargas et al., 2017). However, PBP seemed to have the reverse effect on the propionate shift by systematically altering ruminal metabolism. This suggested that active plant compounds of PBP might have a broad effect on the breakdown in the activity of propionate-producing bacteria during nutrient metabolism via a propionate-generating pathway intermediate. Consequently, this deterioration amounted to less degradation of fermentable cellulose, which was concomitant of a reduced propionic fraction. A lower propionic number may be linked to a lower concentration of valeric acid as well (Andries et al., 1987).

Furthermore, our results addressed the greater concentrations of acetic and butyric acids that may have occurred because of the quercetin and eugenol in PBP. However, these findings contradicted prior studies (Castillejos; Calsamiglia; Ferret, 2006; Oskoueian; Abdullah; Oskoueian, 2013). A possible reason for these different outcomes could be to the interaction between the basal substrate used and the available plant secondary metabolite. For example, Oskoueian, Abdullah and Oskoueian (2013) and Castillejos, Calsamiglia and Ferret (2006) fed the fermenters a 60:40 forage: concentrate diet, whereas in our study, a 50:50 Pangola hay: concentrate diet was used. In addition, those reports used a commercial extract to obtain quercetin and eugenol. These differences in concentrations of acetic and butyric acids could be due to the varied molecular weight content of the plant secondary metabolite used. Hence, response-dependent purity has been suggested to affect substrate fermentation (Petlum; Paengkoum; Liang, 2019). In the present study, organic compounds such quercetin and eugenol might still bind other natural compounds such as phenolic acids (Purba; Paengkoum 2019). Therefore, quercetin and eugenol still

allowed ruminal microorganisms to produce a wide extent of acetic and butyric fractions.

Supplementation of PBP decreased ammonia deposition in the present study. PBP seemed to affect the deamination process through an ammonia-inhibiting feature exhibiting slow affinity due to high amounts of PBP (at least 45–50 mg) supplementations. This result was consistent with other studies involving dietary quercetin (Oskoueian; Abdullah; Oskoueian, 2013) and eugenol (Castillejos; Calsamiglia; Ferret, 2006). Given those references, the reduction in ammonia might be due to eugenol. Eugenol seemed to have stronger antimicrobial properties compared to the other secondary metabolites in inhibiting protein degradability in the rumen (Castillejos; Calsamiglia; Ferret, 2006). Eugenol supplementation dosed to at least 50 mg L^{-1} in the fermenter decreased the ammonia concentration without changing the pH (Castillejos; Calsamiglia; Ferret, 2006). In the present study, the ranges of ammonia concentration and pH were 15.7–18.0 mg 100 mL^{-1} and 6.8–6.9, respectively, which represented the acceptable conditions for ruminal microorganisms to survive for modulating microbial growth and fermentation efficiency (Ørskov; MacLeod, 1982).

Microbial composition

Total bacteria were similar among the treatments (Table 4). Significant effects ($p < 0.05$) of SFO × PBP were observed for rumen protozoa and fungal zoospores ($p < 0.01$). It has been reported that a change of ruminal biogases and fermentation end-products in the rumen might indicate a shift in the ruminal microorganisms (Ungerfeld; Aedo; Martínez, 2019). In the present study, SFO and PBP disturbed ruminal protozoa activity and suppressed ruminal protozoa numbers. It has been previously reported that plants richer in polyphenols, especially tannins, flavonoids, and essential oils, reduced the protozoa community in rumens (Castillejos; Calsamiglia; Ferret, 2006; Oskoueian; Abdullah; Oskoueian, 2013; Cherdthong et al., 2019). In the present study, flavonoids and essential oils present in PBP, either with or without SFO, seemed to have a wider antimicrobial effect, leading to direct deteriorations of protozoa by undertaking cell wall synthesis or nucleic acid synthesis (Cherdthong et al., 2019). Moreover, the decrease in the protozoa did not affect the total number of rumen bacteria. Among flavonoids and essential oils in PBP, quercetin was the predominant active compound (Table 1). This might relate to an earlier report which indicated that supplementing flavonoids like quercetin did not vary the total bacteria in the rumen (Oskoueian;

Abdullah; Oskoueian, 2013). Quercetin has been suggested to have control in maintaining cellulolytic bacteria with lower protozoa and methanogen populations. Although, a possible reason for the escalated fungal zoospores may be related to the altered number of rumen protozoa. Newbold et al. (2015) reported that fungal zoospores slightly increased with a slightly decreased protozoal number, which reflected small competition between fungal zoospores and protozoa regarding consumption of the substrate during fermentation. To support this assessment, fungal zoospores have been reported to have

a strong defense towards interruption by plant-containing polyphenols (Cherdthong et al., 2019). Therefore, fungal zoospores could be assessed as the remaining microorganisms that ingest the ruminal substrate during fermentation.

CONCLUSIONS

This study suggested that flavonoids and their aromatic derivatives obtained from the edible plant *Piper betle* L. dosed at 15–30 mg per incubation on a dry matter basis promotes *in vitro* methane mitigation while improving rumen fermentation. Incorporating *Piper betle* powder with sunflower oil as a dietary fat also showed a positive interaction. These findings might be useful for application in ruminant diets, as they are expected to reduce the existential greenhouse gas emissions from livestock. Furthermore, providing *Piper betle* powder could contribute to limiting agricultural waste.

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Table 4: Effect of treatments on ruminal microorganism.

SFO[1]	PBP[2]	Ruminal microbes (cells mL^{-1})		
		Total bacteria ($\times 10^7$)	Total protozoal ($\times 10^5$)	Total fungal zoospore ($\times 10^5$)
No	S1[3]	6.15x	4.78ax	2.93cx
	S2	6.14x	4.50ax	3.06cx
	S3	6.12x	4.21bx	3.10bx
	S4	6.10x	3.98bx	3.14bx
	S5	6.08x	3.74cx	3.21ax
Low	S1	8.20y	4.19ay	3.15by
	S2	7.28y	3.93by	3.25by
	S3	7.10y	3.69by	3.38ay
	S4	7.26y	3.47cy	3.44ay
	S5	8.15y	3.24cz	3.56ay
High	S1	9.30z	3.71az	3.66bz
	S2	9.18z	3.34cz	3.88bz
	S3	8.89z	3.27cz	4.12az
	S4	8.95z	3.08dz	4.40az
	S5	9.58z	2.89ez	4.69az
SEM[4]		0.344	0.139	0.141
P-values				
SFO		0.016	0.037	0.030
PBP		0.092	0.001	0.022
SFO × PBP		0.051	0.002	0.007

[1]SFO, sunflower oil supplementation per incubation at no (0 mg); low (15 mg); high (30 mg); [2]PBP, *Piper betle* L. powder supplementation per incubation at S1 (0 mg); S2 (15 mg); S3 (30 mg); S4 (45 mg); S5 (60 mg); [3]dedicated as control treatment (only substrate); [4]SEM, standard error of mean. Within a parameter as column, means with different superscript (a, b, c) differ at $p < 0.05$ for the PBP effect in substrate; with different superscripts (x, y, z) at $p < 0.05$ for the SFO effect in substrate.

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