INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20–0873/2021/25–1–231–240
DOI: 10.17957/IJAB/15.1661
http://www.fspublishers.org

Full Length Article

Piper Oil Decreases *In Vitro* Methane Production with Shifting Ruminal Fermentation in a Variety of Diets

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Received 12 June 2020; Accepted 26 September 2020; Published 10 December 2020

Abstract

The objective of this study was to investigate the effect of piper oil (PO), alone or in combination with sunflower oil (SFO), on biogas production, fermentation end-products and microorganism in the rumen of lactating goats using *in vitro* fermentation technique. Basal substrate consisted of pangola hay and concentrate (50:50), which was modified with the experimental treatments. The treatments were organized as a completely randomized 3 × 5 factorial arrangement, whereby 0 (no), 15 (low) and 30 mg (high) SFO were combined with 0, 15, 30, 45 and 60 mg PO on a dry matter basis. Generally, gas accumulation was not affected by supplementation with SFO treated with 0–30 mg PO. However, provision of exceedingly high PO dose at 60 mg decreased gas accumulation. These PO influences were a consistent picture at CO_2 yield, system digestibility and total volatile fatty acid (VFA). Noteworthy, an inclusion of 45 mg PO did not affect total VFA in presence of no, low and high SFO. A significant reduction in CH_4 production was observed when 45–60 mg PO was combined with no SFO (up to 32.1%), low SFO (up to 33.3%) and (up to 33.9%) compared to respective controls. Rumen protozoa were seen to gradually decrease in the presence of SFO and PO. Total bacterial and fungal zoospores varied in numbers following different PO supplementations. SFO and PO supplementations did not change pH, but lowered ammonia levels compared to respective controls. The results of the present study demonstrate that PO (especially at an inclusion of 45 mg) is as effective as other methane mitigation agents such as SFO, in reducing emissions, without negatively impacting rumen fermentation. © 2021 Friends Science Publishers

Keyword: Biogas; Dairy goat; Eugenol; Environmental pollution; Rumen

Introduction

Domestic ruminants are the one of the major contributors to the release of greenhouse gases, so there is mounting interest in reducing such emissions. Enteric fermentation in ruminants contributes to about 18% of CH_4 and 9% of CO_2 global emissions (FAO 2006) and consumes 2–12% of total gross energy intake by these animals (Johnson and Johnson 1995). These values make ruminants highly inefficient and environmentally unfriendly. Vegetable oil, microalgae, organic acids, yeast, and tannin-saponin have been widely used to reduce gas emissions by ruminants (Polyorach *et al.* 2014; Elghandour *et al.* 2017; Naumann *et al.* 2017).

While reducing enteric CH_4 emission, dietary supplementation with vegetable oil such as sunflower oil (SFO) provides documented benefits by improving the lifespan-extending bacterial, especially cellulolytic bacteria (Gao *et al.* 2016). SFO richer in unsaturated fatty acid content inhibits the growth of ruminal ciliate protozoa and interfere engulf activity of bacteria by ruminal protozoa. Dietary SFO, thus, increases total rumen biomass and eventually surpasses rumen fermentation (Gao *et al.* 2016). This activity of oil inclusion in both reducing CH_4 production and providing additional nutrients, therefore, has been suggested as a cost-effective (Beauchemin and McGinn 2006).

Recent strategy by dietary essential oils have shown potential to decrease CH_4 emissions and can alter rumen properties; however, there are self-imposed restrictions such as inconsistency, impermanent and adverse effects in their application as feed additives for ruminants (Newbold and Ramos-Morales 2020). Hence, essential oils that are selected should have a positive impact, at least on fermentation end-products (Benchaar *et al.* 2008). One such

To cite this paper: Purba RAP, C Yuangklang, S Paengkoum, P Paengkoum (2021). Piper oil decreases *In vitro* methane production with shifting ruminal fermentation in a variety of diets. *Intl J Agric Biol* 25:231–240

essential oil component is eugenol (Castillejos *et al.* 2008); however, its effect takes time to be detected and its efficiency depends on primary substrate, dose and incubation (Castillejos *et al.* 2006). Piper oil (PO) is refined essential oil extracted and isolated from *Piper betle* L. leaves using water-steam distillations and PO may have a direct affection on enteric CH_4 emission and ruminal fermentation shift. Eugenol was detected in essential oil of all of the *P. betle* L. varieties at the highest concentration value followed by caryophyllene, safrole and chavicol (Karak *et al.* 2018; Islam *et al.* 2020).

Although dietary vegetable oil and essential oil seem to alleviate enteric CH_4 emission and to achieve the environmentally friendly activities, optimization of their use as a methane inhibitor in ruminant diets is necessary to be investigated, alone and combination (Newbold and Ramos-Morales 2020). For instance, essential oils were added at high dose and this supplementation had deleterious effects on efficiency of rumen fermentation, palatability and possibly cause toxicity (Benchaar and Greathead 2011). Nevertheless, negative effects can be evaded at a lower dose, but the methane mitigation would be dwindled as well (Patra and Yu 2015). A combination of a low amount of methane inhibitors, either using SFO or organic compound of *P. betle* L. leaves, has been reported to reduce enteric methane production and had only a small influence on feed degradation (Purba *et al.* 2020b, c). At present, however, it is not known whether supplementation with PO and SFO has a synergistic effect and can improve animal performance. We postulated that PO could shift the ruminal fermentation pathway. Therefore, the objective of this study was to investigate the effect of PO at five different doses, combined or not with SFO, on biogas release, fermentation end-products, and microbial composition in rumen fluids from lactating goats, as estimated by *in vitro* techniques.

Materials 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).

Substrate, piper oil and treatment

A standard total mixed ration (TMR) commonly fed to ruminant livestock in Thailand consisted of pangola hay (*Digitaria eriantha*) and concentrate (50: 50) was dedicated as basal substrate (Table 1). To obtain piper oil (PO), *P. betle* L. leaves were bought from the local market in Prachinburi in eastern Thailand, collected, dusted and placed into a Clevenger apparatus together with deionized water at a 1:4 ratio and incubated for 2 h. Steam distillation products were rinsed, separated and collected using hexane. Hexane was completely removed using a Rotavapor (R-300; Büchi, Switzerland). To quantify the content of eugenol, 20 μL PO was injected into a 1260 Infinity instrument

Table 1: Ingredients and chemical composition of basal diet (g/kg DM, otherwise stated)

Item	Basal diet
Ingredients	
Pangola Hay	500
Cassava chip	30
Cassava pulp	192
Mineral[1]	8
Molasses	40
Palm meal	130
Premix[1]	2
Rice bran	48
Soybean meal	40
Sulfur	1
Urea	9
Chemical composition	
Organic matter	941
Crude protein	108
Ether extract	24
Neutral detergent fibre	685
Acid detergent fibre	595
Fatty acid (FA) composition (in g/100 g FA)	
C16:0	5.12
C18:0	0.26
C18:2n-6	4.95
C18:3n-3	0.07

[1]Mineral and premix uses a similar commercial product as given by Purba *et al.* (2020b).

(Agilent Technologies, USA) for high-performance liquid chromatography (HPLC) with diode-array detection and mobile phase consisting of 1:9 HPLC-grade acetonitrile: acetic acid (1%) (Purba and Paengkoum 2019). Separation was achieved by a reversed-phase Zorbax SB-C18 column (3.5-μm particle size, i.d. 4.6 mm × 250 mm). A standard stock solution was prepared using commercial eugenol (Sigma-Aldrich, USA). Data collection was performed using OpenLAB CDS v. 1.8.1 (Agilent Technologies). All measurements were performed in triplicate and chemical standards were included in each analytical run as appropriate. Eugenol content in PO was estimated at 20 g/kg DM.

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 PO (0, 15, 30, 45 and 60 mg) on a dry matter (DM) basis. SFO composition (in g/kg 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 PO emulsified in a 1:99 v/v 96% ethanol: aqueous solution, then decanted into a glass syringe. The glass syringes that contained 0 mg of PO in presence of three doses of SFO (0, 15, and 30 mg) were designated as the respective control treatment. Selected doses of SFO and PO in present study were based on the summary of prior studies (Calsamiglia *et al.* 2007; Elghandour *et al.* 2017; Purba *et al.* 2020a, b).

In vitro incubation

Rumen fluids were collected from four lactating Saanen goats (body weight, 41 ± 1.37 kg) *via* oral lavage using a suction pump (CV-SF18; Hitachi, Japan) before morning

feeding time (Tian *et al.* 2018) and following a 15-day adaptation period on the TMR (basal substrate). All preparation and *in vitro* gas production measurements were performed according to the protocol by Menke and Steingass (1988), as modified by Paengkoum (2019), and were conducted in Nakhon Ratchasima, Thailand (14°52'36''N, 102°00'54''E; elevation above 200 m). Briefly, collected rumen fluid was kept in a pre-warmed thermal flask, then strained using a nylon membrane (400 µm; Fisher Scientific S.L., Madrid, Spain) into a conical flask, and mixed with salivary buffer (1:2, mL: mL) under CO_2 and kept at 39°C. 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 and Steingass 1988). Each hundred Hohenheim glass syringes containing the prior SFO and PO treatment combinations were added to 500 mg of basal substrate. For example, the control treatment contained 500 mg of basal substrate, 0 mg of SFO and 0 mg of PO. The glass syringes were then added 30 mL of rumen fluid buffer mixture as a final preparation prior to incubation. Once the glass syringes were locked with three-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, with shaking once per hour. All incubations were completed in ten replications and three runs on separate days, and gas production was corrected for every run with three blanks containing rumen mixture only. Gas production was read after 0, 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h. To calculate the cumulative volume of gas production, the measured value was fitted to the model of Orskov and Mcdonald (1970):

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

Where a (mL/g DM) is gas production from the soluble fraction, b is 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) is the potential gas production, and y is the gas produced at time 't' (mL/g DM).

Laboratory analysis and sampling

DM was prepared (#950.02; AOAC) and analyzed (#925.04; AOAC) from 2.0 g of ground sample after drying in a forced-air oven at 105°C for 4 h (AOAC 2005). Organic matter content was calculated as OM = 100% - ash %; the latter was obtained after incineration in a muffle furnace at 550°C for 5 h (#942.05; AOAC) (AOAC 2005). Total N was measured using the Kjeldahl method and crude protein concentration was calculated as total N × 6.25 (#984.13; AOAC) (AOAC 2005). Ether extract concentration was measured by extraction with petroleum ether (#920.39; AOAC) (AOAC 2005) and fatty acid concentration was calculated from methylation using a gas chromatographer (7890A; Agilent Technologies, USA), with external standards (Supelco 37-Component FAME Mix; Supelco Inc., USA) (Weirdt *et al.* 2013). Concentrations of acid-detergent fiber and neutral-detergent fiber were measured by sequential analysis without amylase (substituted by sodium sulfite) and were expressed by excluding residual ash (Soest *et al.* 1991). Gross energy was determined using a Parr 6200 bomb calorimeter with O_2 as carrier gas (Parr Instruments Co., USA) according to the manufacturer's instructions. All measurements were performed in triplicate and chemical standards were included in each analytical run as appropriate.

Gas production at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, and 72 h was directly read using a pressure transducer and a calibrated syringe as specified by Theodorou *et al.* (1994). Each run of the *in vitro* incubation contained 10 replicated glass syringes. Five replicated glass syringes were used for sample analysis at 24 h and five glass syringes were used for sample analysis after 72 h of incubation. At 24 and 72 h, 10 mL of the gas collected from two glass syringes was dispatched into the gas chromatographer to measure CH_4 and CO_2 levels (mL/g DM). When glass syringes were unplugged, the pH was immediately measured using a pH meter (pH 700; Oakton, USA). The rumen fluid was filtered and rumen content was collected through pre-weighed Gooch crucibles and residual DM was estimated. The percent loss in weight was calculated and *in vitro* DM degradability (IVDMD) was derived. The dried feed sample and remaining residue from above were incinerated in a furnace at 550°C for 5 h to determine *in vitro* OM degradability. Finally, IVDMD samples were observed following the neutral-detergent fiber protocol (Frutos *et al.* 2004) to measure *in vitro* true substrate digestibility.

After 24 h, rumen fluids of remaining glass syringes were filtered through four layers of cheesecloth. Once the glass syringes were unplugged, pH was immediately measured using a pH meter as above. Samples were divided into two aliquots. The first aliquot was centrifuged at 6,000 × g at 4°C for 15 min, and the supernatant was stored at -20°C before NH_3-N analysis using the micro-Kjeldahl method (8100; Foss Kjeltech, USA) (AOAC 2005) and volatile fatty acids (VFA) detection by gas chromatography (HP 6890; Hewlett Packard, USA) (Erwin *et al.* 1961). The second aliquot was prepared and fixed with 10% formalin solution in a sterilized 0.9% saline solution to assess microorganism numbers in a counting chamber (Neubauer-Boeco, Germany). Specifically, the fixed solution was diluted 100 ×, 10 ×, and 10 × with autoclaved deionized water to count total bacteria, fungal zoospores, and protozoa using 10 × 40, 10 × 40, and 10 × 10 (ocular × objective) magnification, respectively (Galyean 1989). The dilution and magnification settings for quantifying microbial composition were different due to varying sizes of bacteria, fungal zoospore and protozoa.

Statistical analysis

Due to outcomes in consecutively runs was similar (we tested in preliminary statistical tabulation; not significant different, $P < 0.05$), data were averaged and subjected to analysis of variance. All data were analyzed as a 3×5 factorial arrangement in a completely randomized design using the PROC GLM of S.A.S. 9.4 software (S.A.S. Institute Inc., 2015, USA). Data were analyzed using the model:

$$Y_{ij} = \mu + A_i + B_j + AB_{ij} + €_{ij}$$

where: Y = observations; μ = overall mean; A^i = effect of factor A (SFO supplementation, i = 1 to 3); B^j = effect of factor B (level of PO, j = 1 to 5), AB_{ij} = interaction between factor A and B, and $€_{ij}$ = the residual effect. Multiple comparisons among SFO supplementation, PO treatment and combination of SFO and PO were assessed using Tukey's honestly significant difference (Kaps and Lamberson 2004). Differences among means were considered statistically significant at $P < 0.05$. The trend of differences in CO_2 yield and CH_4 production were assessed by orthogonal contrast ($P < 0.05$).

Results

Effect of substrate supplemented with or without sunflower oil (SFO) treated by piper oil (PO) on gas cumulative, *in vitro* degradability and *in vitro* true substrate digestibility at 24 and 72 h after incubation is presented in Table 2. In general, gas accumulation was not affected by supplementation with SFO treated with 0–30 mg PO during 72 h of incubation (Fig. 1). However, provision of exceedingly PO dose at 45–60 mg decreased gas accumulation ($P < 0.001$). Substrate supplemented with or without SFO with 0–30 mg PO maintained system degradability and digestibility, but those showed a downward trend ($P < 0.05$) at 45–60 mg PO in all substrates; In addition, providing PO produced a consistent picture at different incubated times. No interaction was found on all parameters in Table 2 ($P > 0.05$).

The effect of treatments on CO_2 is shown in Fig. 2a and can be summarized by no apparent change in CO_2 yield after 24 and 72 h of incubation ($P > 0.05$). A significant drop in CO_2 was observed only with 45–60 mg PO in all substrates 24 and 72 h of incubation ($P < 0.05$). As shown in Fig. 2b, a significant reduction ($P < 0.001$) in CH_4 production was observed at 24 h of incubation with 45–60 mg PO in the presence of no SFO (up to 36.0%), low SFO (up to 38.3%) and (up to 39.8%) compared to respective controls; Furthermore, a significant reduction ($P < 0.001$) in CH_4 production was obtained in similar numbers at terminated incubation with 45–60 mg PO in all substrates (up to 28.0% in all cases) compared to respective controls. Collectively, PO alleviated CH_4 production during substrate 72 h of incubation that was at no SFO (up to 32.1%), low SFO (up to 33.3%) and (up to 33.9%) compared to respective controls.

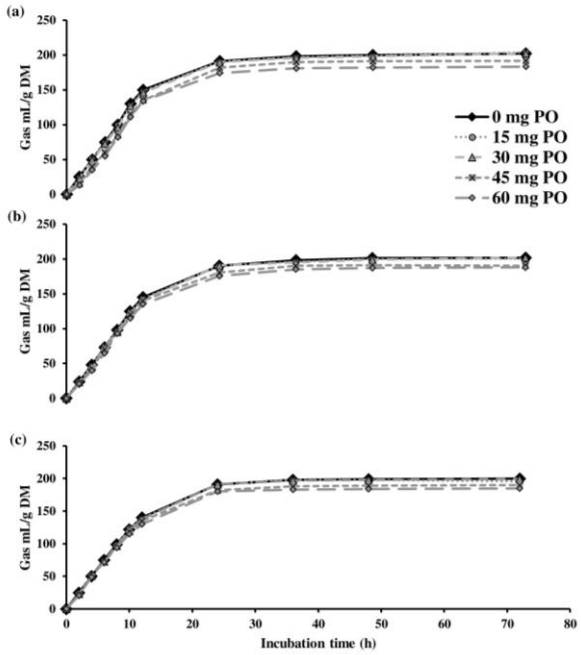

Fig. 1: The cumulative gas production trend of substrates supplemented with or without sunflower oil (SFO) treated piper oil (PO). (**a**) No SFO, 0 mg; (**b**) Low SFO, 15 mg; (**c**) High SFO, 30 mg. Data reported as least-squares ± a standard error of mean (N=30)

Effect of substrate supplemented with or without SFO treated by PO on *in vitro* volatile fatty acid (VFA) is presented in Table 3. SFO increased total VFA ($P = 0.001$). Total VFA remained unchanged after 0–30 mg PO added in substrate ($P > 0.05$). However, a significant decrease in total VFA was observed only with 45–60 mg PO in all substrates supplemented with no, low and high SFO ($P < 0.001$). There was interaction between SFO supplementation and PO dose for ratio of acetate to propionate and propionate fraction as well ($P < 0.001$). PO increased acetate ($P < 0.001$), but the trend was reversed when 45–60 mg PO were added in all substrates. Butyric acid was generally more abundant compared to each respective control after treated with PO ($P < 0.001$) and did not change branched-chain fatty acids (iso fraction of butyric and valeric acids).

SFO and PO supplementation did not generally alter the pH ($P > 0.05$); however, ammonia gradually decreased in conjunction with SFO and PO ($P < 0.05$) towards the respective controls (Table 4). Likewise, composition of the ruminal microbial community was altered by the presence of SFO and PO. SFO modulated total bacteria ($P = 0.029$). While total bacteria remained constant in number after supplementation with SFO and PO ($P > 0.05$), their amount dropped substantially ($P = 0.003$) after treated with PO at 60 mg per DM (Table 4). There was interaction between SFO supplementation and PO dose for protozoa and fungal zoospore ($P < 0.05$). The presence of SFO and PO slightly

Table 2: Effect of substrate supplemented with or without sunflower oil (SFO) treated by piper oil (PO) on gas cumulative, *in vitro* degradability and *in vitro* true substrate digestibility at 24 and 72 h after incubation

Parameter	Time (h)	PO (mg)	Supplementation of SFO[1]			SEM[2]	Comparison		
			No	Low	High		SFO	PO	Interaction
Gas cumulative (mL/g DM)	24	0	190.9^{a}	190.4^{a}	190.9^{a}	0.844	0.177	< 0.001	0.077
		15	190.6^{a}	190.5^{a}	190.9^{a}				
		30	190.0^{a}	190.6^{a}	191.1^{a}				
		45	181.9^{b}	180.4^{b}	181.9^{b}				
		60	174.1^{c}	175.6^{c}	180.1^{c}				
	72	0	202.0^{a}	201.5^{a}	199.8^{a}	0.890	0.063	< 0.001	0.370
		15	204.0^{a}	200.5^{a}	195.5^{a}				
		30	202.4^{a}	201.9^{a}	199.4^{a}				
		45	191.7^{b}	190.2^{b}	189.7^{b}				
		60	183.3^{c}	187.8^{c}	184.8^{c}				
In vitro dry matter degradability (g/100 g DM)	24	0	45.4^{a}	45.5^{a}	45.4^{a}	0.205	0.719	0.010	0.997
		15	45.3^{a}	45.4^{a}	45.6^{a}				
		30	45.3^{a}	45.5^{a}	45.5^{a}				
		45	45.1^{a}	45.0^{a}	45.2^{a}				
		60	44.3^{b}	44.5^{b}	44.5^{b}				
	72	0	48.2^{a}	48.5^{a}	48.3^{a}	0.219	0.612	0.020	0.524
		15	49.0^{a}	48.3^{a}	48.3^{a}				
		30	48.8^{a}	48.5^{a}	48.3^{a}				
		45	48.1^{a}	48.2^{a}	48.2^{a}				
		60	47.6^{b}	47.6^{b}	47.6^{b}				
In vitro organic matter degradability (g/100 g DM)	24	0	52.5^{a}	52.4^{a}	52.5^{a}	0.236	0.961	0.001	0.482
		15	52.3^{a}	52.8^{a}	52.6^{a}				
		30	52.4^{a}	52.5^{a}	52.7^{a}				
		45	51.9^{b}	51.1^{b}	51.4^{b}				
		60	50.2^{c}	50.1^{c}	50.3^{c}				
	72	0	59.5^{a}	59.1^{a}	59.4^{a}	0.267	0.738	0.002	0.818
		15	59.3^{a}	59.5^{a}	59.3^{a}				
		30	59.1^{a}	59.3^{a}	59.2^{a}				
		45	58.3^{b}	57.9^{b}	58.7^{b}				
		60	57.5^{c}	57.2^{c}	57.4^{c}				
In vitro true substrate digestibility (g/100 g DM)	24	0	46.4^{a}	46.5^{a}	46.6^{a}	0.209	0.161	< 0.001	0.665
		15	46.1^{a}	46.4^{a}	46.6^{a}				
		30	45.8^{a}	46.5^{a}	46.5^{a}				
		45	45.6^{a}	46.0^{a}	46.4^{a}				
		60	44.2^{b}	44.1^{b}	44.2^{b}				
	72	0	50.3^{a}	50.5^{a}	50.3^{a}	0.228	0.919	0.007	0.129
		15	50.6^{a}	50.3^{a}	50.3^{a}				
		30	50.5^{a}	50.5^{a}	50.3^{a}				
		45	50.3^{a}	50.2^{a}	50.2^{a}				
		60	49.1^{b}	49.2^{b}	49.1^{b}				

[1] SFO, sunflower oil supplementation per incubation at no (0 mg), low (15 mg), and high (30 mg). Supplementation of SFO treated with 0 mg PO is dedicated as control treatment: [2] SEM – standard error of mean: Means followed by different letters are significantly different at $P < 0.05$

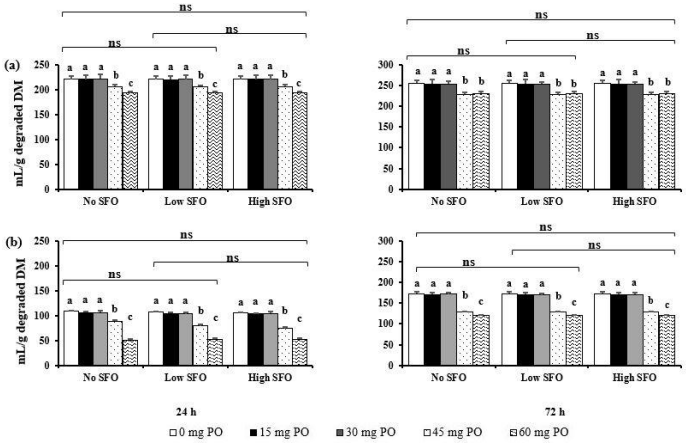

Fig. 2: The carbon dioxide yield (Fig. 2a) and methane production (Fig. 2b) of substrates treated by piper oil (PO) after 24 and 72 h incubation, with different superscript compared to similar time, meaning significantly different ($P < 0.05$; Tukey HSD). Differences among main effects of substrates were performed by Orthogonal contrast ($P < 0.05$) with $P > 0.05$ (ns). Data reported as least-squares ± standard error of mean (N=21)

Table 3: Effect of substrate supplemented with or without sunflower oil (SFO) treated by piper oil (PO) on *in vitro* volatile fatty acid (VFA)

Parameter	PO (mg)	Supplementation of SFO[1]			SEM[2]	Comparison		
		No	Low	High		SFO	PO	Interaction
Total VFA (mmol/L)	0	66.6^{c}	71.9^{b}	76.7^{a}	0.324	0.001	< 0.001	0.222
	15	66.8^{c}	72.0^{b}	76.9^{a}				
	30	67.1^{c}	72.2^{b}	77.1^{a}				
	45	67.1^{c}	72.1^{b}	77.0^{a}				
	60	63.2^{d}	66.5^{c}	72.4^{b}				
Acetate (mol/ 100 mol)	0	54.8^{c}	54.8^{c}	54.8^{c}	0.261	0.372	< 0.001	0.318
	15	57.5^{b}	58.4^{b}	59.7^{b}				
	30	58.9^{a}	59.4^{a}	59.8^{a}				
	45	59.7^{b}	57.8^{b}	56.9^{b}				
	60	57.3^{c}	56.1^{c}	56.0^{c}				
Propionate (mol/ 100 mol)	0	20.7^{Ra}	21.7^{Qa}	22.8^{Pa}	0.088	0.012	< 0.001	< 0.001
	15	18.7^{Qc}	18.7^{Qc}	19.4^{Pb}				
	30	18.5^{Rc}	18.6^{Qc}	18.8^{Pc}				
	45	18.5^{Rc}	18.5^{Rc}	18.7^{Qc}				
	60	18.4^{Rc}	18.5^{Rc}	18.6^{Qc}				
Isobutyrate (mol/ 100 mol)	0	4.5	4.2	4.0	0.019	0.395	0.072	0.105
	15	4.4	4.3	4.1				
	30	4.4	4.2	4.2				
	45	4.1	4.1	4.1				
	60	4.4	4.3	4.1				
Butyrate (mol/ 100 mol)	0	11.0^{c}	10.9^{c}	10.9^{c}	0.052	0.243	< 0.001	0.057
	15	11.5^{b}	11.3^{b}	11.6^{b}				
	30	11.5^{b}	11.6^{b}	11.7^{b}				
	45	11.2^{b}	11.2^{b}	11.1^{b}				
	60	12.1^{a}	12.4^{a}	12.3^{a}				
Isovalerate (mol/ 100 mol)	0	3.3	3.3	3.2	0.016	0.352	0.800	0.997
	15	3.2	3.4	3.0				
	30	3.2	3.3	3.0				
	45	3.1	3.3	3.2				
	60	3.2	3.4	3.1				
Valerate (mol/ 100 mol)	0	5.7^{a}	5.1^{a}	4.2^{b}	0.021	0.026	< 0.001	0.071
	15	4.7^{a}	3.9^{b}	2.2^{c}				
	30	3.4^{c}	2.8^{c}	2.4^{c}				
	45	3.4^{c}	5.1^{a}	5.9^{a}				
	60	4.6^{b}	5.3^{a}	5.8^{a}				
Acetate:Propionate	0	2.6^{Pc}	2.5^{Qc}	2.4^{Rc}	0.014	0.014	< 0.001	<0.001
	15	3.1^{Qa}	3.1^{Qa}	3.1^{Qa}				
	30	3.2^{Pa}	3.2^{Pa}	3.2^{Pa}				
	45	3.2^{Pa}	3.1^{Qa}	3.0^{Ra}				
	60	3.1^{Qa}	3.0^{Ra}	3.0^{Ra}				

[1] SFO, sunflower oil supplementation per incubation at no (0 mg), low (15 mg), and high (30 mg). Supplementation of SFO treated with 0 mg PO is dedicated as control treatment
[2] SEM – standard error of mean
Means followed by different superscript (a, b, c) differ at $P < 0.05$ for the PO effect in substrate; with different superscripts (P, Q, R) at $P < 0.05$ for the SFO effect in substrate

lowered the number of total protozoa ($P = 0.001$); whereas fungal zoospores remained at comparable numbers ($P > 0.05$), except after addition of 45–60 mg PO, whereby they exhibited a slight increase ($P = 0.001$).

Discussion

Modulating rumen fermentation by preventing the release of environmentally damaging biogases derived from domestic ruminants has attracted more attention in recent years. Strategies in this direction include supplementing animal feed with SFO (Elghandour *et al.* 2017; Vargas *et al.* 2017), dietary tannin-saponin (Naumann *et al.* 2017; Cherdthong *et al.* 2019b) and yeast (Polyorach *et al.* 2014). Selected dietary polyphenol compounds, such as flavonoids (*e.g.*, quercetin) and essential oils (*e.g.*, eugenol), seem to play a similar role (Castillejos *et al.* 2006; Lourenço *et al.* 2014; Kim *et al.* 2015). Flavonoids and essential oils of piper powder has been recently shown to modulate rumen fermentation by increasing fermentable organic matter in substrate containing abundance of vegetable oil (Purba *et al.* 2020c). However, the role of a single essential oil component (*e.g.*, eugenol) ingested through feed on ruminal activity remains to be determined. The present discussion, thus, highlighted the use of PO combined with or without SFO in a feeding regimen *via in vitro* measurements.

Rumen perform an aerobic metabolism, which allows their host to derive energy from nutrient fermentation (Olagaray and Bradford 2019). This same fermentation process causes the release of ruminal biogases, measured as total production of CO_2, CH_4, and H_2. Here, total gas production remained unchanged irrespective of increased supplementation with SFO and PO, which could be expected given that these compounds constitute relatively unfermentable nutrient sources. Makkar *et al.* (1995) reported that manipulating rumen fermentation by

Table 4: Effect of substrate supplemented with or without sunflower oil (SFO) treated by piper oil (PO) on pH, NH_3-N (ammonia) and total ruminal microorganism

Parameter	PO (mg)	Supplementation of SFO[1]			SEM[2]	Comparison		
		No	Low	High		SFO	PO	Interaction
pH	0	6.9	6.8	6.8	0.021	0.901	0.937	0.967
	15	6.8	6.8	6.8				
	30	6.8	6.8	6.8				
	45	6.8	6.8	6.8				
	60	6.8	6.8	6.8				
Ammonia (mg/100 mL)	0	17.3^{a}	17.3^{a}	17.4^{a}	0.076	0.107	0.011	0.266
	15	17.1^{b}	16.8^{b}	16.7^{b}				
	30	17.0^{b}	16.7^{b}	16.1^{b}				
	45	16.9^{b}	16.6^{b}	15.7^{b}				
	60	16.1^{b}	16.4^{b}	15.5^{b}				
Ruminal microbes (cells/mL)								
Total bacteria ($\times 10^7$)	0	7.5^{c}	7.8^{b}	8.2^{a}	0.034	0.029	0.003	0.321
	15	7.4^{c}	7.7^{b}	7.9^{a}				
	30	7.3^{c}	7.7^{b}	7.8^{b}				
	45	7.3^{c}	7.6^{b}	7.8^{b}				
	60	6.6^{d}	7.1^{c}	7.2^{c}				
Total protozoal ($\times 10^5$)	0	5.6^{Pa}	4.4^{Qa}	3.7^{Ra}	0.017	0.033	0.001	0.014
	15	4.5^{Qa}	3.7^{Ra}	3.5^{Pb}				
	30	3.6^{Pb}	3.3^{Qb}	3.3^{Qb}				
	45	3.4^{Qb}	3.0^{Pc}	3.0^{Pc}				
	60	3.1^{Pc}	2.8^{Qc}	2.9^{Qc}				
Total fungal zoospore ($\times 10^5$)	0	3.2^{Rb}	3.3^{Qb}	3.5^{Pb}	0.015	0.002	0.001	0.010
	15	3.2^{Rb}	3.3^{Qb}	3.5^{Pb}				
	30	3.2^{Rb}	3.3^{Qb}	3.5^{Pb}				
	45	3.3^{Qb}	3.3^{Qb}	3.6^{Pa}				
	60	3.4^{Qa}	3.4^{Qa}	3.6^{Pa}				

[1] SFO, sunflower oil supplementation per incubation at no (0 mg), low (15 mg), and high (30 mg). Supplementation of SFO treated with 0 mg PO is dedicated as control treatment
[2] SEM – standard error of mean.
Means followed by different superscript (a, b, c) differ at $P < 0.05$ for the PO effect in substrate; with different superscripts (P, Q, R) at $P < 0.05$ for the SFO effect in substrate

supplementing carbohydrate, protein, and fat content in feed substrate resulted in increased gas production, although fats led generally to lower increases compared to carbohydrates and proteins. As a result, more readily fermentable nutrient sources such as carbohydrates were a major contributor to ruminal biogases (Orskov and Mcdonald 1970). In the present study, the carbohydrate content of feed substrate was equal at the onset of each treatment (basal substrate; 500 mg/incubation). Therefore, the lack of change in cumulative gas production resulted solely from inhibition of carbohydrate fermentation by rumen microorganisms during incubation, as evidenced also by prior studies (Elghandour *et al.* 2017; Vargas *et al.* 2017).

In present study, PO supplementation at abundant dose markedly causes substrate disappearance to stagnate during incubation. Adding an excess of PO can inhibit microbial fermentation activity. Castillejos *et al.* (2006) confirmed that eugenol played a major role in suppressing microbial rumen activity in a long-term fermentation study. Cardozo *et al.* (2006), who tested a combination of eugenol and cinnamaldehyde, which had a substantially more severe effect on rumen fermentation, particularly on fermented DM and OM, than eugenol alone. The reduction in *in vitro* degradability of DM and OM in the presence of 60 mg PO per DM, as applied also in the present study, was ascribed to the limited metabolic capacity of rumen microorganisms and, hence, their inability to undertake nutrient fermentation (Polyorach *et al.* 2014). Lower fermentation and degradation activities depleted also the energy supply of the rumen microflora. This pattern was in line with previous studies (Castillejos *et al.* 2006; Lourenço *et al.* 2014), whereby fermentation was strongly inhibited in the presence of elevated doses of eugenol. In present study, large inhibition of fermentation and degradation activity was in consistent with alleviated total gas accumulation in presence of exceedingly high PO dose at 60 mg. The present findings are consistent with earlier *in vivo* studies reporting that SFO and eugenol at acceptable doses did not alter digestibility in any apparent way (Benchaar *et al.* 2012; Atikah *et al.* 2018).

Dietary supplementation with vegetable oil such as SFO provides documented benefits by enhancing the sustainable existence of cellulolytic bacteria (Gao *et al.* 2016). The unsaturated bonding of free fatty acid restricts the growth of ruminal ciliate protozoa and limits the engulfing of bacteria by ruminal protozoa. As a consequence, total rumen biomass augments, favoring fermentation (Gao *et al.* 2016). In line with these observations, the present SFO supplementation led to higher total VFA, confirming an earlier report by Vargas *et al.* (2017) and an *in vivo* study on dietary SFO supplementation in a goat feeding regimen (Atikah *et al.* 2018).

Here, the presence of exceedingly high PO dose reversed the fermentation performance. While abundant PO dose did not alter total VFA and branched-chain fatty acids, it affected the proportions of acetate and butyrate and reduced those of propionate and valerate. These findings were expected given the role of eugenol in mediating

feedback from cellulolytic activity involving prominent fiber-degraders (Vargas *et al.* 2017). Here, the amount of fiber fraction was similar among all treatments. As a result, eugenol derived from PO could alter the proportion of VFA. In a previous study, eugenol successfully suppressed propionic acid without affecting acetic and butyric acid (Castillejos *et al.* 2006); however, Lourenço *et al.* (2014) reported that eugenol supplementation decreased propionic acid, but ensured elevated proportions of acetic, butyric, valeric, and branched-chain fatty acids. The slow substrate disappearance observed in the present study may confirm the inhibitory effect of eugenol on propionic bacteria and particularly on the generation of intermediates by the propionic acid pathway, eventually leading to lower propionic acid accumulation (Mitsumori and Sun 2008; Cherdthong *et al.* 2019b). A possible reason for the difference between previous reports and present results may be related to eugenol purity. Even if eugenol was successfully extracted from PO and quantified, it might nevertheless be contaminated with other volatile compounds, such as caryophyllene (Islam *et al.* 2020; Purba *et al.* 2020d). Hence, PO still contained of caryophyllene may have a direct antimicrobial affection for inhabitant propionic bacteria, but caryophyllene may reduce efficiency of eugenol itself. In this sense, the observed shift of fermentation end-products from acetic to propionic acid is identical to that reported earlier (Busquet *et al.* 2006; Lourenço *et al.* 2014; Joch *et al.* 2016).

Rumen fermentation is accompanied by the release of CO_2, H_2, and CH_4, mostly as a result of hexose hydrolysis (Wolin 1979). In the present study, PO supplementation forced rumen microorganisms to optimize energy consumption, favoring the production of CO_2. Nevertheless, an excessive amount of PO led to a decrease in CO_2 yield. Chaves *et al.* (2008) reported previously that a general mode of action of essential oils was to decrease CO_2 volumes while augmenting the propionic fraction. However, the effect of eugenol itself on reducing the CO_2 volume remains unclear. According to Mitsumori and Sun (2008), CO_2 release is intimately linked to the VFA-producing pathway and particularly acetate, propionate, and butyrate yields. Given that cellulose and hexose content remained constant in this study, the changes in cumulative CO_2 likely reflected VFA production. Mitsumori and Sun (2008) noted also that the increased volume of CO_2 linked to pyruvate metabolism was a consequence of abundant pyruvate-producing bacteria in the rumen, including *Ruminococcus flavefaciens*, *Fibrobacter succinogens*, and *Ruminococcus albus*. Vargas *et al.* (2017) confirmed that SFO supplementation during fermentation maintained in check the number of *F. succinogens* and *R. albus*. Earlier, Cobellis *et al.* (2016) reported that adding essential oils increased *F. succinogens* numbers but had no effect on the population of *R. flavefaciens* and *R. albus.* While the identification of specific ruminal microorganisms was outside the scope of the present study, it is possible that eugenol derived from PO interacted with *R. flavefaciens*, *F. succinogens*, and *R. albus* to affect overall CO_2 yields.

The presence of SFO and PO could alleviate CH_4 production by increasing the ratio of acetic to propionic acid. The extent of methane mitigation by SFO and PO was similar compared to that reported by earlier studies (Joch *et al.* 2016; Elghandour *et al.* 2017; Vargas *et al.* 2017). This result suggested that SFO and PO favored acetogenesis rather than methanogenesis in the rumen. Previous evidence has highlighted that CH_4 formation is a natural outcome of CO_2 and H_2 consumption during methanogenesis (Mitsumori and Sun 2008) and propionic acid plays a major role in the uptake of H_2 (Ochoa-García *et al.* 2019). In other words, increased propionic acid synthesis sequesters H_2 away from methanogenesis, thus lowering CH_4 production (Murali *et al.* 2017). However, a low proportion of propionic acid, as observed here, may mean that CO_2 and H_2 are re-routed towards acetogenesis as in *Blautia* acetogenic bacteria (Greening *et al.* 2019), leading to acetic acid formation via the Wood-Ljungdahl pathway (Ni *et al.* 2011).

Other fermentation end-products such as ammonia were generally lower in the presence of SFO and PO. This fact could be attributed to the high level of eugenol and sunflower oil, as supplementary agents could interfere with the deamination pathway (Cardozo *et al.* 2006; Atikah *et al.* 2018). Busquet *et al.* (2006) reported that higher ammonia inhibition was a result of increased butyric and decreased branched-chain fatty acid accumulation. All rumen fermentation performances in this study, including VFA and ammonia production, appeared independent of pH, confirming similar results from previous studies (Busquet *et al.* 2006; Castillejos *et al.* 2006; Joch *et al.* 2016) and further supporting a role of ruminal microorganisms. The range of pH and ammonia content in the present study was 6.8–6.9 and 15.5–17.3 mg/100 mL, respectively, which was appropriate for microorganisms performing fermentation in the rumen (Ørskov and MacLeod 1982).

The present study detected a change in the composition of ruminal microorganisms, including protozoa, total bacteria, and fungal zoospores following SFO and PO supplementation. The amount of total bacteria remained unchanged following addition of SFO, supporting a previous report by Vargas *et al.* (2017), who suggested that SFO was not capable of enhancing bacterial activity and especially not that of cellulolytic bacteria. Instead, an elevated amount of PO correlated with a reduction in methanogenic bacteria. This could explain the observed reduction in CH_4 formation, as methanogens failed to optimize CO_2 and H_2 consumption. Essential oils such as eugenol have been reported to broadly affect the outer membrane of gram-positive bacteria (Calsamiglia *et al.* 2007). Once this membrane becomes surrounded by essential oils, bacteria lose chemiosmotic control over ion gradients, electron mobilization, phosphorylation cascades, protein translocation, and other enzymatic reactions (Ultee *et al.* 2002). Recently, addition of SFO and PO has been

shown to suppress ruminal protozoa, most likely by inhibiting their nucleic acid synthesis (Wanapat *et al.* 2008; Cherdthong *et al.* 2019a; Patra and Saxena 2009). Fungal zoospores were expected to increase in numbers following a reduction in total protozoa caused by SFO and PO supplementation. However, fungal zoospore numbers remained unchanged contrasting a previous correlation between fungal zoospore abundance and fewer ruminal protozoa (Newbold *et al.* 2015). Fungal zoospores are better equipped than protozoa to cope with plant defenses including secondary compound such as essential oils (Cherdthong *et al.* 2019a). In such instances, fungal zoospores may be the main microorganisms left to ingest the remaining ruminal substrate during fermentation.

Conclusion

This study demonstrates that eugenol, the main compound of piper oil derived from the easily and economically cultivated *Piper betle* plant, could assist in mitigating methane production and in improving feed additive utilization during rumen fermentation by adding 45 mg of piper oil supplementation combined in a variety of diets. Hopefully, utilizing piper oil can be the cheap alternative feed additive to be used further application in animal feeding. Here, sunflower oil is used to provide additional nutrients such lipid as energy through oil inclusion. As a result, solely piper oil use is as effective as other methane mitigation agents such as sunflower oil, in reducing emissions, without negatively impacting rumen fermentation. However, the source of essential oil, type of basal substrate, and incubation time all interact in different ways to affect the final outcome, meaning that further studies are required to determine the optimal combination of these and other factors. Additionally, dietary piper oil supplementation in an animal feeding regimen should be analyzed further, to verify which amount can effectively modulate rumen fermentation while ensuring fail-safe methane mitigation.

Acknowledgments

Authors would like to say thanks to all staffs of the Centre of Scientific and Technological Equipment and Section of Goat and Sheep SUT farm, Suranaree University of Technology, and Nurrahim Dwi Saputra for their valuable helps. Authors would like to extend the heartfelt thanks to the Laboratory of Monogastric Animal Nutrition and Feed Science for use of research facilities. This research was funded by Suranaree University of Technology and by Thailand Science Research and Innovation (TSRI).

Author Contributions

Conceived and designed experiments: RAPP, CY, SP and PP. Performed the experiments: RAPP and PP. Analyzed the data and wrote the paper: RAPP, CY, SP and PP.

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