OPEN ACCESS
Advances in Animal and Veterinary Sciences

Research Article

Dietary Supplementation of Galangal (*Alpinia galangal*) Essential Oil Affects Rumen Fermentation Pattern

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Abstract | The present study aims to evaluate numerous doses of galangal EO (EO) and pure cineole for the relative abundance of rumen microbes and its fermentation parameters *in vitro*. It applies five treatments with six replications and uses completely randomized design to analyze the data. Only if do such differences exist, the Duncan Multiple Range Tests (DMRT) were conducted. The treatments consist of the following galangal EO doses: 0, 30, 60, 120 µL, and 5 µL pure cineole at 300 mg dry matter feed. The experiment proves that gas production, methane (CH_4), dry matter digestibility, and ammonia (NH_3) significantly decrease (P<0.05) at all doses of galangal EO. The addition of cineole results in the significant decrease (P<0,05) of CH_4 (ml/dry matter degraded) while dry matter degradability and gas production shows a significant increase (P<0.05). Also, the addition of cineole results in a more significant increase (P<0.05) of propionate, acetate, total volatile fatty acids, and NH_3 compared to the controls and galangal EO. Propionate significantly increase at the galangal EO dose of 60 and 120 µL. In contrast, protozoa significantly decrease (P<0.05) across all treatments. Furthermore, 30 and 60 µL of galangal EO and cineole does not affect (P>0.05) microbial protein but 120 µL dose of galangal EO significantly decrease the microbial protein. At the genus level, galangal EO increases the abundance of *Succinivibrio* when added cineole showed a higher relative abundance than the controls. Methane production is positively correlated with the relative abundance of *Prevotella*, dry matter degradability, and propionate. As such, the addition of galangal EO can decrease CH_4 and NH_3 productions by inhibiting the nutrients digestibility in the rumen and possibly increasing *Succinivibrio* (a significant actor for methanogenesis) and *Prevotella* (a major actor for ammonia production).

Keywords | Galangal essential oil, Cineole, Decreasing methane, Microbial biodiversity, Rumen fermentations, *In vitro*

Received | October 14, 2021; **Accepted** | December 06, 2021; **Published** | January 05, 2022
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Citation | Daning DRA, Yusiati LM, Hanim C, Widyobroto BP (2022). Dietary supplementation of galangal (Alpinia galangal) essential oil affects rumen fermentation pattern. Adv. Anim. Vet. Sci. 10(2): 323-334.
DOI | http://dx.doi.org/10.17582/journal.aavs/2022/10.2.323.334
ISSN (Online) | 2307-8316

INTRODUCTION

Methane and nitrous oxide released by ruminants contribute to global greenhouse gas emissions (Ricci et al., 2013; Castillo et al., 2000). The emissions also cause a loss of dietary energy and protein that may affect animal productions. Both methane emission and nitrogen excretion are from feed fermentation by the rumen microbiome. Several compounds have been tested as dietary supplements for their ability to modulate the composition and metabolic activity of the rumen microbiome and their lower methane and ammonia productions (Dhanasekaran et al., 2020; Vendramini et al., 2016; Duffield et al., 2008). However, public awareness of antibiotic residue on dairy products encourages stakeholders to use natural plant-based additives that is proven safe (Cobellis et al., 2016).

Plants have various secondary metabolite compounds to

prevent disease, pests, and predators (Wallace et al., 2002). Adding certain secondary metabolites compounds such as tannin, saponin, and EOs can exert positive effects on rumen protein metabolism, volatile fatty acid (VFA) production, and methane and ammonia productions (Klevenhusen et al., 2012; Jouany and Morgavi, 2007; Jafari et al., 2019; Gerlach et al., 2018). However, (Hassan et al., 2020; Kholif et al., 2020) found that such addition may result in the reduction of feed intake, digestion, and rumen fermentation when they are added in sufficiently highly concentration. Thus, new intervention strategies have been developed in dairy nutrition, including the use of different inhibitor combinations where the rumen microbiome deprives methane productions. Determination of novel in the animal feeding requirement balancing of production animals is key to development of animal industry in future trends (Adli, 2021; Sjofjan et al., 2021a, b).

Galangal is a local plant in Indonesia and has the number three production potential after turmeric and ginger for rhizome type (Ministry of Trade, 2011). Galangal (EO) has a promising impact as a rumen modifier because it contains 24% of cineole and is known to have antimicrobial activity on gram positive and negative bacteria (Rialita et al., 2019; Tang et al.,2018). Three studies reported that EO (such as rosemary, eucalyptus, sage, and yarrow EO) with cineole as a main compound positively affects a rumen (Patra and Yu, 2012; Cobellis et al. 2016; Kahvand and Malecky, 2018). The methane production decreases by 42% at 2.0 mL/L eucalyptus EO and lower the number of protozoa compared to the control (Abdelrahman et al., 2019). Rosemary EO (2.0 mL/L) reduces methane production by 9%, and ammonia by 59%–78% compared to the control. Rosemary has significantly decreased *Prevotella* but does not affect *Archaea* (Cobellis et al., 2016). Furthermore, Kahvand and Malecky (2018) reported that both sage and yarrow EOs at the dose of 750 ml/L reduce methane production. Based on the above previous research, a cineole EO as the main compound significantly reduce methane and ammonia. Galangal EO has cineole as the main compound but its effects remain unknown on rumen fermentation *in vitro*. The present research objective is to investigate the effects of galangal EO and their main pure compound (cineole) to reduce methane and ammonia productions in rumen fermentation *in vitro*.

MATERIALS AND METHODS

Galangal EOs preparation

The galangal rhizome harvested from a local farmer in Boyolali district, West Java. The rhizome has been sliced thin as much as 20 Kg and dried at room temperature for three days until the water content decreases by 65%. The dried galangal put into a steam distillation device equipped with a condenser, then heated. The water flowed into the condenser and kept flowing. Condenser temperature kept cool so that all the evaporated oil is condensed and does not escape into the air. The water and oil components separated via Clevenger-type apparatus. The distillation process lasts for 5 hours (Raina and Abraham, 2017).

Bioactive quantification

A quantitative test of bioactive compounds aims to develop the bioactive profile of galangal EO. The quantitative test method for bioactive compounds of Galangal EO is as follows. First, 10l EO samples are taken and then dissolved in 240l methanol. Second, 1l solution is injected into the Gas-Chromatography Mass Spectrometer (GC-MS) system. The flow rate uses a column temperature of 250°C and helium carrier gas with a flow rate of 15 minutes. The column temperature is programmed for the initial temperature for 4 minutes, and slowly raise to 10°C for 30 minutes with the MS detector used. Third, quantitative data is obtained from comparing broad spectrum percentage of EO components with the total area of EO components (Rana et al., 2010). The cineole concentration has been used to determine the dose of additional galangal EO in dairy cattle feed *in vitro*. The reported percentage (Table 1) is the percentage by weight extracted.

Table 1: Bioactive compound of galangal oil by GC-MS.

Bioactive compounds	%
1, 8-Sineol	24.38[2]
cis-ß-Farnesene	12.19
ß-Pinene	8.48
Phenol, 4-(2-propenyl)-, acetate	6.01
(S)-4-(1-Acetoxyallyl) phenyl acetate	5.66
Eugenol	3.00
Geranyl acetate	2.97
Caryophyllene	2.16
Farnesol, acetate	2.21
ß-Bisabolene	1.79
Cyclohexene	2.36
3-Octen-5-yne	1.21
Terpineol	1.27
(E)-Hexadec-2-enal	0.93
Terpinene	0.83
trans-Carveyl acetate	0.73
Bornyl acetate	0.50

[1]Essential oil represented 9.22% of galangal DM; [2]n=3. SD: 0.52

Rumen fermentation by *in vitro* gas production

The feeds tested using *in vitro* gas production from 5 treatments, namely elephant grass: Concentrate 60:40% (S1), 30 µL of galangal oils/300 mg dry matter of feed (S2), 60 µL of galangal oils /300 mg dry matter of feed

(S3), 120 µL of galangal oils /300 mg dry matter of feed (S4), 5 µL of pure cineole/300 mg of dry matter of feed (S5). Cineole (99% purity) was from SIGMALDRICH. Commercial concentrate came from Cooperative Agro Niaga Jabung, Malang. The composition and nutrient content of feed ingredients showed in Table 2. All animal procedures approved by Ethics Committee of Faculty of Veterinary Medicine, Universitas Gadjah Mada Number of Letter: 0055/EC-FKH/Eks./2020.

Table 2: The composition and nutrient content of feed ingredients in treatment.

Nutrient content % (DM basis)	Mott elephant grass	Concentrate
Nutrient compositions		
Dry matter	18.79	91.65
Organic matter	76.21	88.42
Crude protein	13.57	14.38
Ether extract	2.77	2.60
Crude fiber	30.40	29.30
Nitrogen free extract[a]	29.82	42.03
Total digestible nutrient[b]	70.46	70.48

[a]NFE:Nett free extract; calculation results= 100-(ash+crude protein+ether extract+crude fiber). [b]TDN: total digestible nutrient; calculation results forage= 1.6899 + 1.3844(CP)+ 0.7526(NFE) – 0.8279(EE) + 0.3673(CF), concentrates as an energy source= 2.6467 + 0.6964(CP) + 0.9194(NFE) + 1.2159(EE) – 0.1043(CF), source as a protein= -37.3039 + 1.3048(CP) + 1.3630(NFE) + 2.1302(EE) + 0.3618(CF).

Rumen fluid extracted from Bali cattle attached with rumen fistula, fed with 3% body weight DM consisted of 70% *Mott elephant* grass and 30% concentrate given at 08.00 and 15.00 *ad libitum*. Rumen fluid collected before morning feeding, then filtered and added with mixing 474 ml H2O, 0.12 ml micro mineral solution, 237 ml of buffer solution, 237 ml mineral solution macro, resazurin 1.22 ml, and 49.5 ml of reducing solution put into the Erlenmeyer 2 L and during preparation, continuously flushed with CO2 in anaerobic conditions before being put into a syringe glass. The ratio of rumen fluid and the medium is 1:2 (v/v). Approximately 300 mg of each test feed added into the glass syringe, which contains 30 ml of fermentation medium. All glasses were then incubated in a modified water bath at 39°C for 72 hours then its gas production was observed. At 0, 1, 2, 4, 6, 8, 12, 24, 36, 48,72-hour measurement volumes recorded; samples of gases produced were taken in Vacutainer® tubes for CH4 concentration analysis using Gas Chromatography (GC) and then released. At the end of this incubation (72 h), the liquid phase centrifuged at a rate of 3,000 g. Its filtrate used for testing rumen fermentation parameters (ammonia levels, VFAs, pH, methane and CH_4 gas production) and microbial activity (microbial proteins and protozoa). The remaining material filtered through sintered crucibles to determine *in vitro* apparent dry matter and rumen fermentation as ımpacted by supplementation of galangal oils using *ın vitro* gas organic matter degradability. The residual dry matter and organic matter contents determined to refer to the AOAC (2006). Dry matter (DM) and ash contents determined by drying at 105 °C for eight hours and at 550 °C for six hours, respectively.

Methane analysis. Total gas production measured after 72 h incubation with a view on syringes scale based on the increase in gas pressure caused the piston to the top (Getachew et al., 1998). To measure the levels of methane gas, samples gas was analyzed using gas chromatography. Total methane production is known to convert the methane gas levels in a sample of the total gas production. The number of protozoa. Preparation calculation of protozoa by (Diaz, 1993). pH is measured using a pH meter which was calibrated with buffers pH 4 and pH 7. pH measurements made at the end of fermentation. Microbial protein. Measurement of microbial protein by Lowry protein analysis method (Plummer, 1987) ammonia levels. Determination of ammonia using the method of (Marbach and Chaney, 1961).

Rumen microbial abundance analysis

DNA extraction

According to the company protocol, the DNA mini kit (ZymoBIOMICSTM DNA mini kit catalogue No. D4300) is used for the total DNA extraction. Furthermore, electrophoresis on 1% Agarose gel confirms the extracted DNA to determine its concentration and purity in the samples. Phusion® High-Fidelity PCR Master Mix (New England Biolabs) is used to analyze the quality and quantity of the extracted DNA. 16sRNA primer used to amplify prokaryotes (bacteria and archaea) with sequences 5'GTGCCAGCMGCCGCGGTAA, GGACTACHVGGGTWTCTAAT 3', in the V4 region. Then, Qiagen Gel (Qiagen, Germany) is used for PCR purification. The PCR TruSeq® DNA kit has been used to design libraries. Then the sequencing results were calculated using Qubit and Q-PCR through HiSeq2500 PE250.

Sequencing data processing

The paired final reads are combined using FLASH V1.2.7 (Magoč and Salzberg, 2011). Then, Qiime V1.7.0 is used to filter the raw tags to obtain higher quality and cleaner tags (Bokulich et al., 2013). The generated tags are compared with the database via Gold database then the algorithm of Edgar et al. (2011) is used to detect chimaera sequences. The last step to get the effective tags is to remove the chimaera via Chimera formation (Haas et al., 2011).

OTU cluster and species annotation

All effective tags are used to analyze the sequences in the Uparse software v7.0.1001 (Edgar et al., 2013). Similar OTUs are obtained with >97% similarity. The Mothur software is used for the different OTU sequences obtained from the SSUrRNA database via the SILVA Database (Wang et al., 2007). Further phylogenetic relationships of all OTUs are annotated using MUSCLE Version 3.8.31 (Edgar, 2004).

Data analysis

Rumen microbial diversity data are taken from the report generated by Next Generation Sequencing Method. All treatments are replicated six times and the collected data includes total gas production, CH_4, CH_4 per DMD, DMD, pH, NH_3, Microbial protein, Protozoa, Total VFA, Acetate, Propionate, and Butirate. The data obtained were statistically analyzed using a completely randomized directional pattern design using the R program software. Differences were declared as significant at $p<0.05$.

RESULTS AND DISCUSSION

Effect of galangal EO on rumen fermentation

Gas production significantly decreases ($P<0.05$) by 28.91% and 43.77% with the addition of galangal EO at doses of 60 and 120 µL, respectively. However, there are no differences between $P>0.05$ in the dose of 30 µL galangal EO and 5 µL cineole compared to the control (Table 3). The same results also occur in Patra and Yu (2012) research that the use of eucalyptus EO at doses of 40 and 90 µL/300 mg (DM feed) decreases gas production by 3.86% and 10.35%, respectively. Additionally, Cobellis, Trabalza-Marinucci, Marcotullio et al. (2016) found that the addition of eucalyptus EO at doses of 48 and 96 µL/300 mg (DM feed) also causes a decrease in the gas production by 46.32% and 49.75%, respectively. Furthermore, the present study reports that the digestibility of organic matter also decreases by 14.23% and 15.04%, respectively. The production of gas in rumen fermentation *in vitro* is correlated with the digestibility of organic matter (Zijderveld et al., 2011). Table 3 shows that the gas production as similar as those of 60 and 120 µL/300 mg (DM feed) causes a decrease in the digestibility of organic matter. Galangal and eucalyptus EOs have main components under the monoterpene group, with broad-spectrum anti-bacterial properties (Young, 2019). A possible decrease in the gas production and digestibility of organic matter is inhibited by the bacterial rumen activity. Table 4 presents that the abundance of phylum *Bacteriodetes* decreases at doses 60 and 120 µL/300 mg (DM feed). According to Jami et al. (2014) *Bacteriodetes* is the most dominant type of bacteria in the rumen and is responsible for the degradation of several nutrients such as carbohydrates and proteins.

The production of gas in rumen fermentation is also correlated with the level of CH_4 and CO_2 gases. Table 3 shows that at doses 60 and 120 µL galangal EO, the CH_4 level significantly decreases ($P<0.05$) by 39.55% and 53.25%, respectively, compared to the controls. More specifically, the CH_4 production that occurs in the rumen correlates with the mechanism of hydrogen transfer among microorganisms. Protozoa plays a role in hydrogen transfer because of their symbiosis with methanogens converting CO_2 into CH_4 (Kataria, 2016). Table 3 illustrates that the protozoa significantly decreases in all doses of galangal EO and cineole. In addition to protozoa, other bacteria in the rumen also regulate methanogenesis. Greening et al. (2019) explains that methane is formed by hydrogen transfer of the microbes *Methanobacteriales archaeon* and *Wolinella succinogenes*. The effect of decreased methane production on the addition of galangal EO, associated with microbial diversity, can be seen in Table 4. The rumen microbes associated with the methanogenesis process shows that the decrease in the abundance of *Methanobacteriales* and *Wolinella succinogenes*. The addition of cineole is also known to decrease methane production and the abundance of *Methanobacteriales*.

Several studies on EOs with cineole as the main component produce different results depending on the level of EO. Patra and Yu (2012) report almost similar results in eucalyptus EO at doses 20 and 40 µL where there was a decrease in methane by 8.04% and 15.36%, respectively. Moreover, Abdelrahman et al. (2019) report that doses 60 and 80 µL of eucalyptus EO decrease methane by 43.25% and 46.09%, respectively. On the contrary, adding EO by 6 and 12 µL does not affect methane gas production (Wu et al., 2018). Additionally, Colombini et al. (2021) reports that 2 µL of *Archiella moscata* EO does not affect the methane production. Calsamiglia et al. (2007); Benchaar et al. (2008), furthermore, found that the low dose EO in rumen does not affect methane production. Important to note that high dose EO can disrupt fiber digestion. From the data, we can see that adding cineole, although the methane decreases, does not interfere with the digestibility of organic matter. Thus, the use of secondary plant metabolites needs to be taken into consideration that the effectiveness of bioactive compounds is more accessible in pure conditions.

Based on Table 4, the population of *Archaea* and protozoa decreases due to galangal EO and cineole. Cineole compounds may have a mechanism in inhibiting protozoa. According to Nooriyan and Rouzbehan (2017) adding eucalyptus EO at doses of 30, 300, and 3000 µL also decreases the protozoa population by 37.45%, 23.32%, and 34.63%, respectively. EOs and their constituents exhibit biological activity against protozoa (Perez, 2012).

According to Sun et al. (2018) the activity of EO as anti-protozoa is caused by monoterpene compounds such as cineole, which is responsible for the hydrophobic nature of EOs, thus allowing diffusion into the cell membrane of protozoa, affecting intracellular and organelle metabolic pathways. Thus, the cineole found in galangal EO and its pure compound show activity against protozoa. Furthermore, Machado et al. (2014) also explains that there is a possibility of cineole being able to induce cell membrane lysis and causes cytoplasmic leakage to occur.

The VFA level is used as an energy source for livestock and rumen microbes. According to Nozier et al. (2011) the VFA level is correlated with the amount of organic matter degraded in the rumen. The addition of galangal EO decreases the total VFA by 4.10% and 11.52% at 60 and 120 µL doses, respectively. The data are in line with the degradation of organic matter, which also decreases at doses of 60 and 120 µL. Even though the VFA level decreases at the dose of 60 µL, it is to ruminant requirements. According to McDonald et al. (2010) the level of required VFA to support optimal rumen growth is 80-160 mM. However, the VFA level in 120 µL of galangal EO is below the average standard of 78.50 mM. With the addition of cineole, the VFA level increases by 12.94%.

Table 3: The effect of galangal essential oil and cineol on rumen fermentation product.

Parameter	Dosis minyak atsiri lengkuas (µL)				Sineol (µL)
	0	30	60	120	5
Gas production (ml)	51.82^{c}±0.65	51.16^{c}±1.09	41.52^{b}±0,86	29.00^{a}±0.28	53.83^{c}±0.79
DMD (%)	47.54^{c}±0.60	39.34^{b}±0.56	36.89^{b}±0,94	26.59^{a}±1.71	46.14^{c}±0.33
OMD (%)	31.52^{c}±1.32	33.50^{c}±1.83	29.30^{b}±1,11	23.76^{a}±1.04	43.36^{d}±1.79
CH_4 (mL)	5.84^{d}±0.44	6.04^{d}±0.50	3.53^{b}±0,86	2.73^{a}±0.28	4.85^{c}±0.75
CO_2 (mL)	31.48^{d}±0.80	27.42^{c}±1.33	25.28^{b}±1,02	22.27^{a}±0.67	26.15^{bc}±0.71
pH^{ns}	6.90 ±0.07	6.93±0.09	7.07±0,25	7.07±0.07	7.00±0.13
NH_3 mg/100 ml	40.11^{b}±0.48	39.24^{b}±1.15	37.66^{a}±1,31	39.01^{b}±0.64	46.73^{c}±1.18
VFA total mM	88.78^{c}±1.96	89.31^{c}±1.46	85.14^{b}±2,09	78.50^{a}±2.36	101.98^{d}±1.10
Acetate mol/100 mol	66.26^{b}±1.74	61.15^{c}±0.97	62.07^{c}±1,05	57.07^{d}±1.05	69.29^{a}±1.32
Propionate mol/100 mol	23.46^{a}±1.56	22.63^{a}±0.71	30.11^{b}±1,72	29.94^{b}±1.55	23.48^{a}±1.09
Butyrate mol/100 mol	10.27^{c}±1.45	16.20^{d}±0.91	7.81^{a}±0,79	12.98^{b}±1.08	7.22^{a}±1.28
Microbial protein (mg/mL)	0.55^{b}±0.07	0.60^{b}±0.06	0.53^{b}±0,05	0.41^{a}±0.06	0.54^{b}±0.06
Protozoa (10^3/ml)	8.94^{d}±0.05	6.07^{c}±0.33	2.62^{a}±0.23	3.60^{b}±0.22	7.08^{e}±0.11

$^{a, b, c, d, e}$ Means within rows and subtitles followed by distinct superscripts different (Duncan test at 5%).

Table 4: Genus-level composition of the rumen samples from dosage of galangal oils and cineole (% total observation).

Phylum	Genus	Dose µL/300 mg dry matter of feed				
		0	30	60	120	5 (cineole)
Bacteriodetes		52.03	55.47	43.95	34.66	58.17
	Rikenellaceae_RC9_gut_group	12.39	11.70	12.17	12.19	11.69
	F082-uncultured_rumen_bacterium	4.75	5.21	2.33	1.82	5.93
	Bacteroidales_BS11_gut_group	3.28	3.94	2.44	2.12	3.58
	SP3-e08	2.85	2.70	3.76	3.02	2.76
	Prevotellaceae_UCG-003	0.61	0.36	0.35	0.26	0.86
Proteobacteria		7.85	8.60	12.08	16.59	3.67
	Sutterella	0.77	1.42	2.78	7.81	0.44
	Succinivibrio	0.86	1.79	2.93	1.85	0.63
	Ruminobacter	0.40	0.99	0.86	0.57	0.34
Firmicutes		14.71	15.89	20.73	26.53	15.49
	Ruminococcaceae_UCG-011	0.86	0.98	1.30	2.37	0.83
	Clostridium_sensu_stricto_1	0.003	0.002	0.008	0.006	0.224
	Streptococcus	0.07	0.05	0.10	0.44	0.06
	Lachnospiraceae_XPB1014_group	0.70	0.69	0.89	0.94	0.54

Euryarchaeota	*Methanobrevibacter*	0.50	0.20	0.10	0.20	0.20	

Several studies on EOs with cineole as a source, i.e., Eucalyptus and Rosemary with the same dose of 60 µL, result in differences in the VFA level. Eucalyptus EO which is equivalent to 47.21% cineole does not affect the VFA level while Rosemary which is equivalent to 16.8% cineole content causes the VFA level to decrease by 12.35% (Cobellis et al., 2016). However, other studies Wang et al. (2018) also report that the addition of eucalyptus EO increases the VFA level compared to the controls with doses ranging from 20 to 80 µL, which was equivalent to the cineole level of 35 to 75%. Viewing from the patterns of cineole in galangal and rosemary EOs, which are almost the same (20 to 30% lower than Eucalyptus EO). In that case, the influence of other compounds may be possible. According to Kahla et al. (2017), the bioactive component of Eucalyptus EO is 85% cineole, and the rest are compounds such as pinene and camphene. In contrast to galangal EO, its constituent elements consist of 24% cineole, and the remaining consists of other components such as phenolic compounds. Based on the data on the abundance of microbes in Table 4, it can be drawn that cineole does not affect bacteria that degrade carbohydrates such as cellulose and starch. Therefore, the increase in the VFA level can be concluded that the cellulose-degrading bacteria are not affected.

The acetate level in rumen decreases at all doses of galangal EO. However, the propionate level increases by 22.21% and 21.23% at 60 and 120 µL doses, respectively, compared to the controls. Furthermore, the butyrate level decreases at the dose of 60 µL and increased at 30 and 120 µL doses. At the dose of 5 µL cineole causes an increase in acetate compared to the controls and galangal EO. However, the level of propionate and butyrate decreases compared to galangal EO and are not significantly different from the control. In line with Soroor and Rouzbehan (2017) research, the addition of eucalyptus EO also causes the acetate level to decrease by 26.38%, 22.15%, and 30.32%. Likewise, the propionate level also increases by 18.02%, and at doses of 3.30 and 300 µL, respectively. The acetate and butyrate productions from pyruvate is accompanied by the H_2 production while the propionate production utilizes hydrogen as the primary substrate for methanogenesis (Bharanidharan et al., 2018). Table 3 shows that methane decreases at doses of 60 and 120 µL, therefore there is a possibility of propionate to increase. Although the methane level also decreases with the addition of cineole, the reduction is not as much as that of the galangal EO treatment. It is primarily because the hydrogen from the acetate and butyrate productions has not been optimally converted to propionate.

The addition of galangal EO and cineole do not affect the rumen pH after 48 hours of *in vitro* fermentation. At the dose of 60 µL galangal EO, the NH_3 level decreases significantly by 6.10% lower than the control, while at the dose of 30 µl there was no difference compared to the control. Furthermore, at the cineole dose, the NH_3 level increases by 14.16% compared to the control. The decrease in the NH_3 level in galangal EO indicates a reduction in protein degradation in the rumen. However, there is no significant difference in the microbial protein level in the rumen compared to the controls. The decrease in microbial protein occurs at the addition of 120 µL dose of galangal EO due to a reduction of organic matter digestibility, the VFA level, and the NH_3 level. The main precursor for the microbial growth is NH_3 and energy in ATP, produced from the feed degradation process by rumen microbes (Hristov et al., 2013). The nitrogen needed for rumen microbial synthesis is NH_3, amino acids, and peptides (Bach et al., 2005). Microbial protein synthesis can still be achieved optimally if NH_3 and VFA are available in sufficient conditions for 24 hours (Widyobroto et al., 2007).

The decrease in the NH_3 level at the dose of galangal EO is correlated with the reduction in the genus *Prevotella*, a type of proteolytic bacteria. A similar study reported by Cobellis et al. (2015) using eucalyptus EO and rosemary at the dose of 60 µl in rumen fermentation decreases the relative abundance of *Prevotella* and the NH_3 level. Further Chouchen et al. (2018); Wang et al. (2018) found that the use of eucalyptus EO reduces the NH_3 level. The relative abundance of the genus *Prevotella* correlates with the production of NH_3 because it inhibits the amino acid deamination process and reduces protein degradation (Liu et al., 2020). The relative abundance of *Prevotella* decreases at the dose of galangal EO which at the same time results in a lower NH_3 level, while cineole produced higher NH_3 than the controls. According to Chaves et al. (2008); Liu et al. (2020) Proteolytic bacteria in the rumen produce proteases and peptidases, which convert proteins into peptides and amino acids. These two then are converted into microbial cells synthesized into microbial proteins or deaminated into ammonia. Excess ammonia production leads to a lower efficiency of N sources and microbial protein synthesis. Bacteria play a role in the deamination of amino acids in the rumen and are associated with the production of NH_3 in the genus *Prevotella* (Bekele et al. 2010). The decrease in rumen NH_3 concentration and the effect of galangal EO supplementation is related to the protozoa population in this study which is also decreased. Rumen protozoa play a role in utilizing protein as food and then releasing NH_3 as a metabolic product.

Effects of galangal EO on rumen microbial abundance

Phylogenetic analysis of metagenomic data at the domain level contains 96% of sequences binned to bacteria and 4% of *Archaea*. At the genus level, the most predominant genera are *Rikenellaceae*, *Prevotella, Sutterella*, *Ruminococcaceae*, *Bacteroidales* SP3, *Succinivibrio*, *Ruminococcaceae*_NK4A214_group. The above genera represent 48.26%, 40.64%, 44.20%, 46.47%, and 44.22% of the total sequences at doses of galangal EO 0, 30, 60, 120 µL and cineole. Across all treatment, *Rikinellaceae* which is the most predominant genera has a similar abundance. Across all doses of galangal EO, *Prevotella* and *Bacteriodales* have a lower abundance, but the cineole dose has similar abundance than the controls. As such, all doses of galangal EO intervenes the abundance of *Archaea* while the 5 µL cineole does not do so. The addition of galangal EO at the dose of 60 µL caused abundant phylum *Bacteriodetes*. In line with Colombini et al. (2021) research, *Archiella moscata* EO at the dose of 20 l is equivalent to 11% cineole which also causes a decrease of *Bacteriodetes* by 16.71% and *Firmicutes* by 12.35%. Cobellis et al. (2015) report that adding eucalyptus and Rosemary EOs both at the dose of 60 µL is equivalent to cineole 47.1% and 16.8%, respectively. The two also causes *Prevotella* to significantly decrease compared to the controls. From several studies using EOs with cineole as the main component, it can be concluded that the addition of EOs from cineole sources has the same pattern, namely reducing the type of bacteria of the genus *Bacteriodetes* in the rumen.

Interestingly, some studies of galangal EO points out noticeable results i.e., a decrease in *Prevotella ruminicolla* bacteria. These bacteria are proteolytic bacteria that are responsible for protein degradation in the rumen (Jewell et al., 2015). The reduction in the abundance of *Prevotella* bacteria is correlated with the efficiency of feed protein sources for dairy cattle (Xue et al., 2020). The decrease in the relative abundance in the species *Prevotella ruminicolla* is associated with Mcintosh et al. (2003) results, a mixture of high doses EOs consisting of thymol, eugenol, vanillin, and limonene inhibiting the growth of pure cultures of rumen bacteria (*Prevotella ruminicola, Clostridium sticklandii*, and *Peptostreptococcus anaerobius)*. Additionally, Patra and Yu (2014) reports that cinnamon and oregano EOs at the dose of 30 µL/300 mg (DM feed) also reduce the abundance of *Prevotella* bacteria. Furthermore, Cobellis, Cobellis er al. (2015) study adds 7 g/head/day of rosemary leaves, which are equivalent to 24% cineole in sheep and reduce the population of *Prevotella ruminicolla*. Meanwhile, rosemary EO at the dose of 70 l/300 mg (DM Feed) on sheep feed does not affect the abundance of *Prevotella ruminicolla* bacteria (Cobellis et al., 2016).

The use of galangal EO, which mainly decreases the abundance of Gram-negative bacteria in the phylum *Bacteriodetes* and the genus *Prevotella,* is in congruent with those of Jirovetz et al. (2005) their research evaluates that rosemary EO with cineole component is 45% more effective against Gram-negative bacteria (*Escherichia coli*) compared to Gram-positive bacteria (*Staphylococcus aureus*). However, pure cineole compounds are also reported to be effective as antibacterials compared to rosemary EO. The present study has explained that the cineole contained in rosemary was lower than the pure compound (Jirovetz et al., 2005). Therefore, it is possible that compounds such as pinene, camphene, and limonene included in rosemary EO may cause different results with pure compounds. Another study also shows that eucalyptus EO with the concentration of 45% cineole, 35% spathulenol, and 20% cymen is more effective against Gram-negative bacteria (Limam et al., 2020).

The addition of pure cineole, which is the main component of galangal EO, has a different pattern. The abundance of rumen bacteria is as same as the controls. According to Hendry et al. (2009) the antimicrobial effectiveness of eucalyptus EO is higher than those of cineole against *Staphylococcus aureus* bacteria. Antibacterial EO activity is affected by the synergism effect of multiple compounds. Many other compounds work as antimicrobials in galangal EO, such as phenol, eugenol, frankincense, pinene, and other minor parts (Table 1). According to the research of (Schären et al., 2017; Kim et al., 2019; Lee et al., 2020) EO as a rumen modifier has inconsistent effects on microbial diversity because it depends on the type of bioactive component and the dose used.

Compared to galangal EO, the insignificant effect of cineole in reducing the *Prevotella* population possibly be due to the loss of some volatile antimicrobial compounds. Furthermore, Malecky et al. (2009) also report that monoterpene can be easily degraded in the rumen than phenolic compounds. Although both have properties as antibacterial, some of the compounds in EOs are chemically unstable and volatile (Turek and Stintzing, 2013). In galangal EO, the phenol content is 9.16% thus it is possible to play a role in reducing the population of *Prevotella* bacteria. Chen et al. (2021) reports that tannins, which are phenolic compounds, can reduce the abundance of *Prevotella* by 53.5% compared to the controls.

According to Miklasińska-Majdanik et al. (2018) against pathogenic bacteria, the toxicity of cineole compounds is lower than those of phenol. Tang et al. (2018) further explain that the highly concentrated phenol can penetrate and disrupt bacterial cell walls and then precipitate proteins in bacterial cells. In addition, phenol can cause protein coagulation, change the permeability of bacterial membranes, and eventually, cell membranes undergo lysis

(death). Meanwhile, phenol can form both protein and phenol complex bonds at lower concentrations. Phenol penetration into cells follows which causes precipitation and protein denaturation, thereby inactivating essential enzyme systems in bacterial cells. Phenol activity as an antibacterial is higher in Gram-positive than Gram-negative species (Miklasińska-Majdanik et al., 2018). According to Santos et al. (2012), the effectiveness of galangal EO as antibacterial works well against both Gram-positive and Gram-negative bacteria.

Possible future research is to examine the effect of galangal EO on microbial diversity, particularly on Succinivibrio because it can use hydrogen, which is converted into succinate. Furthermore, succinate will be carboxylated to propionate (Iqbal et al., 2018; Ungerfeld, 2020). Propionate production competes with methanogens for the metabolic hydrogen consumption. Under these conditions, the more propionate produced, the less methane. The activity of *Succinivibrio* bacteria also influences methanogenesis and hydrogen transfer in the rumen. In galangal EO, there is a higher relative abundance of *Succinivibrio* bacteria than the controls and cineole. In contrast, adding the pure cineole to the abundance of *Succinivibrio* bacteria leads to similar results as those of the controls. According to research of Zhao et al. (2018); Joch et al. (2019); Hassan et al. (2020) the mechanism of methane production has a negative correlation with the relative abundance of *Succinivibrionaceae* bacteria and a positive correlation with the relative abundance of *Ruminococcaceae*. This finding is essential for further research on *Succinivibrio* as an inhibitor of methanogenesis to boost feed efficiency.

In contrast to bacteria, all *Archaea* do not have a muramic acid-based cell walls, and the most common *Archaea* cell walls consist of a single glycoprotein. Also, in some *Archaea*, the cell wall is composed of polymers such as pseudomurein (Jarrell et al., 2013). Galangal EO does not affect *Archaea* abundance, but cineole does. In line with Colombini et al. (2021) research, the addition of pure cineole with a dose of 20l is equivalent to 20% cineole causing a decrease in the genus *Euryarchaeota* by 35.26%. Further, Cobellis et al. (2015) report that the addition of eucalyptus EO at the dose of 60 l is equivalent to 47.1% cineole, and does not affect the total *Archea*. Patra and Yu (2012) explain that eucalyptus EO at the dose of 40l is equivalent to 34.5% of cineole, which also does not affect the abundance of *Archaea* and protozoa. According to Ohene-Adjei et al. (2008) the diversity of methanogenic *Archaea* such as *Methanosphaera stadtmanae* and *Methanobrevibacter smithii* correlates with the protozoan species such as *Isotricha* sp. and *Dasytricha* sp. As can be seen in Table 4, both galangal EO and cineole cause a total reduction of protozoa. According to Le et al. (2018) pure cineole compounds effectively inhibit the growth of *Trypanosomal*. The present study also describes significant changes in the plasma membrane and mitochondrial swelling. These two effects are similar to the use of antibiotics that can inhibit the biosynthesis of protozoan sterols (Le et al., 2018).

CONCLUCIONS AND RECOMMENDATIONS

Based on the above elaboration, it can be concluded that the EO from galangal can reduce methane and ammonia at the dose of 60 µL. Moreover, the increasing abundance of *Succinivibrioceae* leads to the reduction of methane while *Prevotella* contributes to the ammonia production.

ACKNOWLEDGEMENTS

The present publication was supported by Ministry of Research and Technology/National Research and Innovation Agency (Indonesia). Grant number is 6/E1/KP.PTNBH/2021 and 2292/UN1/DITLIT/DIT-LIT/PT/2021.

NOVELTY STATEMENT

This study is the first to evaluate an EO originating from Indonesia, namely Galangal. Galangal is known to have bioactive components such as cineol and phenol. The use of essential oils is expected to be able to increase the function of bioactive compounds in modifying rumen microbes and optimizing rumen fermentation products.

AUTHOR'S CONTRIBUTION

DRAD, LMY, CH & BPW: Idea and research design.
DRAD: In Vitro
collection and lab analysis. DRAD and BPW: Write the manuscript.
LMY and CH: Revision.

Conflict of interest

The authors have declared no conflict of interest.

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