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Effect of galangal essential oils on rumen microbial population and biodiversity on *in vitro* rumen fermentation

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ABSTRACT The study aimed to evaluate the effect of administering galangal essential oil (EO) on the abundance of rumen bacteria using the 16s rRNA method. The treatments included a control (no EO addition), galangal EO (30, 60, 120 µL), and cineole (5 µL). The treatments were assessed using a 48-hour *in vitro* batch culture of rumen fluid containing a 60:40 ratio of forage to concentrate. For amplification of the prokaryotes (bacteria and archaea) in region V4, 16s rRNA primer 5'GTGCCAGCMGCCGCGTAA, GGACTACHVGGGTWTCTAAT3' was employed. The data for rumen microbial abundance were analysed descriptively, while the data for rumen microbial diversity were obtained from the report on the Next Generation Sequencing Method. The microbial composition of each sample was tested for operational taxonomic units (OTUs) with a 97% identity rate on a valid label. The 16S rRNA gene sequencing yielded a total of 3,977 OTUs. Adding galangal and cineole EOs resulted in the same variation of the Shannon index. The population index (chao1 index) was highest when 60 µL of galangal EO was added, compared to 30 and 120 µL of galangal EO and cineole. In addition, providing 60 µL of galangal EO decreased the abundance of *Prevotella ruminicola* compared to the control and cineole doses. The addition of galangal EO also led to a decline in the number of *Methanobacteriales*. The population of the fibre-degrading bacteria group (*Ruminococcus albus* and *Ruminococcus flavefaciens*) was higher in a dose of galangal EO than the control and cineole. Therefore, it can be concluded that the effective dose of galangal EO, i.e. 60 µL/300 mg (DM feed) *in vitro*, can reduce the abundance of *Prevotella* bacteria and methanogens.

KEYWORDS galangal essential oil; microbial diversity; rumen fermentation; 16sRNA

1. Introduction

The rumen has been manipulated to optimise protein quality. Consequently, high-quality protein is not degraded in the rumen and can be digested post-rumen and utilised by ruminants (Leondro et al. 2019). Utilising secondary plant metabolites, such as phenolic compounds, to prevent protein breakdown in the rumen has been widely documented as a strategy for reducing protein degradation in the rumen (Patra 2012). According to research conducted by Carrasco et al. (2017), one of the phenolic compounds, such as tannin, can minimise protein degradation in the rumen by inhibiting bacterial activity. In addition, Rawel et al. (2005) and Young (2019) revealed that phenolic compounds in the presence of hydroxyl groups could penetrate bacterial cells and interfere with the cytoplasmic membrane's function, including active transport and proton strength. Furthermore, it has been demonstrated that phenol inhibits the activity of protease enzymes and binds to feed protein (Fitriastuti et al. 2019; Lavrenčič and Levart 2021).

Essential oils contain various bioactive compounds, of which over 200 types have been identified (Young 2019). Phenolic compounds are also common in essential oils and have strong antibacterial properties (Faleiro 2011). The mechanism of action of essential oils depends on the major compounds, but it is likely that minor compounds also impact their efficacy (Young 2019). In addition, the mechanism of action of essential oils varies greatly depending on the chemical composition (both the components present and their proportions), the structure of the chemical elements, and the interactions between the components of the essential oil and is very different when compared to the pure compound (Chouhan et al. 2017). The complexity of the rumen microbial ecosystem and volatile oil composition contribute to varying results on rumen fermentation parameters (Kim et al. 2019; Sofyan et al. 2019). Few studies have demonstrated an association between certain groups of bacteria and fermentation products in the rumen. For example, the abundance of the phylum *Bacteroidetes* correlated significantly with enhanced propionate produc-

tion, whereas *Firmicutes* correlated with butyrate production (Jewell et al. 2015). Moreover, Iqbal et al. (2018) reported that *Prevotella* spp. significantly reduced protein degradation in the rumen.

In Indonesia, galangal is an aromatic plant that ranks third in rhizome production after turmeric and ginger (Ministry of Trade of the Republic of Indonesia 2011). Galangal can be planted in dry land for all seasons with a three-month planting age and has been utilised optimally for the presence of essential oils (Lely et al. 2017). Because it inhibits pathogenic bacteria, galangal essential oil is widely used as a food preservative (Oonmetta-aree et al. 2006). Cineol, the primary component of galangal essential oil, is also known to inhibit Gram-positive and Gram-negative pathogenic bacteria (Pereira et al. 2018). By exploiting the conserved 16S rRNA gene, molecular methods can be used to determine the existing composition of bacteria (Morgavi et al. 2013), predicts its development, or calculates microbes targeted in complex ecosystems without the need to cultivate (Chaucheyras-Durand and Ossa 2014). Therefore, this study aimed to evaluate the effects of administering galangal essential oil on the abundance of rumen bacteria using the 16s rRNA method. Furthermore, understanding the taxonomy of rumen bacteria through the metagenomic approach aids in determining the optimal dose of galangal essential oil.

2. Materials and Methods

2.1. Galangal oils extraction

The galangal rhizome was harvested from a local farmer in Boyolali district, Central Java. The rhizome has been thinly sliced up to 20 kg and air-dried at room temperature for three days until the water content reduces by 65%. The dried galangal was then put in a steam distillation device equipped with a condenser and then heated. The water flowed into the condenser and kept flowing. The condenser must be kept at cold temperatures so that all evaporating oil is condensed and does not escape into the air. Finally, the water and oil components were separated using the Clevenger-type apparatus. The distillation lasts five hours (Tang et al. 2018).

2.2. Bioactive quantification

The resulting essential oil was evaluated using gas chromatography-mass spectrometry (GC-MS). Quantitative data were obtained from the % area of the essential oil component spectra to the total area of the essential oil components (Apel et al. 2017). The cineole content in galangal essential oil is used to determine the *in vitro* dosage of galangal essential oil added to dairy cattle feed. Table 1 presents the percentage of bioactive compounds as a percentage by weight in the extracted oils.

TABLE 1 Screening of bioactive compounds of galangal oils by chromatography gas

Bioactive compounds	%
Cineol	6.14
Phenol	9.82

2.3. Rumen fermentation by in vitro gas production

In vitro gas production techniques were designed to evaluate five types of treatment consisting of elephant grass: concentrate 60% galangal EO + 40% dry feed matter (S1), 30 µL galangal EO + 300 mg dry feed matter (S2), 60 µL galangal EO + 300 mg dry feed ingredients (S3), 120 µL galangal EO + 300 mg dry matter feed (S4), and 5 µL pure cineole + 300 mg feed dry matter (S5). Cineole (purity level of 99%). Pure cineole compounds were obtained from SIGMALDRICH. The grain feed was obtained from Jabung Agro-Commerce Cooperative (Koperasi Agro Niaga Jabung), Malang. The Ethics Committee of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, has approved all veterinary procedures in this research (approval number 0055/EC-FKH/Eks./2020).

The source of rumen fluid was a fistulated Bali cow fed by 3% dry matter of bodyweight with a composition of 70% *Pennisetum purpureum* and 30% grain with distribution periods at 8 am and 3 pm on an ad libitum basis. The rumen fluid was collected before morning feeding, then filtered using two layers of Muslin cloth. The filtered rumen was then added to the mixture medium, which consisted of 474 mL of H_2O, 0.12 mL of micro-mineral solution, 237 ml of buffer solution, 237 ml of macro solution, 1.22 mL of resazurin, and 49.5 mL of reducing solution. The medium is supplied with CO_2 during preparation to create an anaerobic condition. The ratio of rumen fluid and the medium used was 1:2 (v/v). Each treatment consisted of 300 mg and 30 ml medium in a syringe glass. All treatments were incubated in a water bath at 39 °C for 72 hours, then the fermentation process stopped and was used for further DNA analysis.

2.4. DNA extraction

Total DNA extraction utilised the ZymoBIOMICSTM DNA mini kit No. D4300. Furthermore, to determine the concentration and purity of DNA in the sample, the extracted DNA was validated by electrophoresis on 1% agarose gel using the Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Region V4 of prokaryotes (bacteria and archaea) is amplified by the 16sRNA primer 5'GTGCCAGCMGCCGCGGTAA, GGACTACHVGGGTWTCTAAT3'. PCR amplification was performed as follows: 98 °C for 1 min (1 cycle), 98 °C for 10 sec, 50 °C for 30 sec, 72 °C for 30 sec (35 cycles), and 72 °C for 5 sec. For PCR purification, Qiagen Gel (Qiagen, Germany) was utilised. The TruSeq® DNA PCR kit was used to design the sequence library. Then the sequencing results were calculated using Qubit and Q-PCR through HiSeq2500 PE250.

2.4.1 Sequencing data processing

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

2.4.2 OTU cluster and species annotation

All effective tags are used to analyse the sequences in the Uparse software v7.0.1001 (Edgar 2013). Similar OTUs are obtained with greater than 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).

2.5. Data analysis

The data for rumen microbial abundance were analysed descriptively, while rumen microbial diversity data were taken from the report generated by Next Generation Sequencing Method.

3. Results and Discussion

The microbial composition of each sample was tested for operational taxonomic units (OTU) with 97% identity on a valid label from all samples. A total of 3,977 OTUs were obtained from 16S rRNA gene sequencing. In Figure 1, the curve tends to be flat when the number of sequences reaches 140,000 and is declared valid. However, the curve obtained from the image on each sample is greater than 130,000, and it is stated that the sequence data is sufficient (Edgar 2013).

Adding galangal and cineole essential oils had the same variation in the Shannon index (Figure 2a). The dose of 60 µL of galangal essential oil had the largest population index (chao1 index) compared to the dose of 30 and 120 µL of galangal essential oil and the dose of cineole. Meanwhile, the population index was lowest for the dose of cineole compared to the dose of galangal essential oil and the control. The decrease in the chao1 index in cineole levels was attributable to one of the most dominant types of bacteria. Figure 3 demonstrates that the phylum *Bacteriodetes* was more abundant at the genus level than the dose of galangal essential oil and the control. On the other hand, at a dose of 60 µL of galangal essential oil, the abundance of *Bacteriodetes* phylum was lower than the control and cineole doses. According to Chaucheyras-Durand and Ossa (2014), species diversity is low if the community contains a small number of species and only a few species are dominant.

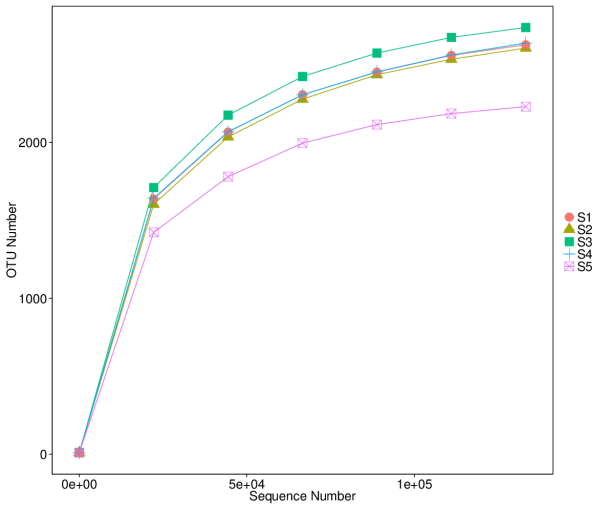

FIGURE 1 Sample feasibility analysis. Each curve represents a sample. Rarefaction curves depict the effect of sequences on the number of identified OTUs in the rank abundance curve. S1: 0, S2: 30, S3: 60, S4: 120, and S5: 5 (pure cineole) µl/300 mg DM of feed

The results of the evolutionary tree analysis at the genus level contained the top 100 selected genera (Figure 3). The relative abundance of each genus within each group is shown outside of the circle, and different colors represent different phyla. In the phylogenetic tree, it has been found that bacteria with high genetic similarity strengthened each other in colonisation in each treatment, e.g., *Prevotella_1* and *Provotellaceae_UCG_001*. Both bacteria had a higher abundance in the control and cineole treatment than all galangal essential oil doses. All doses of galangal essential oil resulted in a greater bacterial population of *Succinivibrio* and *Ruminobacter* than the control and cineole. Closely related genera have the same abundance and activity in the rumen (see Table 2). According to Wang et al. (2019), rumen fermentation is regulated not only by a single type of bacteria but also synergistically by several closely related bacteria. Furthermore, the bacteria form colonisation to degrade feed.

The addition of galangal essential oil at a dose of 60 µL lowered the abundance of the *Prevotella ruminicola* compared to control and cineole doses (Table 2). In this study, 60 µL of galangal essential oil and 5 µL of cineole were equivalent to 24% cineole. However, both had different results on the abundance of *Prevotella* bacteria. In Table 1, the concentration of cineole and phenol is the dominant compound of galangal essential oil. According to Bouarab-Chibane et al. (2019), phenol compounds work more strongly as an antibacterial against Gram-negative bacteria than Gram-positive bacteria. Furthermore, *Prevotella* is a group of Gram-negative bacteria (Zhu et al. 2021), and the hydroxyl group of phenol binds lipids and proteins to the bacterial cell membrane (Álvarez-Martínez et al. 2021). This research is also consistent with Hendry et al. (2009), who found that eucalyptus essential oil has higher antibacterial activity against *Escherichia coli* than cineole compounds which are the main components.

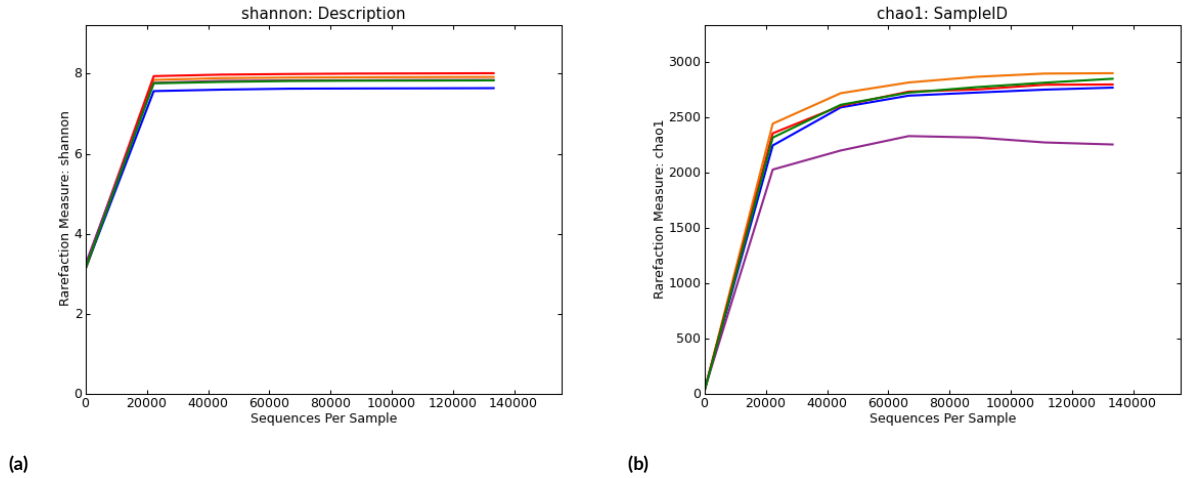

FIGURE 2 Collation results of DNA sequence data and microbial diversity index analyses. (A) Shannon index, (B) Chao1. The numbers represent the unique or common OTUs of each group. S1: 0, S2: 30, S3: 60, S4: 120, and S5: 5 (pure cineole) µl/300 mg DM of feed

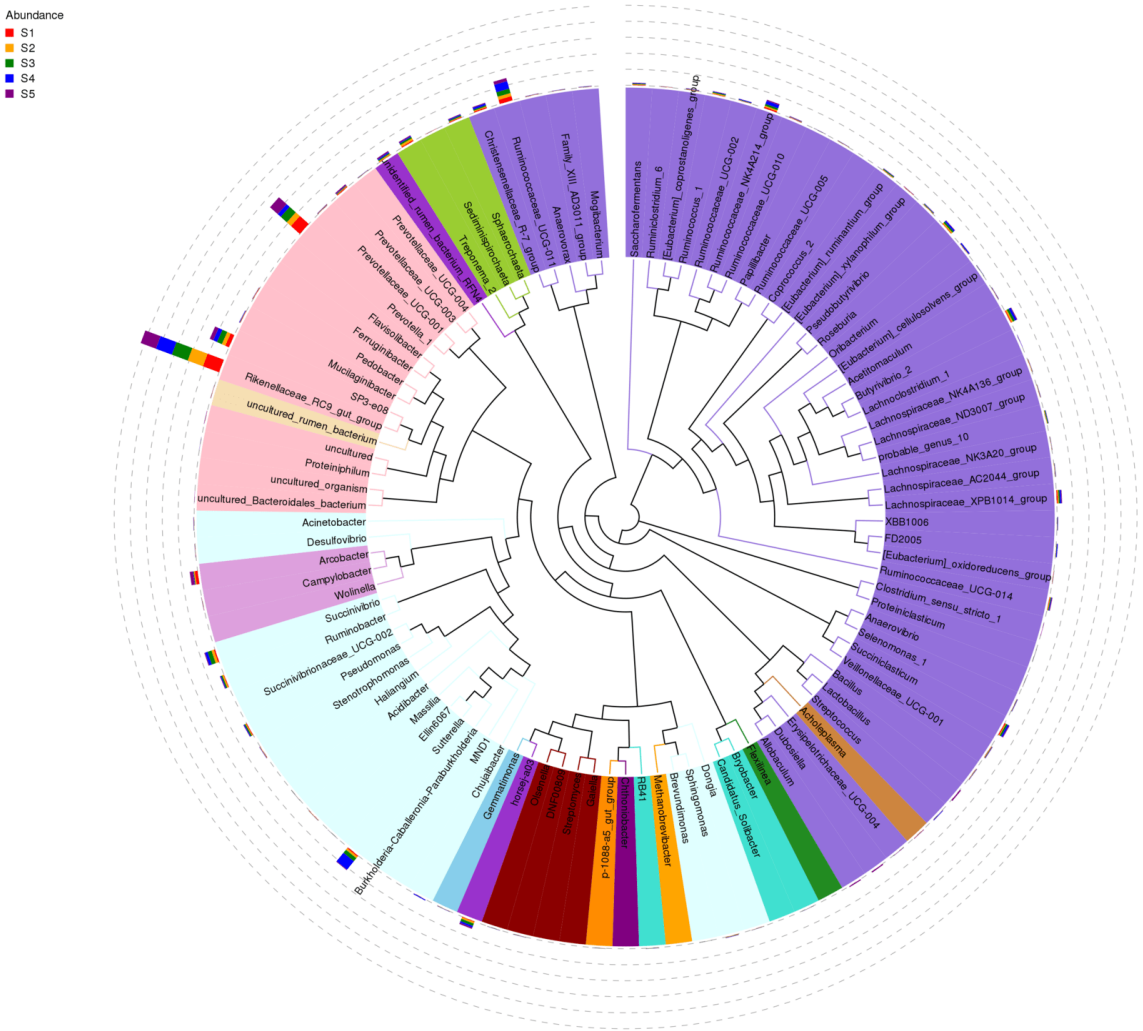

FIGURE 3 The phylogenetic tree on the genus level. Each branch in the phylogenetic tree represents a species, and the branch length is the evolutionary distance between the two species. The histogram outside the circle shows the relative proportion of reads belonging to different species in each group. Different colors of circles represent different phyla

TABLE 2 Effect of galangal essential oil on the relative abundance of the species level (%)

Species	Dose µL/300 mg (DM feed)				
	0	30	60	120	5 (cineole)
Prevotella_ruminicola	3.50	1.47	1.06	1.09	3.01
Arcobacter_skirrowii_CCUG_10374	1.97	0.73	0.33	0.29	2.29
Mucilaginibacter_sp.	0.09	0.02	0.01	0.16	0.02
Pseudomonas_stutzeri	0.14	0.14	0.14	0.12	0.06
Campylobacter_fetus_subsp._fetus	0.12	0.04	0.07	0.07	0.08
Ruminococcus_albus	0.04	0.11	0.06	0.05	0.05
uncultured_*Methanobacteriales_archaeon*	0.04	0.02	0.01	0.01	0.05
Acholeplasma_sp.	0.07	0.04	0.05	0.04	0.08
Wolinella_succinogenes	0.06	0.05	0.05	0.07	0.07
Selenomonas_ruminantium_AB3002	0.02	0.02	0.03	0.05	0.04
Ruminococcus_flavefaciens	0.01	0.02	0.05	0.02	0.01
Lachnospiraceae_bacterium_CG55	0.01	0.02	0.03	0.05	0.01
Ochrobactrum_intermedium	0.02	0.02	0.04	0.04	0.02

It was further explained that the linalool compound had higher antibacterial properties than cineole (Zengin and Baysal 2014). NH_3 levels decreased significantly by 6.10% compared to the control, while there was no difference at a dose of 30 µL compared to the control. The decrease in NH_3 levels in the addition of galangal essential oil indicated a decrease in protein degradation in the rumen by proteolytic bacteria (Kim et al. 2017; Daning et al. 2022b). This result is in line with the population of proteolytic bacteria (*Prevotella ruminicola*) and the level of protease enzymes (Daning et al. 2022a) which decreased with the addition of galangal essential oil. Although there was a decrease in NH_3, there was no significant difference in microbial protein levels in the rumen compared to controls. The decrease in microbial protein occurred with the addition of 120 µL of galangal essential oil dose. This result is due to a decrease in the digestibility of organic matter, VFA levels, and NH_3 levels (Daning et al. 2022b). In almost the same study, namely the addition of eucalyptus essential oil with a cineole content of 85%, NH_3 levels decreased compared to controls (Chouchen et al. 2018).

The addition of galangal essential oil at a dose of 60 L decreased the abundance of the phylum *Bacteriodetes*. The ratio of *Bacteroidetes*: *Firmicutes* has been shown to affect milk yield and is increased in cows fed a high grain diet (Jewell et al. 2015). Among the *Bacteroidetes* lineages, *Prevotella* is the dominant genus, accounting for 40 to 60% of the total 16S rDNA gene sequence (Kim et al. 2017). According to Sofyan et al. (2019), *Prevotella* increased significantly in the high-producing dairy cows. However, according to Xue et al. (2020), a low population of *Prevotella* bacteria correlates with the efficiency of feed protein sources for dairy cows. The use of galangal essential oil can be used as a protein bypass because it can reduce *Prevotella bacteria*, a type of rumen proteolytic bacteria (Jewell et al. 2015). According to Jami et al. (2014), *Bacteriodetes* is the most dominant type of bacteria in the rumen, responsible for degrading several nutrients such as carbohydrates and protein. As a result, gas production decreased significantly ($p < 0.05$) by 28.91% and 43.77% with the addition of galangal essential oil at doses of 60 and 120 µL, respectively. However, there was no difference between $p > 0.05$ in the dose of 30 µL galangal essential oil and 5 µL of cineol compared to the control (Daning et al. 2022b). The same results also occurred in the research of Patra et al. (2017), who found that using eucalyptus essential oil at doses of 40 and 90 µL/300 mg (DM feed) also decreased gas production by 3.86% and 10.35%, respectively. Furthermore, in the study of Chouchen et al. (2018), adding eucalyptus essential oil at doses of 48 and 96 µL/300 mg (DM feed) also caused a decrease in gas production by 46.32% and 49.75%, respectively.

In Table 1, it can be seen that the population of the fiber-degrading bacteria group (*Ruminococcus albus* and *Ruminococcus flavefaciens*) is higher in the dose of galangal essential oil than the control and cineole. Fiber digestibility is not only related to the number of cellulase microbial populations, but the inhibition of cellulase enzyme activity enzymes (Daning et al. 2022a) is also a factor. Although there was no change in the group of fiber-degrading bacteria, the digestibility of fiber decreased at the dose of galangal essential oil (Table 2). The addition of galangal essential oil reduced the total VFA by 4.10% and 11.52% at 60 and 120 µL doses, respectively (Daning et al. 2022b). With the addition of cineol, the levels of acetate and VFA increased. Phenolic compounds from galangal essential oil could affect enzyme activity in degrading feed. According to Surendran et al. (2018), phenol can inhibit cellulase enzyme activity by binding to the active site of the enzyme protein. Phenol also can bind cellulose and hemicellulose (Qin et al. 2016). The acetate content formed results from fiber fermentation in the rumen by *Ruminobacter* and *Ruminococcus* bacteria (Nagaraja 2016).

The production of gas formed in rumen fermentation also correlated with the levels of CH_4 and CO_2 gases. It is known that at doses of galangal essential oil 60 and

120 µL, CH_4 levels decreased significantly ($p < 0.05$) by 39.55% and 53.25% compared to controls, respectively (Daning et al. 2022b). Furthermore, the low methane production correlated with a decrease in the population of uncultured *Methanobacteriales* archaeon with the addition of galangal essential oil (Table 2). According to Kataria (2016), protozoa symbiotically with methanogens convert CO_2 into CH_4. The same effect between cineol and galangal essential oil on protozoa and *Archea* is possible because cineol with a cyclic ether structure can bind polysaccharides and *Archea* cell wall proteins (Jarrell et al. 2013). Furthermore, with its hydrophobic nature, cineole more easily crosses the plasma membrane of the protozoa and causes mitochondrial swelling. This study is almost the same as Colombini et al. (2021) that adding Achillea moschata essential oil with cineole as the main compounds caused a decrease in the genus *Euryarchaeota* by 35.26%.

4. Conclusions

This study concludes that galangal essential oil with the dose of 60 µL/300 mg (DM feed) *in vitro* can effectively reduce the abundance of Prevotella bacteria and methanogens.

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Authors' contributions

DRAD, LMY, BPWB, and CHN designed the study. DRAD contributed in sample collection and reagents. DRAD carried out the laboratory works. DRAD, LMY, BPWB, and CHN analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript

Competing interests

The authors declare no competing interests.

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