The effect of a candidate feed additive derived from the essential oils of Pinus merkusii (jungh. & de vriese) and Melaleuca leucadendra (l.) on the kinetics of gas production and methane emitted during in-vitro ruminal fermentation

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Abstract. The study was designed to determine the effect of a candidate natural feed additive on the kinetics of gas production as a representation of feed degradability and methane produced during rumen fermentation. Three blends of essential oil (BEO) as candidates for feed additives were formulated using pine and eucalyptus essential oils in the following ratios: 75:25, 50:50, and 25:75 for BEO1, BEO2, and BEO3, respectively. Every BEO was added to the batch fermentation system at dosages of 0, 100, and 200 l/l in the medium. Furthermore, an in vitro gas production technique was used to simulate rumen feed fermentation. According to the gas production kinetics, all BEO additives did not affect the total potential gas produced, as well as the potential gas produced from the soluble and insoluble substrate. The rates of gas production were similar among treatments. Furthermore, the addition of BEO did not affect the total volume of gas produced during fermentation. Meanwhile, BEO1 at 200 l/l dose and BEO 3 at 100 l/l dose significantly reduced methane production (P0.05). In conclusion, the BEO1 and BEO 3 at dosages of 200 and 100 l/l, respectively, had the potential as a feed additive to reduce methane production without a negative effect on nutrient digestibility.

1 Introduction

Feed fermentation in the rumen is an essential part of the ruminant feed digestion process. Fermentation of feed nutrients, especially carbohydrates and protein, results in fermentation products such as volatile fatty acids (VFA), microbial biomass, ammonia, and gases, including carbon dioxide and methane. The rumen is one of the compartments of the ruminant digestive tract [1,2]. Ruminants contribute to greenhouse gas emissions that cause global warming since one of the fermentation products is methane.

Methane production indicates a loss of feed energy, which reduces feed efficiency [3,4], therefore, efforts to reduce methane production from feed fermentation in the rumen

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offers two benefits, namely reduced greenhouse gas emissions and increased feed efficiency. The fermentation of feed nutrients in the rumen is carried out by microbes such as bacteria, protozoa, and fungus. Changes in rumen microbial activity, population, and composition will affect the fermentation process, both positively and negatively.

Several studies were conducted to reduce methane and increase feed efficiency, including one on an antimicrobial compound. These studies show that using essential oils (EO) reduces methane production during rumen fermentation. Furthermore, Thymol, a bioactive compound obtained from oregano (Origanum vulgare), was found to reduce methane emissions. However, supplementation up to 300 mg/L decreased nutrient utilization in an in-vitro study [5,6]. Eugenol, a bioactive chemical found in clove (Syzigium aromaticum), was tested for its effects on ruminal fermentation at the same concentration as thymol but did not affect reducing methane emissions or nutrient utilization [7,8]. The efficiency of EO as rumen fermentation modulators varies depending on the source, type and level used in the studies due to high variations in the bioactive compound and each distinct limiting level to modify rumen fermentation [9,10].

The essential oils of eucalyptus and pine contain antimicrobial activity. However, the action of the main components of EO is closely related to its bioactivities [11].

The results of laboratory analysis revealed that the main components of eucalyptus and Pine EO is 1,8 cineole and pinene. Furthermore, 1,8-Cineole, another name for eucalyptol, is a cyclic monoterpene [12], whereas pinene is a monoterpene hydrocarbon [13]. Interaction between EO compounds can have four different effects: indifferent, additive, antagonistic, or synergistic. The combination of 1,8-cineole with monoterpene hydrocarbons and sesquiterpene has shown synergistic and additive effects, respectively [11]. As a result, the combination of eucalyptus and pine essential oils containing 1,8 cineole, and pinene, respectively, is expected to have a synergistic effect on rumen fermentation. The application of a blend EO in rumen fermentation is expected to reduce methane in a lower dosage than applying a single EO because of the synergistic effect, potentially reducing the negative effect on nutritional digestibility. The purpose of this study was to evaluate the effects of a candidate natural feed additive on gas production kinetics as a representation of feed degradability and methane produced during rumen fermentation.

2 Materials and methods

Ruminally cannulated Bali crossbred cows were employed as rumen microbial donors for in vitro feed fermentation and rumen fluid was collected before feeding. Animals were fed Pennisetum purpureum and commercial beef cattle concentrate in a dry matter diet of ratio 60:40. In addition, Pennisetum purpureum, rice bran, and pollard in a 60:20:20 DM base ratio are used as feed material for fermentation substrate.

Three BEO were formulated from pine (Pinus merkusii (Jungh. and de Vriese)) and eucalyptus (Melaleuca leucadendra (L.)) essential oils in the following proportions: 75:25, 50:50, and 25:75 for BEO1, BEO2, and BEO3, respectively. Pure essential oils were purchased from Lansida, a local essential oil store in Yogyakarta, Indonesia.

2.1 In vitro gas production

Feed material in the amount of 300 mg was placed in a 100 ml calibrated syringe glass and mixed with the BEOs according to the treatments. Each treatment was tested in triplicate. Menke [14] described an in-vitro gas production technique for ruminal fermentation. Rumen fluid was collected from fistulated Bali cattle, fed ration of 60:40 forage: concentrate ration. Rumen fluid was collected before morning feeding and carried to the laboratory in prewarmed thermos flasks at 39 °C. It Rumen was filtered through four layers of cheesecloth and flushed with CO2, then mixed with buffered mineral in a 1:2 (v/v) ratio. The buffered mineral solution was prepared by mixing solutions as described by [14]. In addition, a total of 30 mL of buffered rumen fluid was anaerobically transferred to a 100 mL syringe containing feed substrate and incubated for 24 hours in a water bath at 39°C. The volume of gas produced was measured at intervals of 0, 2, 4, 6-, 12-, 24-, and 42-hours during incubation. The Fit Curve program was used to analyze the kinetics of gas production and obtain data on the intercept value at initial gas production (a), gas production from the insoluble fraction (b), potential extent of gas production (a+b), and fractional rate constant of gas production for the insoluble fraction (**c**).

At the end of the incubation period, 5 ml of gas sample was collected for further methane concentration analysis using gas chromatography. Residual feed was collected to measure the remaining organic matter for the calculation of degraded organic matter (DOM)

2.2 Data analysis

The data was analyzed using a randomized complete design and one way ANOVA. Furthermore, Post hoc and statistical analyses were performed using Duncan's Multiple Range Test (DMRT) and SPPSS 18 software, respectively.

3 Results and discussion

According to the data in Table 1, the addition of BEO1, BEO2, and BEO3 at all levels did not affect the total volume of gas produced during the fermentation. It is assumed that the addition of BEOs did not negatively affect nutrient digestibility in the rumen. Since there is a slight correlation between feed digestibility and gas production, the volume of gas produced during in vitro fermentation could represent feed degradability [14]. Data from laboratory analysis revealed that the main active compound in eucalyptus essential oil was 1,8 cineol (43.43 %), whereas in pine essential oil were pinene (24.49 %) and 2-Methyl-3-ethylheptane (19.51 %). The addition of San wormwood essential oil with the main component 1,8 cineole (56,7 %) at a concentration of 125 µl/l did not affect digestibility in the fermentation of soybean meal as a feed substrate. However, it increased digestibility in the fermentation of Bermuda grass, which was observed when the dose was increased to 250 µl/l [15]. The addition of a single essential oil, with pinene as the main component, did not hamper nutrient fermentation in the rumen but instead improved feed degradability. Furthermore, a single essential oil containing pinene has a minor effect on methane production at a dose of 50 µl/l. However, when used as a single essential oil at 1000 µl/l, both pinene and 1,8 cineole reduced the concentration of methane produced during fermentation [17]. In this study, combining both components of the essential oils by mixing eucalyptus and pine oil reduced methane production (P0.05). Table 1 demonstrated that the addition of BEO1 and BEO3 reduced methane production at doses of 200 µl/l and 100 µl/l, whereas BEO2 increased methane production at doses of 100 µl/l.

This study also showed that pinene and 1,8 cineole did not affect feed degradation. According to the gas production kinetics in table 2, the addition of BEO1, BEO2, and BEO3 at all doses did not affect the total potential gas produced (a+b) or the potential gas produced from soluble (a) and insoluble substrate (b). Furthermore, all fermentation produces the same amount of gas. Rosemary essential oil with high concentrations of pinene (23.02 %) and 1,8 cineole (19.08 %) did not reduce dry matter digestibility at doses up to 500 µl/l. However, at higher doses up to 2000 µl/l, dry matter degradability was significantly reduced. Rosemary essential oil reduced methane at higher doses (1500 and 2000 µl/l) [18].

Table 1. Cumulative gas and methane production from in vitro rumen fermentation added with three blends of essential oil.

BEO	Control	BEO1		BEO2		BEO3
Doses (µl/l)	0	100	200	100	200	100
Vol Gas (ml/ 200mg DM)	37,83±2,47	39,17±1,89	36,67±5,03	38,67±2,36	38,67±2,08	36,67 ±2,08
CH_4 (ml/g DOM)	20,16^{ab}±2,66	22,04^{ab}±2,38	14,78^{a}±4,72	26,27^{b}±7,41	20,01^{ab}±0,92	15,76^{a} ±2,47

[ab] different superscript in the same row indicated significant difference ($P<0.05$)
BEO: blend essential oil of pine (*Pinus merkusii) and* eucalyptus (*Melaleuca leucadendra* in ratio BEO1: 75:25, BEO2: 50:50, and BEO3: 25:75

The reduction in methane due to the addition of rosemary essential oil might be caused by the lower substrate concentration for methanogenic bacteria due to the reduction of nutrient degradation. However, those tendencies did not occur in this research. The decreasing of methane production probably caused by the direct effect of BEO on methanogen and methanogenesis.

Table 2. Parameters of gas production kinetics of in vitro rumen fermentation added with three blends of essential oil.

BEO	Control	BEO1		BEO2		BEO3
Doses (µl/l)	0	100	200	100	200	100
Parameter of gas production kinetic						
a	-2,10±1,11	-2,85±1,52	-4,45±1,07	-2,85 ±1,22	-2,75±1,89	-5,35±2,91
b	64,83 ±7,30	74,44±1,52	61,83±2,07	64,55 ±8,85	69,90±4,63	63,76±2,91
c	0,05±0,02	0,04±0,01	0,05±0,01	0,05 ±0,01	0,04±0,01	0,05±0,01
a+b	62,73±6,36	71,59±2,26	57,38±9,98	61,71 ±8,60	67,16±5,43	58,41±1,17

4 Conclusion

In general, the addition of BEO has no negative impact on gas production, as demonstrated by the unaffected nutrient digestibility. BEO 1 at 200 µl/l and BEO 3 at 100 µl/l have the potential to be used as feed additives for methane mitigation.

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