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Research Article
Effects of Lemongrass Leaves as Essential Oil Sources on Rumen Microbial Ecology and Nutrient Digestibility in an *in vitro* System

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Abstract

Background and Objective: Modifying rumen fermentation using plant secondary metabolites has long been used as a nutritional strategy to improve feed efficiency and it leads to higher livestock productivity. An essential oil (EO) is a plant secondary metabolite with volatile characteristics that has various bioactive compounds and plays an important role in manipulating the fermentation process in the rumen and modifying feed efficiency. The aim of the study was to observe the effect of lemongrass leaves (*Cymbopogon citratus*), which are Indonesian herbs containing an essential oil (EO), as potential agents to manipulate rumen fermentation and to observe their effects on the rumen microbial population and nutrient digestibility in an *in vitro* system. **Methodology:** Five inclusion levels of lemongrass leaves (LEM), which were equal to the EO levels of 0, 25, 50, 75 and 100 mg L^{-1} on a DM basis, were added to a diet consisting of king grass and rice bran (40:60). An *in vitro* rumen fermentation method was used to determine nutrient digestibility and anaerobic inoculations were used to determine the total microbial count. All of the treatments were replicated five times and the collected data included the total rumen microbial population count, total protozoa count, dry matter digestibility (DMD), organic matter digestibility (OMD), crude protein digestibility (CPD) and crude fiber digestibility (CFD). **Results:** No difference was observed in the total protozoa count; however, an increase in EO supplemented with LEM decreased the total ruminal microorganism count ($p<0.001$). Furthermore, the inclusion of LEM in the diet affected DMD, OMD and CPD. The inclusion of LEM at 50, 75 and 100 mg L^{-1} decreased ($p<0.01$) OMD by 5.54, 5.24 and 6.17% and reduced DMD ($p<0.01$) by 10.02, 9.26 and 11.38%, respectively. Furthermore, there was considerable evidence that supplementation with LEM inhibited deamination, which resulted in low CPD. Interestingly, CFD was not affected by the addition of LEM. **Conclusion:** The study suggested that LEM caused a reduction in the total number of rumen microbes and that it potentially decreased ruminal digestibility; thus, EO supplemented with LEM should be limited to 25 mg L^{-1}.

Key words: Essential oil, lemongrass leaves (*Cymbopogon citratus*), nutrient digestibility, rumen fermentation, rumen microbial ecology

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Essential oil (EO) has been known to have anti-microbial properties[1] and is likely to beneficially modify rumen fermentation activities to reduce methane production and improve the efficiency of nutrient utilization[2,3]. The hydrophobic characteristics of EOs cause interactions within the cell membrane and cause the EOs to accumulate in the phospholipid bilayer. Prolonged interactions with the membrane cause alterations and an enlargement of membrane structure, they decrease ion transfer and they eventually decrease the ruminal bacterial population[4-6]. However, the anti-microbial activity of an EO varies depending on its chemical structure, its plan origin and its inclusion level, which have different mechanisms in manipulating ruminal fermentation[7,8]. The addition of the EO from thymol to the diet at 2.2 mg L^{-1} reduced methane emissions; however, increased inclusion to the extent of 300 mg L^{-1} reduced not only methane production but also feed efficiency[9]. In addition, the addition of the EO from eugenol did not affect dry matter digestibility (DMD) and crude fiber digestibility (CFD); however, the addition of the EO from thymol decreased DMD and CFD[8,10]. Few studies have investigated the effects of the EO from the lemongrass leaf (*Cymbopogon citratus*) (LEM) on ruminal fermentation[11]. LEM has a 2.1-2.34% EO content that consists of three main EO compounds: geranial (48.1%), neral (34.6%) and myrcene (11.0%)[12], on which further research to identify the effect of the EO addition from LEM on ruminal fermentation is warranted. The current study expected that the addition of the EO from LEM would modify rumen fermentation but would not reduce nutrient digestibility as shown by an *in vitro* method. The objective of the research was to evaluate the effects of lemongrass leaves on ruminal microbial population and nutrient digestibility using *in vitro* ruminal fermentation.

MATERIALS AND METHODS

Chemical analysis: Each feedstuff in the diet and the LEM were milled through a 1 mm sieve in a Wiley mill and the dry matter (DM) content of each was determined by drying the samples at 105°C for 24 h (overnight) while organic matter (OM) was determined by burning the samples in a furnace at 550°C for 8 h. Ether extract (EE) was determined by weighing the samples before and after extraction using petroleum-benzene and drying at 105°C overnight. Crude fiber (CF) was determined by the difference in the sample weight after drying at 105 and 550°C after being dissolved in 1.25% H_2SO_4 and 1.25% NaOH, respectively. The nitrogen (N) content was determined using the Kjeldahl method to obtain the crude protein (CP) content according to AOAC[13]. The EO concentration of the LEM was determined using the steam distillation method described by Bassole *et al.*[12]. The distillation was conducted in a Clevenger apparatus (SIGMA-ALDRICH, Z147893) containing 200 and 500 mL of distilled water that boiled for 6 h. The apparatus was then cooled with cold water until it reached 80°F. The steam and EO that had evaporated remained in the Clevenger apparatus. The steam was cooled to become water and separated from the EO. The concentration of the EO collected from the apparatus was then calculated using the following calculation[14]:

$$\text{Essential oil concentration}(\%)=\frac{\text{Weight of the oil produced}(g)}{\text{Weight of raw material taken}(g)}\times 100$$

The calculated EO concentration was then used to estimate the number of LEM added to each treatment (each calculation is shown in Appendix 3).

***In vitro* ruminal nutrient digestibility:** Samples of each feedstuff and lemongrass leaves were ground and milled through a 1 mm sieve and incubated with rumen fluid in calibrated tubes following the procedures of Theodorou *et al.*[15]. Two ruminally cannulated Ongole crossbred cows were used as donors of the ruminal inoculum. The cows were fed twice daily with a rice bran beef diet containing king grass and rice bran at a ratio of 40-60 (9.94% CP and 88.50% TDN on a dry matter basis). The incubation was conducted in a 125 mL module tube containing 100 mL of equilibrated buffer-rumen solution and five replicates of the samples that each weighed 0.75 g dry weight in the presence (25, 50, 75 and 100 mg L^{-1}) or in the absence of the essential oil (EO) from lemongrass leaves (LEM). The buffered mineral solution[16] was prepared by mixing solutions (listed in appendix 1), flushing them with CO_2, mixing them with the diet samples and prewarming them at 39°C overnight. The buffer-sample solution was then mixed with rumen fluid at a ratio of 9:1 (v/v). The module was flushed with CO_2, sealed with rubber stoppers and incubated at 39°C. The pressure was relieved every 2 h and the incubation was terminated after 24 h. The solid feed residue was filtered and collected to be analyzed in proximate analyses to obtain the DMD, OMD, CPD and CFD data; furthermore, the liquid was separated and used for the total protozoa count.

Total protozoa: Total protozoa were counted using the counting chamber method as described by Diaz *et al.*[17], where 1 mL of the filtered liquid sample after *in vitro* incubation was added into 0.8 mL of formaldehyde saline solution. The ratio of formaldehyde to NaCl was 1:9 (v/v). A 20 µL sample was observed using a 4×4 counting chamber under a light microscope (Tension, Osaka, Japan) coupled to an Optilab and fitted to a computer for counting.

Total ruminal microbial population (TMC): The ruminal microbial population count was conducted using the Hungate[18] procedure. The rumen culture was obtained from two Ongole crossbred cows. The culture media was mixed using the solutions listed in Hungate[18] (listed in appendix 2). Next, 4.5 mL of the culture media was added in five replicates to 10 mL anaerobic tubes in the presence (25, 50, 75 and 100 mg L^{-1}) or the absence of the EO from LEM. The mixture with the culture media was then sterilized at 121°C for 15 min. The culture media was then added to a 10^4 dilution series of 10% rumen culture and incubated at 39°C for 48 h.

Statistical analysis: One-way analysis of variance (ANOVA) was carried out to determine the effect of the EO from LEM on the *in vitro* ruminal nutrient digestibility and ruminal microbial ecology. A post hoc analysis using Duncan's multiple range test (DMRT) was conducted to determine mean differences and significance was declared at $p<0.05$ or $p<0.001$.

RESULTS AND DISCUSSION

Microbial ecology: The total microbial population count decreased ($p<0.001$) with the increasing inclusion level of LEM (Table 1). A greater inclusion level of the EO from LEM, particularly for 100 mg L^{-1} medium, decreased the total microbial population count in the rumen by more than 90%. Similarly, Benchaar *et al.*[19] reported that the total microbial population count decreased with the addition of EOs (thymol, *eugenol*, *guaiacol*, *limonene* and vanillin mixture) to the diet. The results showed that the EO concentration of the lemongrass leaves in the study was 1.30%, which is lower than that reported by Bassole *et al.*[12].

The decrease in the microbial population count in this study is probably due to the presence of *geranial* and *neral*, which are anti-microbial compounds in LEM and have the same characteristics as *thymol*, as both are listed as belonging to *monoterpene* groups. Although the level of ruminal microbe inhibition by *geranial* and *thymol* seems different, there is solid evidence that both have the same anti-microbial mechanism against ruminal microbes, in which the bioactive compounds in the *monoterpenoids* lower the number of ruminal microbes by entering the bacterial cell membrane; hence, the conformation of the membrane structure is modified, resulting in the loss of membrane stability[8,19,2].

EO supplementation from LEM did not affect the total protozoa count ($p = 0.451$) (Table 1). The unaffected protozoa count in this study is consistent with that of previous *In vitro* studies[20], which reported that EO supplementation had no effect on protozoa numbers or activity. This outcome is presumably because the EO components from LEM, *geranial* and *neral* are dose-dependent against protozoa numbers and, presumably, because more than 100 mg L^{-1} LEM is required to act against protozoa. Further studies are needed to test the effect of LEM on the predominant species of rumen bacteria and protozoa to classify which species are affected or unaffected.

***In vitro* ruminal nutrient digestibility:** Significant differences were observed in the dry matter digestibility (DMD), organic matter digestibility (OMD) and crude protein digestibility (CPD) among the treatments (Table 2). However, there was no difference in crude fiber digestibility with LEM supplementation. Increasing LEM inclusion significantly decreased ($p<0.01$) DMD. In this study, the addition of LEM at as much as 25, 50, 75 and 100 mg L^{-1} decreased DMD by 7.54, 15.9, 14.75 and 18.12%, respectively, compared with the control. LEM addition of as much as 50, 75 and 100 mg L^{-1} decreased OMD by 9.71, 9.14 and 10.79%, respectively. Despite the reduction in DMD, the addition of LEM at as much as 25 mg L^{-1} did not decrease OMD compared to the control. The decreases in DMD and OMD are most likely because increasing level of EO from LEM inhibited most ruminal microbial degradation of the nutrient content in the feed, which resulted in a lower DMD and OMD. A similar result

Table 1: Effects of the level of supplementation of LEM on the total number of microbial colonies and protozoa

	LEM supplementation (mg L^{-1} medium)						
Parameters	0	25	50	75	100	SEM	p-value
TMC ($\times 10^4$ CFU mL^{-1})	132.00^e	40.66^d	27.33^c	18.00^b	5.33^a	0.93	0.001
Protozoa ($\times 10^3$ mL^{-1})	100.97	131.44	128.06	137.63	131.20	8.94	0.451

LEM: Essential oil from lemongrass leaves, TMC: Total microbial population in the rumen, $^{a-e}$Means with different superscripts in the same row differ ($p<0.001$)

Table 2: Effect of the level of supplementation of LEM on ruminal nutrient digestibility in an *in vitro* system

	LEM supplementation (mg L^{-1} medium)						
Digestibility (%)	0	25	50	75	100	SEM	p-value
Dry matter	62.76[c]	58.03[b]	52.78[a]	53.50[a]	51.39[a]	1.95	0.001
Organic matter	57.29[b]	55.12[b]	51.73[a]	52.05[a]	51.11[a]	1.41	0.003
Crude protein	41.55[c]	48.88[d]	40.50[b]	41.27[bc]	29.23[a]	0.42	0.000
Crude fiber	50.76	51.31	49.83	50.54	51.76	1.35	0.680

LEM, Essential oil from lemongrass leaves, [a-d]Means with different superscripts in the same row differ ($p<0.01$)

was reported by Kamalak *et al.*[21] who stated that increasing orange oil as an EO source resulted in lower DMD and OMD. Higher concentrations of EO in the diet affect fiber, starch and protein degradability consequently decrease rumen dry matter digestibility[22,23].

There were various differences ($p<0.01$) in CPD among the treatments. CPD increased by 17.64% with the addition of 25 mg L^{-1} LEM, while the addition of LEM at 50 and 100 mg L^{-1} reduced CPD by 2.52 and 29.65%, respectively. There are contradictory results on the effect of EO inclusion on crude protein digestibility[24,19,2]. The reason for the inconsistency of the effect of EOs on CPD is presumably that the effect is dose-dependent and varies depending on the bioactive compound contained in the EO[19,2,25]. From this study, there was a considerable decrease in CPD because of the decreasing concentration of ammonia. Most studies have suggested that the reduction in protein degradation with the addition of EOs is due to their selective antimicrobial action against certain rumen microorganisms, specifically some proteolytic bacteria[26]. Wallace *et al.*[20] also suggested that the possible mechanism of action of EO is the inhibition of amino acid deamination of hyperammonemia-producing bacteria.

There was no significant difference in the crude fiber digestibility (CFD) among the inclusion levels of LEM. Most studies have shown no difference in crude fiber degradability with the supplementation of EO in the diet[10,19]. Hart *et al.*[26] suggested that fibrolytic bacteria may be resistant to the antimicrobial action of EOs. McIntosh *et al.*[27] reported that there was no inhibition of EO on several fibrolytic bacteria, such as *Fibrobacter succinogenes*, *Eubacterium ruminantium* and *Butyrivibrio fibrisolvens*, resulting in no effect on crude fiber digestibility.

This study discovered that LEM supplementation as an EO source improved digestibility and had a minimal impact on the microbial population when supplemented at a 25 mg L^{-1} inclusion level. Furthermore, a greater inclusion level of more than 50 mg L^{-1} reduced DMD, OMD and CPD. The reduction in digestibility was supported by a reduced number of total ruminal microbes; however, which microbial groups are affected by EO supplementation has not yet been identified in the study. Thus, further investigations on the effect of LEM on specific microbial populations should be conducted to identify which microbial groups were affected by LEM as well as *In vivo* studies to determine the proper amount of LEM that should be offered in the diet.

CONCLUSION

Essential oil (EO) supplementation from lemongrass leaves (LEM) had a significant effect on decreasing the total number of rumen microbial colonies, DMD, OMD and CPD. Nevertheless, the EO from LEM had no effect on the total protozoa count and CFD. A lower number of microbial colonies, DMD, OMD and CPD were observed with higher levels of supplementation of the EO from LEM.

Several additional studies should be conducted to provide a better explanation of which specific microbes were affected by LEM and the supplementation level that should be added to the diet, thus providing a valid strategy to manipulate rumen fermentation without affecting digestibility. An *in vivo* study can also be conducted to understand the feasibility of LEM supplementation in cattle diets for the same purpose.

SIGNIFICANCE STATEMENT

This study discovered that lemongrass leaves, which are local herbs from Indonesia containing 1.30% essential oils, improved nutrient digestibility in the rumen and had a minimal impact on the rumen microbial population at the 25 mg L^{-1} inclusion level. However, lemongrass leaf supplementation with an essential oil concentration greater than 50 mg L^{-1} should be limited, as this amount began to alter rumen fermentation and reduce nutrient digestibility by lowering the total number of ruminal microbes. This study will help research on modifying rumen fermentation to improve nutrient digestibility and feed efficiency. Thus, supplementation with EOs has been determined as another nutritional strategy to improve livestock productivity for practical purposes.

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APPENDIX

Appendix 1: Buffer solution in menke and steinngas[23]: Mineral A solution [$Na_2HPO_4.2H_2O$ (MERCK, Darmstadt, Germany), 1.7 g+KH_2PO_4 (Hopkins and Williams Ltd, Essex, England), 1.5 g+$MgSO_4.7H_2O$ (MERCK, Darmstadt, Germany), 0.15 g diluted in 237 distilled water]; mineral B solution [$CaCl_2.2H_2O$ (MERCK, Darmstadt, Germany), 15 mg+$MnCl_2.4H_2O$ (MERCK, Darmstadt, Germany), 1.2 mg+$CoCl_2.6H_2O$ (MERCK, Darmstadt, Germany), 1 mg+$FeCl_3.6H_2O$ (MERCK, Darmstadt, Germany), 0.9 mg diluted in 0.12 mL distilled water] and buffer solution [$NaHCO_3$ (MERCK, Darmstadt, Germany), 8.3 g+NH_4HCO_3 (MERCK, Darmstadt, Germany), 0.95 g diluted in 237 distilled water) raised to 1000 mL with distilled water. The pH and anaerobic conditions were adjusted to 6.5 with 1.2 mL 1% resazurin (SIGMA, Steinheim, Germany) and 49.5 mL reduction solution [2 mL NaOH (MERCK, Darmstadt, Germany), 1 N+285 mg $Na_2S.7H_2O$ (MERCK, Darmstadt, Germany)].

Appendix 2: Culture media in hungate[17]: A total of 0.5 g yeast extract (Merck); glucose, 10 g L^{-1} (Merck); 2 g agar (Merck); 70 mL mineral solution [K_2HPO_4 (MERCK, Darmstadt, Germany), 5.9 mg+KH_2PO_4 (Hopkins and Williams Ltd, Essex, England), 45 mg+$(NH_4)_2SO_4$ (MERCK, Darmstadt, Germany), 90 mg+NaCl (MERCK, Darmstadt, Germany), 90 mg+$MgSO_4.7H_2O$ (MERCK, Darmstadt, Germany), 18.5 mg+$CaCl_2.2H_2O$ (MERCK, Darmstadt, Germany), 11.9 mg diluted in water q.s to 70 mL]; 30 mL clear rumen liquid; 0.5 g cysteine-HCl, 3% (MERCK, Darmstadt, Germany); 1.7 mL Na_2CO_3, 12% (MERCK, Darmstadt, Germany) and 1.7 mL resazurin, 1% (SIGMA, Steinheim, Germany).

Appendix 3: Calculation of lemongrass leaf supplementation: The essential oil content of the lemongrass leaves was 1,3%.

$$1,3\% = 1,3\ g/100\ g = 0,013\ g\ g^{-1} = 0,013\ mg\ mg^{-1}$$

The essential oil concentration in the medium should be equal to 25, 50, 75 and 100 mg L^{-1}.

$$1,3\% = 1,3\ g/100\ g = 0,013\ g\ g^{-1} = 0,013\ mg\ mg^{-1}$$

The supplementation of the lemongrass leaves (on a DM basis) was calculated as follows:

$$Z = (B/A) \times Y$$

Z = $(B/A) \times Y$
Z = Weight of lemongrass leaves added to the medium (mg)
B = Desired essential oil concentration (mg L^{-1})
A = Essential oil concentration of the lemongrass leaves (mg mg^{-1})
Y = Total volume of medium for incubation (mL)

i.e., calculation for LEM supplementation equal 25 mg L^{-1} EO

$$Z = \left(\frac{25}{0.013}\right) \times 100 = 192.3\,mg = 0.1923\,g$$

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