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Strategy to Reduce Methane to Increase Feed Efficiency in Ruminants Through Adding Essential Oils as Feed Additives

Valentine Dhe Brenda[1], Mardiati Zain[2] and Fauzia Agustin[2]

[1]Postgraduate Student, Department of Animal Nutrition, Animal Science Faculty, Universitas Andalas, Kampus Limau Manis, Padang, West Sumatera, Indonesia
[2]Department of Animal Nutrition and Feed Technology, Animal Science Faculty, Universitas Andalas, Kampus Limau Manis, Padang, West Sumatera, Indonesia
***Corresponding author:** mardiati@ansci.unand.ac.id

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ABSTRACT

Increased emissions of methane gas (CH_4) have an effect on global warming and are predicted to continue to increase in line with increasing livestock productivity. This research aimed to obtain the optimum level of using feed additive essential oil (EO) and to know the effect of EO to increase feed efficiency so as to reduce methane gas emissions. The addition of EO to cattle rations was tested experimentally *in vitro*. This research used a Randomized Block Design (RBD) with five treatments and three replications. The treatments were 0, 50, 100, 150, and 200µL citronella oil/200mL of buffered rumen fluid. The variables observed were methane gas production, protozoa population, microbial protein synthesis, rumen fluid characteristics (pH, NH_3, VFA), digestibility of dry matter (DMD), organic matter (OMD), crude protein (CPD) and fiber fractions (Neutral Detergent Fiber, Acid Detergent Fiber, cellulose, and hemicellulose). The results showed that supplementation of citronella oil in rations *in vitro* had no significant differences ($P>0.05$) in ruminal pH, significant differences ($P<0.05$) on VFA, NH_3, NDF, ADF, cellulose, hemicellulose, had a highly significant difference ($P<0.01$) on methane production, protozoa population, DMD, OMD, CPD. Based on the results of the study it was concluded that the addition of essential oil, 50 µL citronella/200 mL buffered rumen fluid (P2), can be used as a rumen modification to reduce methane production and protozoa populations and to increase digestibility *in vitro*.

Key words: Methane, Essential oil, Citronella, Rumen fermentation, *In vitro.*

INTRODUCTION

The high imports of meat and milk to date are due to the low productivity of the ruminants themselves. Various efforts need to be made to overcome these problems such as improving the quality of feed. According to Thomassen et al. (2009) if nutrients are not converted into milk and meat production, it will cause environmental pollution in the form of methane emissions. Production of methane gas from ruminants contributes to 95% of total methane emissions produced by livestock and humans, and about 18% of the total greenhouse gas in the atmosphere (Zain et al. 2011).

Increased emissions of methane gas (CH_4) will have an impact on global warming and get serious attention from environmentalists (Martin et al. 2008). Increased emissions of methane gas (CH_4) are predicted to continue to increase in line with increasing livestock productivity (Restitrisnani et al. 2022). Reducing methane gas production from ruminants is a means to increase feed efficiency. Therefore, it is necessary to approach feed management and rumen manipulation so as to reduce methane gas emissions and improve the efficiency of feed energy use (Ningrat et al. 2017). One way to increase feed efficiency is by using feed additives as rumen modifiers. The rumen modifier is defined as a "feed additive" that alters rumen fermentation, and microbial growth, and has a positive impact (Daning et al. 2020).

One of the rumen modifiers which is used in ruminant animal feed, namely monensin. However, the use of monensin in ruminant livestock rations is feared to result in the production of residues (Daning et al. 2020). Therefore, there is a need for other alternatives as a substitute for antibiotics that are natural and do not cause product residues by utilizing secondary metabolites of plant origin such as saponins, tannins, and essential oils. Secondary metabolites of plant origin have an antimicrobial activity that can fight various microorganisms, such as bacteria,

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viruses, and fungi (Bakkali et al. 2008). Among these metabolites, the use of essential oils has been widely studied in the field of animal nutrition as an alternative to antibiotics (Monteny et al. 2001; Froehlich et al. 2017).

Essential oils are complex mixtures of secondary metabolites and volatile compounds extracted by steam distillation or solvent extraction methods derived from several plant parts, such as leaves, flowers, stems, or seeds (Radwan et al. 2022). The main components of essential oils are terpenoids (α-pinene, α-phellandrene, p-cymene, m-cymene, γ-terpinene, and limonene) and phenolic compounds (carvacrol, thymol, and eugenol) (Patra 2011; Cobellis et al. 2016). Essential oils are known to be used as rumen modifiers which have a positive impact on starch and protein degradation, production of ammonia, volatile fatty acids (VFA), and methane (Tekippe et al. 2013). Essential oil (EO) has been known to have anti-microbial properties and the hydrophobic characteristics of EOs cause interactions within the cell membrane and cause the EOs to accumulate in the phospholipid bilayer (Zulfa et al.2019). Prolonged interactions with the membrane cause alterations and enlargement of membrane structure, decrease ion transfer and eventually decrease the ruminal bacterial population (Dorman and Deans, 2000). The content of bioactive compounds in essential oils can increase the efficiency of energy use by reducing methane gas emissions (Lee et al. 2018; Jimayu 2022).

Until now, the use of essential oils as a rumen modifier is still dominated by those from temperate plants (Daning et al. 2020). Citronella (*Cymbopogon nardus*) is one of the many herbal and aromatic plants in Indonesia and has several active compounds such as geraniol, citronellol, geranyl acetate, and citronellal acetate (Hermawan et al. 2007). Usage of citronella oil in ruminant feed needs to be investigated because there are still limited studies on citronella oil as a feed additive. Based on the description above, it is necessary to conduct research to evaluate essential oil which comes from citronella as a feed additive to increase feed efficiency in ruminants.

MATERIALS AND METHODS

Ethical Approval

Ethical approval was not required because this study did not use any live animals.

Study Period and Experimental Site

This experiment was carried out at the Ruminant Nutrition Laboratory, Faculty of Animal Science, Andalas University, Padang, Indonesia from January to March 2023.

Sampling

The ingredients for the ration consist of ammoniated rice straw and concentrate (rice bran, cassava flour, palm kernel cake, minerals). An experimental method was conducted having a Randomized Block Design (RBD) with five treatments (P1:0µL, P2:50µL, P3:100µL, P4:150µL and P5:200µL citronella oil/200mL of buffered rumen fluid) and replicated three times (Table 1).

The following parameters were observed:

1) Methane gas production, 2) Total population of protozoa, 3) Ruminal pH, 4) NH_3, 5) VFA Total, 6) Microbial Protein Synthesis, 7) Digestibility of Dry Matter (DM), Organic Matter (OM) and Crude Protein (CP), 8) Digestibility of NDF (Neutral Detergent Fiber), ADF (Acid Detergent Fiber), Cellulose and Hemicellulose.

The composition and nutritional contents of the experimental ration have been presented in Table 2.

Table 1: Details of five treatments

Treatment	Citronella oil (µL)
P1	0
P2	50
P3	100
P4	150
P5	200

Every treatment contained a ration and 200mL buffered rumen fluid along with various quantities of citronella oil.

Table 2: Composition and nutritional content experimental ration

Item	%
Rice straw ammoniated	50
Rice bran	22
Cassava flour	12
Palm kernel cake	15
Mineral Mix	1
Nutrition (%)	
DM	90.49
Ash	8.23
Organic matter	91.76
Crude Protein	10.6
TDN	62.72
ADF	49.8
NDF	68.95
Cellulose	15.71
Hemicellulose	19.15

In vitro Method

The *in vitro* trial was carried out according to the procedure set out in the Tilley and Terry method (1963). The *in vitro* process began with taking rumen fluid at the abattoir. Then the rumen fluid was filtered and separated from the dregs. Furthermore, rumen liquor was mixed with buffer in a ratio of 1:4 (McDougall, 1947). A sample of 2.5 grams was weighed and put into a 250mL Erlenmeyer flask. After that added the mixture of rumen fluid (50mL) and buffer (200mL) and graded concentrations of citronella oil were added to the respective bottles. Then the Erlenmeyer tube was tightly closed while flowing CO_2 gas so that it remained anaerobic. The sample was then placed in a shaking incubator at 39°C at 90rpm for 48 hours. When the incubation period ends, the Erlenmeyer tube was immersed in ice cubes to stop microbial activity, followed by measuring the pH. Then centrifugation was carried out to separate the supernatant and residue at 3000rpm at 40°C. The supernatant was used for the analysis of total NH_3 and VFA. To determine the NH_3 concentration, we used the method of Conway and O'Malley (1942), and the measurement of total VFA contents was measured by the steam distillation method (Procedure, 1996). The residue that was filtered with Whatman paper No. 41 and dried in an oven at 60°C in 48 hours, was used to determine the digestibility of DM, OM, and CP. To establish the contents of Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), and cellulose, Van Soest analysis was used

(Goering and Van Soest 1970). The calculation of the protozoa population was done using the methods by Ogimoto and Imai (1981).

Statistical Analysis

The gained data were examined with analysis of variance and if the results were significantly different, they were analyzed with Duncan Multiple Range Test (DMRT) based on the instructions of Steel dan Torrie (1993).

RESULTS

Effect of Citronella Oil Supplementation on Protozoa Population and Methane Gas Production

The addition of citronella oil in rations *in vitro* showed a significant effect (P<0.01) on methane gas production and protozoa populations. Data in Table 3 shows total protozoa ranged from $1.00x10^5$-$3.53x10^5$ cells/mL and methane gas production ranged from 8.7-20.6mL.

Table 3: Effect of citronella oil supplementation on protozoa population and methane (CH_4) gas production

Groups	Protozoa (cells/mL)	Methane gas (mL)
P1 (0)	$3.53\ x10^5$±0.07a	20.6±8.36a
P2 (50)	$2.85\ x10^5$±0.01b	13.8±5.59b
P3 (100)	$2.10\ x10^5$±0.02c	11.7±4.60c
P4 (150)	$1.13x10^5$±0.04d	10.3±3.58d
P5 (200)	$1.00x10^5$±0.04e	8.7±3.06e

DM=Dry Matter, ADF=Acid Detergent Fiber, NDF=Neutral Detergent Fiber, TDN=Total Digestible Energy.

Effect of Citronella Oil Supplementation on Rumen Characteristics (pH, NH_3, VFA Total) and Microbial Protein Synthesis

Based on the observations (Table 4), supplementation of citronella oil with different levels in rations *in vitro* showed no significant difference (P>0.05) in ruminal pH and showed a significant difference (P<0.05) on NH_3, VFA (Volatile Fatty Acid) total and significant difference (P<0.01) on microbial protein synthesis.

Effect of Citronella Oil Supplementation on Dry Matter Digestibility (DMD), Organic Matter Digestibility (OMD), and Crude Protein Digestibility (CPD)

Based on the observations (Table 5), the addition of citronella oil with different levels in rations *in vitro* showed significant difference (P<0.01) in DMD, OMD, and CPD. Data in Table 5 shows DMD ranged from 50.54-62.06%, OMD ranged from 51.78-63.02%, and CPD ranged from 52.09-63.89%.

Effect of Citronella Oil Supplementation on Digestibility of Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), Cellulose and Hemicellulose

The addition of citronella oil in rations *in vitro* showed significant differences (P<0.05) among treatments regarding the digestibility of NDF, ADF, cellulose, and hemicellulose (Table 6).

DISCUSSION

Effect of Citronella Oil Supplementation on Methane Gas Production and Protozoa Populations

Supplementation of citronella oil until a dose of 200µL (P5) can reduce methane gas production and the population of protozoa (Table 3). Essential oils can reduce methane gas production (Joch et al. 2019; Ortiz et al. 2022; Batool et al. 2023) and protozoa population (Singh et al. 2018). Gallegos-Flores et al. 2019 and Ruiz et al. 2021 report that the secondary metabolites of plants are recognized as antimicrobial agents that act against bacteria, protozoa, and fungi. The rumen protozoa population is closely related to CH_4 production in the rumen (Onel et al. 2021). The use of essential oils decreased the population of protozoa, according to research findings from the past (Pawar et al. 2014; Singh et al. 2018). Essential oil from citronella oil (*Cymbopogon nardus*) contains bioactive substances such as citronellal, geraniol, and citronellol, which could lower methane levels. These bioactive compounds are able to reduce gas production by inhibiting the growth of protozoa by binding to cell membrane proteins and then disrupting membrane permeability and damaging membranes by lysing cells resulting in nutrient deficiency cells (Soroor and Rouzbehan 2017). According to Ningrat et al. (2017), the protozoa population is directly proportional to methane gas production. Methanogen bacteria symbiosis with protozoa in the rumen. The decrease in protozoa populations reduces the production of hydrogen gas which most likely disrupts the symbiotic mechanism between methanogens and protozoa cilia due to reduced substrate for hydrogen formation. This indicates that the antibacterial activity has the potential to positively reduce the formation of methane gas by reducing the protozoa population.

Effect of Citronella Oil Supplementation on Rumen Fluid Characteristics (pH, NH_3, VFA) and Microbial Protein Synthesis

Rumen pH did not significantly change with citronella oil supplementation and was in the range of 6.79-6.9. This value is within the normal range of 5.5-7 (Puniya et al. 2015). This study's pH value is within the range of values necessary for optimal rumen microbial activity. In a previous study, an increased essential oil supplementation did not affect pH (Gunal et al. 2013; Kurniawati et al. 2018).

The results showed that citronella oil (*Cymbopogon nardus*) supplementation had a significant (P<0.05) effect on ammonia levels (NH_3). Table 4 shows that the concentration of NH_3 was highest in the P2 treatment of 19.55mg/100mL and the lowest concentration was in the P5 treatment of 12.6mg/100mL. A decrease in NH_3 implies that the rumen is not degrading feed proteins as much. The cause of the decrease in NH_3 concentration in this study was suspected by two things, namely the strong antibacterial activity of essential oils that inhibited the growth of NH_3-producing microbial groups which causes a decrease in the rate of deamination of amino acids (Castillejos et al. 2006) or due to their propensity to coat feed protein, essential oils increase the amount of protein bypass (rumen undegradable protein) and decrease the amount of rumen degradable protein in the rumen (Daning et al. 2020). From the results of this study, the concentration of NH_3 produced from all treatments produces NH_3 above the minimum requirement which ranges from 12.6-19.55mg/100mL and this value is still optimal for rumen microbial growth.

Table 4: Effect of citronella oil supplementation on rumen characteristics (pH, NH_3, VFA Total) and protein microbial synthesis

roups	pH	NH_3 (mg/100mL)	VFA Total (mM)	MPS (mg/mL)
P1 (0)	6.79±0.04	19.12±0.73a	123.3±5.8a	76.77±2.96a
P2 (50)	6.8±0.37	19.55±1.70a	125.0±10a	81.16±6.21b
P3 (100)	6.82±0.06	15.3±2.55b	111.7±2.9b	71.17±2.57c
P4 (150)	6.85±0.06	13.03±3.43c	108.3±10.4c	62.93±2.72d
P5 (200)	6.9±0.06	12.6±2.49c	105.0±5.0c	60.62±0.87e

Each group contained a ration and 200mL buffered rumen fluid along with various quantities (µL) of citronella oil (P1=0, P2=50, P3=100, P4=150, and P5=200). Values (mean±SD) having different superscript letters in a column differ significantly (P<0.01): NH_3=Ammonia, VFA=Volatile Fatty Acid, MPS=Microbial Protein Synthesis

Table 5: Effect of citronella oil supplementation on DMD, OMD and CPD

Groups	Digestibility (%)		
	DMD	OMD	CPD
P1 (0)	61.85±0.86a	62.80±0.84a	63.11±1.78a
P2 (50)	62.06±1.53a	63.02±1.49a	63.89±1.52a
P3 (100)	58.84±0.86a	59.87±0.83a	60.41±2.27a
P4 (150)	55.61±2.51b	56.72±2.44b	57.03±1.48b
P5 (200)	50.54±0.72c	51.78±0.70b	52.09±4.09c

Each group contained a ration and 200mL buffered rumen fluid along with various quantities (µL) of citronella oil (P1=0, P2=50, P3=100, P4=150, and P5=200). Values (mean±SD) having different superscript letters in a column differ significantly (P<0.01): DM= Dry Matter Digestibility, OMD= Organic Matter Digestibility, CPD= Crude Protein Digestibility

Table 6: Effect of treatments on digestibility of NDF, ADF, cellulose and hemicellulose

Groups	Digestibility (%)			
	ADF	NDF	Cellulose	Hemicellulose
P1 (0)	52.44±1.43a	55.96±1.27a	57.36±1.64a	65.12±1.75a
P2 (50)	53.15±2.67a	56.80±2.81a	58.76±2.69a	66.29±3.20a
P3 (100)	50.74±2.00b	53.92±1.85a	55.32±1.46a	62.18±1.63a
P4 (150)	49.36±2.51b	52.37±1.78b	54.19±1.39b	60.21±2.10b
P5 (200)	46.44±2.54c	50.00±1.68c	50.70±2.99c	59.27±2.78c

Each group contained a ration and 200mL buffered rumen fluid along with various quantities (µL) of citronella oil (P1=0, P2=50, P3=100, P4=150, and P5=200). Values (mean±SD) having different superscript letters in a column differ significantly (P<0.01): ADF=Acid Detergent Fiber, NDF=Neutral Detergent Fiber

According to Satter and Styler (1974), the concentration of NH_3 minimum for rumen microbial reproduction requires NH_3 5mg/100mL.

Volatile Fatty Acid (VFA), which mostly consists of acetic acid, propionate, and butyrate, is the product of the fermentation of carbohydrates (Lamid 2010). Based on the results obtained, it is indicated that the addition of citronella oil had a significant impact (P<0.05) on VFA levels. From the research results, it was found that the resulting average VFA ranged from 105-125 mM.

These results corresponded to a linear decrease in VFA production, to the extent that a high citronella oil inclusion rate can be detrimental by reducing VFA concentrations. The decrease in VFA production with the addition of EO is similar to the findings of Zhou et al. 2019. The VFA production results are in the normal range for rumen microbial growth and development. According to Sutardi (1980), the suitable VFA values for optimal growth of ruminal microorganisms are 80-160mM. The decrease in total VFA at P3, P4, and P5 (Table 4) compared to P1 (0µL citronella oil) was thought to be due to the antimicrobial activity of essential oils against starch-degrading amylolytic bacteria. This is consistent with Şahan et al. (2021) stated that supplementation of essential oils could significantly inhibit the fermentative activity of rumen microbes and interfere with the entire feed fermentation process. Essential oils are mixtures of numerous compounds of variable chemical identities, and their effectiveness as rumen fermentation modulators is strongly associated with their composition (Garcia et al. 2020).

The present study shows that increasing the EO level of citronella oil had a significant impact (P<0.01) on microbial protein synthesis (Table 4). According to Table 3, the average microbial protein synthesis ranged from 60.62-81.16mg/mL with the highest microbial protein synthesis in treatment P2 (50µL/200mL of liquid buffer*)* of 81.16mg/mL compared to P1 (0µL/200mL of liquid buffer). Increased ammonia use, fiber digestibility, and microbial protein synthesis may result from an increased rumen microbial population. As a result, the feed can be degraded more effectively, increasing the total digestibility of the feed (Zain et al. 2020). However, there was a decrease in microbial protein synthesis at P3, P4, and P5 compared to P1. This decrease is thought to be due to decreased levels of NH_3 and VFA in the rumen (Table 4) resulting in less optimal microbial protein synthesis. The microbes require ammonia (NH_3) from protein degradation to form protein components of the cell wall (Putri et al. 2021) and VFA as a carbon skeleton for microbial protein formation.

Effect of Citronella Oil Supplementation on Nutrient Digestibility

Citronella oil supplementation had a significant effect (P<0.01) on the digestibility of DMD, OMD, and CPD. Based on Table 5, DMD, OMD, and CPD in treatment P2 (50µL) were higher than all treatments. However, there is a decrease in nutrient digestibility at P3, P4, and P5 together with the addition of citronella oil compared to without citronella oil supplementation (P1). Significant decreases in DMD, OMD, and CPD indicate that only a small amount of nutrients can be digested. According to

Suharti et al. (2018), the greater the digestibility value of the dry matter of the diet, the more nutrients will be digested to satisfy the nutritional demands of livestock. Conversely, the lower the digestibility of dry matter, the fewer nutrients the livestock can use to meet their needs. The decrease in DMD, OMD, and CPD along with the increase in citronella oil levels is thought to be due to inhibition of the activity of bacteria fermenting feed in the rumen. The bioactive compounds contained in citronella as antimicrobials not only have the potential to eliminate harmful microbes such as methanogens and protozoa but also affect the activities of a wide range of microbial groups, such as starch-degrading amylolytic bacteria and protein-degrading proteolytic bacteria (Cobellis et al. 2015).

ADF, NDF, cellulose, and hemicellulose were significantly ($P<0.05$) different (Table 6). From Table 6 we know, treatment P2 (50µL citronella oil) showed the maximum digestibility of the fiber components of all the treatments. Giving up to a level of 50µL citronella essential oil did not interfere with the growth and activity of cellulolytic bacteria so the digestibility at P2 increased. However, there was a decrease in the digestibility of the fiber fraction at an additional dose of >50µL citronella oil (P3, P4, and P5). It is suspected that the threshold for supplementing citronella oil is only up to 50µL citronella oil. The presence of active compounds contained in citronella oil influences the growth and activity of rumen microbes because active compounds such as citronella, geraniol, and citronellol have antimicrobial properties that can interfere with microbial activity digesting fiber such as cellulose. According to Dorman and Deans (2000), citronella oil contains an active ingredient, which has antibacterial properties against gram-positive and gram-negative bacteria. Cellulose-digesting (cellulolytic) bacteria such as *Ruminococcus flaveciens* and *R. albus* including gram-positive bacteria, and in general gram-positive bacteria are more susceptible to the effects of essential oil than gram-negative ones (Davidson and Naidu 2000).

Conclusion

The study's findings suggest that adding essential oil from *Cymbopogon nardus* to ammoniated rice straw-based diets can reduce methane gas production and protozoa populations. Supplementation of the best citronella essential oil at 50µL/200mL of buffered rumen liquid increased digestibility and fermentation characteristics. The continuation of *in vivo* research needs to be investigated to see the effect of adding essential oil from citronella (*Cymbopogon nardus*) for livestock.

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Author's Contribution

Valentine Dhe Brenda, Mardiati Zain and Fauzia Agustin formulated the experimental design and experimental work at the laboratory. Valentine Dhe Brenda drafted the manuscript and did data analysis under the guidance of Mardiati Zain and Fauzia Agustin. The final version of the manuscript was read and approved by all authors.

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