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in

Papachristou T.G. (ed.), Parissi Z.M. (ed.), Ben Salem H. (ed.), Morand-Fehr P. (ed.). Nutritional and foraging ecology of sheep and goats

Zaragoza : CIHEAM / FAO / NAGREF
Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 85

2009
pages 267-272

Efficacy of eucalyptus oil on *in vitro* ruminal fermentation and methane production

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Abstract. The *in vitro* semi automated gas production (GP) technique has been used to evaluate the potential of different levels of eucalyptus (*Eucalyptus citriodora*) oil (EuO, 25, 50, 100 and 150 µl) on total gas and methane production, as well as on rumen fermentation parameters. A total mixed ration (50% roughage:50% concentrate) was incubated with buffered rumen fluid. The EuO decreased GP by 5.3, 24.2, 44.6 and 56.7% with increasing level of oil. Also, the EuO at 25, 50, 100 and 150 µl linearly decreased CH_4 production by 26.0, 46.8, 77.3 and 85.3%, respectively. There were no significant differences among investigated levels of EuO on the dry and organic matter degradation. The efficiency of microbial protein production was estimated by the partitioning factor (PF, estimated as the ratio of truly organic matter degradation and the gas volume produced at 24 h of incubation), expressed as mg/ml. The PF values were 3.79, 3.66, 3.58, 4.97 and 6.92 for control, 25, 50, 100 and 150 µl of eucalyptus oil, respectively. The ammonia-N concentration linearly decreased ($P < 0.05$) when the EuO was included. At 24 h of incubation, protozoa counts were reduced by 29.0, 38.7, 62.9 and 64.5%, respectively by adding 25, 50, 100 and 150 µl of EuO compared to the control. It is suggested that EuO could modify the rumen fermentation and has a potential in methane mitigation, which may be beneficial (at low level) for improving nutrient utilization and animal growth.

Keywords. Gas production – Methane – Rumen protozoa – Degradation – Sheep.

Effet de l'huile d'eucalyptus sur la fermentation ruminale* in vitro *et la production de méthane

Résumé. *La production de gaz (PG)* in vitro *a été étudiée en utilisant la technique semi-automatique afin d'évaluer le potentiel de différents niveaux d'huile d'eucalyptus (*Eucalyptus globulus*) (HEu, 25, 50, 100 et 150 µl) sur la production de gaz total et de méthane, ainsi que d'autres paramètres de fermentation ruminale. Une ration complète (50% de fourrage:50% de concentré) a été incubée en présence du liquide de rumen tamponné. Les résultats ont montré que l'augmentation du niveau de HEu se traduit par une diminution de la PG de 5,3, 24,2, 44,6 et 56,7%. D'autre part, l'HEu aux niveaux 25, 50, 100 et 150 µl a linéairement diminué la production de méthane (26,0, 46,8, 77,3 et 85,3%, respectivement). L'administration d'HEu n'a pas affecté la dégradation de la matière sèche et de la matière organique. L'efficacité de la production de protéines microbiennes a été estimée par le facteur de division (FD, rapport entre la dégradation réelle de la matière organique et le volume de gaz produit après 24 h d'incubation), exprimé en mg/ml. Les valeurs de FD étaient de 3,79, 3,66, 3,58, 4,97 et 6,92 pour le témoin, 25, 50, 100 et 150 µl de l'HEu, respectivement. La quantité de l'HEu ajoutée était inversement proportionnelle aux concentrations d'azote ammoniacal ($P < 0,05$). Par comparaison au témoin (sans HEu), le nombre de protozoaires après 24 h d'incubation a chuté de 29,0, 38,7, 62,9 et 64,5%, respectivement avec les niveaux 25, 50, 100 et 150 µl de l'HEu. Il ressort que l'HEu pourrait modifier la fermentation ruminale et avoir un potentiel pour la réduction du méthane, ce qui encourage à l'utiliser à faible dose pour améliorer l'utilisation des nutriments et la croissance de l'animal.*

Mots-clés. *Production de gaz – Méthane – Protozoaires – Dégradation – Mouton.*

I – Introduction

The eucalyptus is a tall evergreen tree native with many species available which produce a wide variety of oils. Eucalyptus trees can be found in many parts of the world, including Algeria, Australia, China, Egypt, India, Portugal, Spain, the southern United States, and South America. It

is, therefore, important to refer to the actual tree species from which the essential oil is derived to properly understand the properties of an eucalyptus essential oil. For example eucalyptol (1,8-cineole) is the main active ingredient in Eucalyptus oil from *E. globulus.*

Many feed additives have been developed to improve the efficiency of nutrient use by decreasing the total amount of methane or ammonia N produced, among which ionophore antibiotics have been very successful (Hutjens, 1992). However, the risk of the presence of antibiotic residues in milk and meat and its effects on human health have led to its prohibition for use in animal feeds in the European Union in 2006. Industry is seeking alternative additives that would improve the efficiency of nutrient use in the rumen, e.g. essential oil which have received more attention as potential alternatives to growth promoters for animal production. Recently, the *in vitro* studies have demonstrated that essential oil or their components have the potential to favorably alter rumen metabolism (McIntosh *et al.*, 2003; Busquet *et al.*, 2006). McIntosh *et al.* (2003) showed that a commercial blend of essential oils inhibited the rate of deamination of AA and the number of hyper-ammonia-producing bacteria in 48-h *in vitro* batch cultures. There are few experimental data on effects of the eucalyptus essential oil on rumen digestion and fermentation patterns, including rumen methanogenesis. Therefore, the objective of this study was to evaluate the effects of different levels of eucalyptus essential oil on anti-protozoal and methanogenic activities and fermentation pattern *in vitro*.

II – Materials and methods

1. Treatments and experimental design

The Eucalyptus oil (EuO) was obtained from the Distillery Tres Barras Company, Torrinha city, Sao Paulo, Brazil. The EuO was extracted by steam distillation of fresh eucalyptus leaves. Four levels of EuO were investigated: control (no additive), 25, 50, 100 or 150 µl of EuO per 75 ml buffered rumen fluid. A total mixed ration (50% roughage:50% concentrate) was used as substrate incubated with buffered rumen fluid (2:1, v/v) in 160 ml serum bottles for 24 h. The chemical composition of the used total mixed ration was 922.4, 131.0, 718.0, 343.0 and 20.0 g/kg for dry matter, crude protein, neutral-detergent fibre, acid-detergent fibre and ether extract, respectively. Five adult rumen cannulated sheep grazing tropical grass pasture and a supplement based on maize and soybean meal (0.7 kg/100 kg of live weight, 20% crude protein) plus a mineral mixture were used as inoculum donor.

The *in vitro* gas production (GP) assay was carried out using a pressure transducer and data logger (LANA/CENA-USP, Piracicaba, SP, Brazil) for measuring the gas produced in 160 ml serum bottles incubated at 39°C (Mauricio *et al.*, 1999). Ground samples (0.5 g) were incubated in 75 ml of diluted rumen fluid (25 ml mixed rumen fluid + 50 ml of Menke's buffered medium) in 160 ml serum bottles (Longo *et al.*, 2006). Once filled, all the bottles were closed with rubber stoppers shaken and placed in the incubator at 39°C. The bottles were shaken manually after the recording of the gas headspace pressure at 6, 14 and 24 h incubation using a pressure transducer (Theodorou *et al.*, 1994). Methane determination was done in a Shimadzu 2014 gas chromatography equipped with a thermal conductivity detector. Separation was achieved using shincarbon ST micro packed column, helium was the carrier gas with a flow rate of 10 ml/min. The detector and column temperature were 250 and 60°C, respectively. The test of linearity and calibration were accomplished using the standard gas curve in the range of probable concentration of the samples. Methane production at the end of incubation period was estimated from the volume of gas and the gas composition data as "CH_4 = [GP + HS] × Conc"; where CH_4 is the volume (ml) of methane, GP is the volume (ml) of gas produced at the end of the incubation, HS is the volume (ml) of the headspace in the serum bottle and Conc is the percentage of methane in the gas sample analyzed (Tavendale *et al.*, 2005). After termination of the incubation, two bottles content were used for determination of *in vitro* dry and organic matter degradability (DMD, OMD). Another two bottles content were used for determining the partitioning factor (PF, truly OM degradation/gas volume produced in 24 h)

according to Blümmel and Becker (1997) and Blümmel *et al.* (1997). The NH_3-N concentration was measured according to Preston (1995) and protozoa were counted microscopically following the procedure described by Kamra *et al.* (1991).

2. Statistical analysis

Data were subjected to analysis of variance (ANOVA), using the General Linear Model procedure, and significant differences between individual means were identified using Tukey test (SAS, 2000).

III – Results and discussion

The effect of different levels of EuO on gas and methane production is presented in Table 1. The results showed that the increase in EuO concentration resulted in a linear decrease in GP (ml/g DM) from 5.3 to 56.7%. Methane concentration decreased in a linear manner with EuO supplementation at 25, 50, 100 and 150 µl by 26.0, 46.8, 77.3 and 85.3% relative to control, respectively, while the reduction in methane volume was 29.9, 59.8, 82.4 and 90.3% with increasing level of EuO.

Table 1. Effect of different levels of EuO on gas (ml/g DM) and methane production (ml/g DM) *in vitro* for 24 h incubation

Parameters	No additive	Treatments				
		25 µl	50 µl	100 µl	150 µl	SEM†
GP (ml/g DM)	127.3[a]	120.6 [a]	96.6[b]	70.6[c]	55.2[c]	6.4
% GP depression	–	5.3	24.2	44.6	56.7	–
CH_4 (ml/g DM)	13.0[a]	9.1[b]	5.2[c]	2.3[d]	1.3[d]	0.99
CH_4 concentration	10.21	7.5	5.43	2.83	1.79	–
% CH_4 depression	–	29.9	59.8	82.4	90.3	–

†SEM: standard error of difference between means.
[a,b,c,d]Means with different superscripts, within row, are different (Tukey test; $P < 0.05$).

The high degree of unsaturation of EuO likely made it toxic to methanogens (Prins *et al.*, 1972) and resulted in a strong decrease of CH_4 production. The EuO consists of a volatile oil distilled from the fresh leaves and branches top of the eucalyptus tree. The EuO is a rich source of an antiseptic (cineole) and contains substances with strong antibacterial properties. Eucalyptus leaves contain tannins, flavonoids and volatile oils. Inhibitory interactions between terpenes, as well as other plant secondary compounds, may inhibit the activity of rumen protozoa and methanogenic bacteria, therefore, explaining the mitigation of methane emission with EuO. It has been shown that the medium chain fatty acids (FA, C8-C16) cause the greatest reduction in CH_4 production (Dohme *et al.*, 2000). Indeed, besides the specific inhibitory effect on rumen methanogens of saturated medium chain FA (Dohme *et al.*, 2000), reduction of rumen methanogenesis by higher chain FA (mainly C18) was established to be closely related to the degree of unsaturation (Demeyer and Henderickx, 1967). Current results confirm this relationship for long chain FA ($C \geq 20$) present in EuO.

The decrease in CH_4 production with addition of unsaturated fatty acids has been attributed to the fact that these fatty acids can serve as electron acceptors during biohydrogenation in the rumen (Hegarty, 1999). Long-chain fatty acids are non-fermentable and, therefore, may decrease the percentage of CH_4 in the gas produced (Johnson and Johnson, 1995). Van der Honing *et al.* (1981) observed a decrease of 10-15% in CH_4 produced from dairy cows fed 5% tallow or soybean oil. This reduction in CH_4 production was attributed to a decrease in the fermentable substrate rather than to a direct effect on methanogenesis.

The effect of different levels of EuO on PF, DMD and OMD, NH_3-N concentration and protozoa count is given in Table 2. There were no significant differences among investigated levels of EuO on the *in vitro* dry and organic matter degradation. The dry mater degradation was 423.8, 426.2, 436.4, 384.2 and 407.6 g/kg DM for control, and 25, 50, 100 and 150 µl of EuO, respectively. The organic matter degradation was slightly decreased at the high levels of EuO but it did not differ significantly in comparison with the control. There was a wide variation among the EuO supplementation levels in PF values. The highest PF values (4.97 and 6.92) were observed with 100 and 150 µl of EuO, respectively, while the lowest levels of EuO did not differ significantly from the control. The high values indicate a solubilization of feed contributing to the dry matter loss but without contributing to the gas produced, and inhibition of rumen fermentation. Ammonia-N concentration linearly decreased without significant differences. At 24 h of incubation, protozoa counts decreased from 2.2 to 0.8 $\times$ 10^5/ml. The decrease in protozoa count ranged from 29.0 to 64.5% with supplementation of 50 or 150 µl EuO, respectively. The PF was regarded as an index of efficiency of microbial biomass synthesis (EMBS) *in vitro* (Blümmel *et al.*, 1997). The PF reflects substrate-dependent variation in the *in vitro* partitioning of degraded substrate between short chain fatty acids, gases and microbial biomass. Further, it was observed that the PF of the mixed diets had a significant relationship with the microbial efficiency *in vivo*, indicating the possibility of using PF of the diet to influence EMBS *in vivo* (Blümmel *et al.*, 2003). The results of the *in vitro* incubations suggested that EuO concentration of 50 or 200 µl of EuO per 75 ml buffered rumen fluid revealed the maximal CH_4 inhibition without reducing ruminal apparent OMD. As in the current study, fish oil supplementation has been shown to reduce rumen methanogenesis (van Nevel *et al.*, 1974) without effect on ruminal (Keady and Mayne, 1999) or whole tract (Doreau and Chilliard, 1997) fibre digestibility. On the other hand, fat-induced reduction of rumen methanogenesis is accompanied with a shift to increased propionate production (Demeyer and van Nevel, 1995) but often with negative side effects such as reduced level of intake or fibre digestibility (Doreau *et al.*, 1997). However, some researchers suggest that dramatic CH_4 reduction by dietary fat feeding will only occur when fibre digestion is inhibited (Johnson and Johnson, 1995). As a consequence, Mathison *et al.* (1998) concluded that ration-induced reduction in methanogenesis could not be justified when supplementation of these higher cost ingredients does not increase the metabolisable energy content of the diet.

Table 2. Effect of different levels of EuO on partition factor (PF, mg truly digested organic matter/ml gas at 24 h), dry and organic matter degradability (DMD, OMD), NH_3-N concentration (mg/l) and protozoa count (×10^5/ml)

Parameters	Treatments					
	No additive	25 µl	50 µl	100 µl	150 µl	SEM†
PF	3.79^{b}	3.66^{b}	3.58^{b}	4.97^{a}	6.92^{a}	0.65
DMD	423.8^{a}	426.2^{a}	436.4^{a}	384.2^{a}	407.6^{a}	37.8
OMD	421.7^{a}	425.9^{a}	418.8^{a}	361.5^{a}	384.7^{a}	35.4
NH_3-N	160.6^{a}	138.6^{b}	112.2^{ab}	123.9^{ab}	125.2^{ab}	9.32
Protozoa	2.2^{a}	1.7^{b}	1.4^{b}	0.9^{c}	0.8^{c}	7544

†SEM: standard error of difference between means.
^{a,b,c,d}Means with different superscripts, within column, are different (Tukey test; $P < 0.05$).

Decreased methane production is usually accompanied by a reduction in the number of protozoa resulting from the increased dietary fat concentration. Similar results were presented by Machmüller and Kreuzer (1999) who observed depressed methane production when coconut oil was supplemented to the diet. However, the decrease in CH_4 caused by feeding oils also occurs in defaunated animals, implying that the elimination of methanogens associated with protozoa is not the principal cause of the inhibition of CH_4 production (Nagaraja *et al.*, 1997). Indeed, direct toxicity

of oils and pure fatty acids on methanogens has been shown in pure (Prins *et al.*, 1972) and mixed (Dong *et al.*, 1997) cultures studies. Hristov *et al.* (2004) observed a strong antiprotozoal effect of C18:3 derived from linseed oil on a wide range of protozoa species.

IV – Conclusion

The results of this experiment indicate that the EuO have a potential to manipulate rumen fermentation favourably with antimethanogenic and defaunating properties.

Acknowledgements

This work was supported by the Third World of Academic Science (TWAS), the National Council for Scientific and Technological Development (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

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