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Effect of Supplementation of Different Essential Oils on *In-Vitro* Methanogenesis, Fermentation and Digestibility of Finger Millet Straw based Diet in Rumen Liquor of Crossbred Cattle.

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ABSTRACT

In vitro studies were conducted to determine the effect of graded supplementation of commercially available essential oils (Eos) such as Lemon grass oil (*Cymbopogon citrates-LGO)* Gauthiria oil (*Gaultheria fragrantissima - GO)and* Clove oil (*Syzygium aromaticum- CO),* , on *in vitro* methane production and fermentation on high roughage finger millet straw based diet. These three Eos were tested at 11 levels @ 0, 0.66, 1.32, 2.00, 2.67, 3.33, 4.00, 4.67, 5.33, 6.00, 6.67/ µl/ml of incubation medium equivalent to 0, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µl/30 ml of incubation medium . The total gas production increased quadratically (P<0.0001) at a decreasing rate from LGO and GO, while it was decreased at a decreasing rate (quadratic: P<0.0001) from CO. The methane production linearly decreased (P<0.0001) with increasing level of dosage of all the oils used in the study. The total volatile fatty acids (TVFA) concentration increased quadratically (P<0.0001) at a decreasing rate with LGO, while it was linearly increased (P<0.0001) with increasing level of dosage of GO and CO, respectively. The in vitro tru dry matter digestibility (IVTD) was increased quadratically (P<0.0001) at a decreasing rate with increasing level of dosage with LGO, while it was decreased at a decreasing rate (quadratic: P<0.0001) with GO and CO. The methane emission was reduced to the extent of 39 and 4% without compromising the digestibility and fermentation, when compared to control with supplementation of 2.67 and 0.66 µl/ml of incubation medium of LGO and GO, respectively. Hence, LGO can be graded as the best among the three oils tested on high fibre straw based diets in ruminants. The data indicated that LGO was the most promising feed additive which caused maximum methane inhibition and had the minimum adverse effect on feed digestibility.

Keywords: Essential oils, Methane, Rumen fermentation, Finger millet straw based ration, *in vitro* studies

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INTRODUCTION

Rumen is considered to be an anaerobic fermentation vat and is inhabited by various microorganisms viz., bacteria, fungi, protozoa etc., All these organisms participate in the process of digestion in one way or the other and ultimate end products of digestion are volatile fatty acids, microbial protein and different gasses. One among such gasses is methane. Methane is a potent greenhouse gas and has 23 times higher global warming potential than carbon dioxide [1]. Methane represents a loss of 2–12% dietary energy [2], in addition to causing greesn house effect. Therefore, it is essential to minimize methane emission, protect the environment from its greenhouse effect and to divert dietary energy from feeds that is wasted in methanogenesis for production purposes. Plant extracts have been used for centuries for various purposes due to their anti-microbial properties [3]. Several efforts were made to inhibit methane emission in the ruminants by using various feed additives and met with varying degree of success [4-9]. The use of essential oils is in general considered to be safe for modifying rumen fermentation as they are the natural products [10]. The use of essential oil/rich products due to its antimethanogenic and antiprotozoal activity have been reported with encouraging results [11-13]. The *in vitro/ in vivo* studies carried out on methanogenesis on high fiber diets (>70% roughage) simulating most of the feeding pattern under village conditions in many developing countries is either scanty or very limited. Therefore, an attempt has been made in this study to determine the effect of graded supplementation of commercially available essential oils such as Lemon grass oil (*Cymbopogon citrates)* Gauthiria oil (*Gaultheria fragrantissima) and* Clove oil (*Syzygium aromaticum),* on *in vitro* methane production and fermentation on finger millet straw based diet.

MATERIAL AND METHODS

Experimental design with substrates and essential oils

Commercially available essential oils (Eos) such as Lemon grass oil (LO) (*Cymbopogon citrates)* Gauthiria oil (GO)(*Gaultheria fragrantissima) and* Clove oil (CO) (*Syzygium aromaticum),* were used for the study. These three Eos were tested at 11 levels , 0, 0.66, 1.32, 2.00, 2.67, 3.33, 4.00, 4.67, 5.33, 6.00, 6.67 µl/ml of incubation medium equivalent to 0, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µl/30 ml of incubation medium . Three adult crossbred steers of about 300 kg body weight fitted with rumen cannulae maintained in a well-ventilated shed were used in the study for collection of rumen liquor.. Animals were individually fed concentrate mixture (maize 30, groundnut cake 25, wheat bran 42, mineral mixture 2 and common salt 1 parts), finger millet straw (FMS) and green grass (para grass) to meet their nutrient requirement for maintenance [14]. The FMS contained the organic matter (OM) of 933.30 ; crude protein (CP), 52; ether extract (EE), 7.60; neutral detergent fibre (NDF), 694.90; and acid detergent fibre (ADF), 506.80 g kg-1 on a dry matter (DM) basis. The para grass contained (g kg-1) 898.30g of OM; 113.50g CP; 13g EE; 706.20g NDF; and 585.40g ADF on DM basis. The concentrate mixture contained OM content of 936.20; CP, 180.30; EE, 45.30; NDF, 453.90; and ADF, 320.00 g kg-1 on DM basis. Rumen liquor was collected just prior to feeding from the 3-fistulated animals by placing the probe in different positions inside the rumen. The rumen liquor was collected in sterile bottles flushed with CO_2 in a thermos flask maintained at a temperature of 39°C. The rumen liquor was then strained through four layers of muslin cloth and the strained rumen liquor was continuously bubbled with CO_2 to maintain anaerobic conditions for *in vitro* study. The time gap maintained between collection of rumen liquor and its use for *in vitro* incubation was 5 minutes.

The substrate used in the study contained the roughage and concentrate mixture in the ratio of 80:20. The roughage portion of substrate contained FMS and Para grass (90:10).The substrate was milled to pass through 1mm sieve and 200±10 mg was weighed in glass syringes of 100 ml capacity. The incubation medium was prepared and 30 ml was dispensed anaerobically in each syringe [15]. Syringes were incubated at 39°C for 24 h. Syringes with substrate and without any oil was served as control. Four replicates were used for each treatment/dose. Syringes with only medium without any substrate were taken as blank to nullify the fermentation due to the inoculum. Syringes were incubated at 39°C for 24 h, with thorough mixing of the contents manually every one hour from the start of the incubation up to 10 hours, thereafter at 12 and 18 h.

Determination of total gas and methane

The total gas production was estimated after 24 h incubation by the displacement of piston during incubation. The gas produced due to fermentation of substrate was calculated by subtracting gas produced in

blank syringe (containing only the inoculum and buffer without any substrate) from total gas produced in the syringe containing substrate and inoculum. The gas produced in standard syringe (containing standard FMS) was used to check day to day variation in the quality of inoculum. For methane estimation 25 µl gas was sampled from the headspace of syringe in an airtight syringe and injected into Gas Chromatograph (Perkin Elmer, Claurus 500 model) fitted with Flame Ionization Detector (FID) and capillary column (Elite FFAP, Perkin Elmer, USA, 30 meter length and 250 micrometer diameter). Nitrogen was used as a carrier gas with oven temperature at 60°C, injector temperature at 100°C and detector temperature at 110°C.

Determination of Volatile fatty acids

The VFA was analysed as per the method described by Cottyn and Boucque [16]. One ml of the supernatant in each syringe was collected at the end of incubation (24 h) in a microfuge tube containing 0.20 ml metaphosphoric acid (25 ml/100 ml). The mixture was allowed to stand for 2 h at room temperature and centrifuged at 5000×*g* for 10 min. The clear supernatant was collected and stored at −20°C until analyzed. For VFA estimation, 10 µl supernatant was injected in a Gas Chromatograph (Perkin Elmer, Claurus 500 model) fitted with Flame Ionization Detector (FID) and capillary column (Elite FFAP, Perkin Elmer, USA, 30 meter length and 250 micrometer diameter). Nitrogen was used as a carrier gas with oven temperature at 170^{o} C, injector temperature at 270^{o}C and detector temperature at 270^{o}C.

Determination of true dry matter degradability

After 24 h of incubation the contents of the syringes were digested with neutral detergent solution and the feed undigested was recovered on crucibles, washed and dried at approximately 90°C for 16 h. The true dry matter degradability was calculated by subtracting this value from the dry matter of the total substrate incubated in the syringe [17].

Protozoa counts

After termination of incubation, the contents of the syringe were mixed properly and 1 ml of effluent fluid was diluted to 5 ml with 10 % formal saline. Eosin stain (2 %) was added to the rumen fluid at the rate of one drop per 5 ml. After allowing for 5-10 minutes, the contents were mixed thoroughly and the haemocytometer was charged. Total protozoal count was calculated in the entire eight WBC chamber using the technique described by Moir [18].

Chemical analysis

Dry matter, ash, ether extract of the feed samples was analyzed using Association of Official Analytical Chemistry [19] procedure numbers 930.15, 942.05 and 920.39. Total N in samples was determined with an N gas analyzer (LECO FP-528, LECO Corporation, Italy) using an induction furnace and thermal conductivity (procedure number, 990.03). For analysis of fibre fractions, NDF and ADF were assayed without a heat stable amylase and sodium sulphite and expressed inclusive of residual ash, as per Van Soest *et al*., [20].

Statistical analysis

The statistical analysis of data was carried out in accordance with Snedecor and Cochran [21]. The data were analyzed by PROC GLM procedure using the statistical package SAS 9.2 [22] (SAS Institute Inc., USA). Significance of linear and quadratic terms was computed to assess the linear or quadratic effect on study variables.

RESULTS AND DISCUSSION

Effect on total gas and methane production

The results of gas production as affected by different oils viz., Lemon grass oil (LO) (*Cymbopogon citrates)* Gauthiria oil (GO)(*Gaultheria fragrantissima) and* Clove oil (CO) (*Syzygium aromaticum),*are presented in Table 1, 2 and 3. The total gas production (ml/g DM) differed significantly (P<0.0001) among different doses within the oils. The total gas production increased quadratically (P<0.0001) at a decreasing rate

from LGO and GO, while it was decreased at a decreasing rate (quadratic: P<0.0001) from CO. The methane production linearly decreased (P<0.0001) with increasing level of dosage of all the oils used in the study.

The methane production was not affected at the lowest level with all the oils, but there after methane production was decreased with increasing level of inclusion, irrespective of the oil used in the study. The methanogenesis was inhibited to the extent of 8, 10, 39, 36, 40, 49, 50, 51, and 51% with LGO; 4, 5, 9, 18, 21, 30, 49, 50 and 52% with GO and 3, 4, 8, 11, 12, 15, 18, 21 and 25% with CO at 1.32, 2.00, 2.67, 3.33, 4.00, 4.67, 5.33, 6.00, 6.67 µl/ml levels, respectively with LGO, GO and CO. Similar type of observation with gas production and methanogenesis was made with extract of the berries of *Sapindus mukorossi and* peppermint oil ((*Mentha piperita*) at different doses [13, 23].

Total protozoa

The total protozoal numbers decreased at a decreasing rate as the dosage level increased with LGO, CO (quadratic: P<0.0001) and GO (Linear: P<0.0001). The total protozoal numbers were decreased with increasing dose of all the oils and were accompanied with inhibition in methane production indicating that methanogens were associated symbiotically and affecting adversely the methane production. According to Newbold *et al.,* [24] and Hess *et al.,* [25] only a small portion of total methane production is due to the presence of methanogens attached with the ciliate protozoa. Dohme et al. [26] also reported inhibition of *in vitro* methane emission both in defaunated and faunated rumen liquor with coconut oil. However, Machm¨uller *et al.,* [27] demonstrated an increased number of methanogens in defaunated sheep, and suggested that association between protozoa and methanogens does not play an important role in methanogenesis in rumen.

Volatile fatty acids

The TVFA concentration increased quadratically (P<0.0001) at a decreasing rate with LGO, while it was linearly increased (P<0.0001) with increasing level of dosage of GO and CO, respectively. The acetate production (%) decreased (P<0.05) and the propionate production (%) was increased quadratically (P<0.0001) and A/P ratio was decreased (quadratic: P<0.0001) with increasing level of dosage from 0 to 6.67 µl of LGO. The TVFA concentration increased linearly (P<0.0001) at a decreasing rate with increasing level of dosage of GO and CO, respectively. The reduction in methane emission was 39, 9, and 3 % along with increase in in TVFA, propionate production and a decrease in acetate to propionate (A/P) when compared to control with supplementation of 2.67µl of LGO and GO and 0.66 µl of CO, respectively. The rumen VFA was not affected by feeding of lemongrass leaf at the rate of 5 % of diet [28]. The results of these experiments indicated that lemon grass as such could be fed safely to the animals, whereas lemongrass oil can be used only at very low level (167 µl l^{-1}) as feed additive, since higher levels are detrimental for feed fermentation

The methane synthesis in rumen is usually associated with increased propionate production and reduced acetate to propionate ratio [29] and this observation is corroborated well with the results of the present study. However, the methane synthesis with higher *A*/*P* ratio was observed [30], which might be due to accumulation of molecular hydrogen. The stoichiometric calculation of VFA and methane production data [30] also indicated that with increasing concentration of peppermint oil in the reaction mixture, there was a reduced efficiency of hydrogen utilization for the synthesis of VFA and methane. Higher *A*/*P* ratio accompanied with a reduction in *in vitro* methane emission with the extracts of *Syzygium aromaticum* which is rich in essential oils also reported by Patra *et al.,* [12]. Inclusion of extracts resulted in a significant reduction in methane emission, but acetate to propionate ratio increased with no change in TVFA concentration (8). *Busquet et al.,* [31] also observed a decreased TVFA production by inclusion of clove bud oil at 300 and 3,000 mg l^{-1} levels, but at lower levels (3, 30 mg l^{-1}), TVFA concentration was not changed when the substrate used was forage: concentrate in 50:50 ratio. In contrast, Castillejos *et al.,* [32] with 10:90 forage /concentrate ratio reported that clove leaf oil at 5, 50 and 500 mg l^{-1} doses increased total VFA concentration by 32.8–52.3 % and decreased acetate, butyrate proportions and acetate to propionate ratio. According to *Calsamiglia et al.,* [33], the response of EOs was diet dependent and this might be the reason for differences in the response of oils used in the present experiments. The three oils affected rumen fermentation in different manner might be due to some active component(s) differing in these oils which due to their synergistic effects gave different characteristics to these oils.

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Table 1: Effect of lemongrass (*Cymbopogon citrates)* oil on in vitro gas production, methanogenesis and fermentation of finger millet straw based diet with rumen liquor of crossbred cattle.

Parameters	Lemongrass oil (µl ml^{-1} of incubation medium)											SEM	Significance level	
	C	0.66	1.32	2	2.67	3.33	4	4.67	5.33	6	6.67		L	Q
Gas(ml g-1DM)	142.78	187	172	172	169.8	163.3	163.3	161.2	159	152.6	143.9	1.90	P<0.0001	P<0.0001
Methane (ml g-1DM)	24.66	24.52	22.62	22.18	15	15.72	14.88	12.53	12.41	12.2	12	0.94	P<0.0001	0.114
IVTD (%)	55.01	57.92	56.46	55.79	53.68	47.52	45.92	42.32	40.6	39.73	38.91	1.25	P<0.0001	P<0.0001
TVFA (mmol/dl)	3.15	4.34	4.52	4.34	3.6	3.66	3.59	3.33	3.23	2.68	1.78	0.13	P<0.0001	P<0.0001
Acetate (%)	83.17	71.43	71.9	69.98	70.89	69.67	70.19	70.57	64.71	69.5	66.92	0.52	P<0.0001	P<0.05
Propionate (%)	15.56	21.43	20.58	13.54	20.78	19.95	20.61	20.42	22.6	21.77	22.29	0.38	P<0.0001	P<0.0001
Butyrate (%)	10.5	7.14	7.52	16.5	8.33	10.4b	9.19	9.01	12.7	10.7	10.8	0.31	P<0.0001	0.233
A/P ratio	5.38	3.33b	3.49	5.19	3.41	3.49b	3.41	3.46	2.86	3.19	2.99	0.11	P<0.0001	P<0.0001
Total Protozoa (×10^4 per ml)	15.3	14.3	12.8	11.8	9.1	8.9	8.1	7.9	8	7.8	7.6		P<0.0001	P<0.0001

IVTD *in vitro* true dry matter digestibility, TVFA total volatile fatty acids, SEM standard error of mean, L, Q, linear, quadratic effects, respectively, A/P ratio acetate/propionate ratio

Table 2: Effec of gaultheria (*Gaultheria fragrantissima)* oil on in vitro gas production, methanogenesis and fermentation of finger millet straw based diet with rumen liquor of crossbred cattle.

Parameters	Gaultheria oil (µl ml^{-1} of incubation medium)											SEM	Significance level	
	C	0.66	1.32	2	2.67	3.33	4	4.67	5.33	6	6.67		L	Q
Gas(ml g-1DM)	141.78	159.9	147.8	145.8	143.8	137.8	133.7	127.7	125.7	117.6	117.6	2.01	P<0.0001	P<0.0001
Methane (ml g-1DM)	24.66	26.16	23.78	23.32	22.46	20.3	19.56	17.27	12.56	12.44	11.84	0.95	P<0.0001	0.196
IVTD (%)	55.01	54.83	49.16	46.79	46.84	44.47	43.14	45.41	44.78	42.7	42.28	1.25	P<0.0001	P<0.0001
TVFA (mmol/dl)	3.15	4.03	3.44	3.3	3.3	3.06	3	2.9	2.49	2.51	1.45	0.11	P<0.0001	0.079
Acetate (%)	83.17	70.93	69.7	70.58	71.52	70.71	69.25	71.49	69.1	69.28	72.1	0.14	0.988	0.071
Propionate (%)	15.56	19.2c	18.2	20.3	20	19.7	20.9^b	19.4	20.4	20.2	20.1	0.52	0.458	0.091
Butyrate (%)	10.5	9.88	12.1	9.09	8.52	9.59	9.84	9.13	10.5	10.5	7.69	0.38	0.358	0.463
A/P ratio	5.37	2.64	2.99	3.47	3.57	3.59	3.31	3.68	3.38	3.42	3.57	0.31	0.069	0.002
Total Protozoa (×10^4 per ml)	15.3	14.9	13.8	12.8	11.2	11.5	10.8	7.9	6.8	6.8	6.1^a		P<0.0001	0.355

IVTD *in vitro* true dry matter digestibility, TVFA total volatile fatty acids, SEM standard error of mean, L, Q, linear, quadratic effects, respectively, A/P ratio acetate/propionate ratio

Table 3: Effect of clove oil (*Syzygium aromaticum)* oil on in vitro gas production, methanogenesis and fermentation of finger millet straw based diet with rumen liquor of crossbred cattle

Parameters	Clove oil (µl ml^{-1} of incubation medium)											SEM	Significance level	
	C	0.66	1.32	2	2.67	3.33	4	4.67	5.33	6	6.67		L	Q
Gas(ml g-1DM)	141.78	141.8	139.9	138	128.4	126.5	120.7	118.8	111.2	105.5	103.5	2.01	P<0.0001	P<0.0001
Methane (ml g-1DM)	24.66	23.91	23.84	23.56	22.7	22	21.82	20.86	20.16	19.48	18.49	0.63	P<0.0001	0.706
IVTD (%)	55.01	52.01	48.48	48.12	47.5	46.85	45.24	43.23	43.48	42.59	41.6	0.35	P<0.0001	P<0.0001
TVFA (mmol/dl)	3.15	4.3	4.25	4.29	3.7	3.62	3.35	3.36	3.21	3.1a	2.06	0.13	P<0.0001	0.003
Acetate (%)	83.17	74.19	73.41	67.6	74.73	72.65	74.33	74.11	71.03	72.26	66.02	0.60	P<0.0001	0.080
Propionate (%)	15.56	18.6	18.5	15.38	17.2	16.85	15.82	16.96	16.2	13.55	16.02	0.48	0.015	0.009
Butyrate (%)	10.5	7.21	8	17	8.06	10.5	9.85	8.93	12.8	14.2d	18	0.65	P<0.0001	P<0.0001
A/P ratio	5.38	3.99	3.95	4.4	4.35	4.32	4.71	4.38	4.39	5.36	4.12	0.09	0.727	0.065
Total Protozoa (×10^4 per ml)	15.3	13.8	12.9	11.8	11.7	11	10.8	11.2	10.8	10.9	10		P<0.0001	P<0.0001

IVTD *in vitro* true dry matter digestibility, TVFA total volatile fatty acids, SEM standard error of mean, L, Q, linear, quadratic effects, respectively, A/P ratio acetate/propionate ratio

Increase in propionate (per cent) and decrease in acetate (per cent) and consequently decrease in acetate and propionate ratio by these oils could be due to the presence of anti-microbial compounds which could have inhibitory effect on protozoa. The reduced protozoa numbers is sometimes associated with increase in propionate (per cent) and decrease in A:P ratio [25, 27]. The changes in the VFA pattern due to reduction in protozoa population is not always consistent because nature of diet also plays an important role in VFA pattern (Jouany *et al.,* [34].

Effect on true dry matter digestibility

The IVTD was increased quadratically (P<0.0001) at a decreasing rate with increasing level of dosage with LGO, while it was decreased at a decreasing rate (quadratic: P<0.0001) with GO and CO. The suppression in degradability varied and it was to the extent of 14, 17, 23, 26, 28, and 29% with LGO at 3.33, 4.00, 4.67, 5.33, 6.00, 6.67 µl/ml levels; 11, 15, 15, 19, 22, 17, 19, 22 and 23% with GO at 1.32, 2.00, 2.67, 3.33, 4.00, 4.67, 5.33, 6.00, 6.67 µl/ml levels and 5, 12, 13, 14, 15, 18, 21, 21, 23 and 25% with CO at 0.66,1.32, 2.00, 2.67, 3.33, 4.00, 4.67, 5.33, 6.00, 6.67 µl/ml levels, respectively in comparison to control. It seems that these oils had some anti-microbial compounds which might be detrimental to one or the other important rumen microbes. Tannins have been implicated for their inhibitory effect on feed digestion, microbial population and enzymes activity in many experiments [35, 36].

The antimicrobial activity of lemongrass oil has been established by Hammer *et al.,* [37]. Inclusion of LGO in the incubation medium at 0.66 ul/ml level did not induce methane inhibition with positive effect on feed digestibility and other fermentation parameters. The methane emission was reduced to the tune of 39.17% at 2.67 ul level with no adverse effect on fermentation when compared to control. Further increase in the dosage of LGO adversely affected feed fermentation by lower feed digestibility, reduced TVFA, and increased acetate to propionate ratio though it reduced the methane emission.

CONCLUSIONS

Therefore, the results of present study indicated that the Eos have good antimethanogenic activity, but their efficiency varies. The EO which is proposed to be used as feed additive should inhibit methanogenesis without affecting feed digestibility and fermentation. In the present study, the LGO could inhibit methanogenesis without affecting feed digestibility and fermentation at the level of 2.67 µl, but at higher than this concentration, LGO exhibited adverse effects on feed digestibility. Hence, LGO can be graded as the best among the three oils tested on high fibre straw based diets in ruminants. Though these Eos appear to have a potential for commercial exploitation in livestock production, further detailed screening and identification of the active principles is essential since the benefits are demonstrated *in vitro* that too with higher concentrations. However, it remains to be seen whether these concentrations can be used *in vivo* without affecting palatability, rumen fermentation and causing toxicity. The main challenge need to be addressed is the adaptation of rumen microbes to these anti-microbial compounds and their degradation capacity.

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