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Impact of feeding essential oils on feed fermentation and rumen microbial profile in crossbred cattle calves

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ABSTRACT

The essential oils (EOs), viz. ajwain (*Trachyspermum copticum,* AjO), lemongrass (*Cymbopogon citratus,* LO), clove (*Syzygium aromaticum*) oleoresin (CO) and a blend (BEO) of the 3 were screened using *in vitro* gas production test for their potential to mitigate methane production. Inclusion of all the 3 EOs and BEO at the rate of 1.0 µl/ml of incubation medium resulted in a depression in total gas and methane production with a maximum reduction of 91.6 and 97.2% by BEO. Feed digestibility was significantly reduced by all the 3 EOs and BEO as compared to control but was similar among the 4 treatments. The level of total volatile fatty acids, acetic and propionic acids in fermented medium was significantly decreased by inclusion of EOs and BEO. Based on the results of these results, BEO was selected for the feeding trial. Ten crossbred cattle calves (average body weight, 68±5 kg) were divided into 2 groups of 5 animals each and subjected to 2 treatments i.e. diet without (control) and with BEO @ 0.1 mL/kg BW (treated). The diet comprised concentrate mixture (21.9%, CP and 71.1% TDN) and wheat straw in 50:50 ratio. There was no impact of BEO feeding on rumen metabolites and enzyme profile. As assessed by real time PCR, the population of protozoa and *Ruminococcus albus* reduced, whereas, bacteria, fungi, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* remained unaffected by BEO feeding. Methanogen population density tended to reduce by BEO feeding. The results clearly indicated that BEO exhibited antimethanogenic activity in *in vitro* system. By feeding BEO, the decreased population density of protozoa and methanogens in the rumen, the 2 major microbial groups involved in methanogenesis, also supported antimethnogenic property of BEO.

Key words: Calves, Crossbred cattle, Essential oils, Methane, Microbes, Rumen

Methane is an important greenhouse gas (GHG) and its accumulation in the atmosphere is thought to be a key factor in global anthropogenic warming besides carbon dioxide and nitrous oxide (IPCC 2013). Enteric methane emission is one of the main sources of GHG from the livestock sector. This contribution is likely to increase over the next few decades as the population of livestock is growing to meet the increasing demands for meat and milk due to growth of human population and urbanization (Caro *et al.* 2014, Patra and Yu 2015, Abbasi *et al.* 2016). Scarcity of good quality feed in tropics enforces the livestock to thrive on the poor quality roughages like wheat straw, rice straw, sugarcane bagasse etc. so the animal needs more feed per unit of production and emits higher amount of CH_4. Therefore, there is an urgent need to check the methane production with suitable rumen modifiers. Presently, use of plant secondary metabolites (PSMs) as rumen modifiers seems to be one of the most promising strategies to mitigate methane production. PSMs are socially acceptable as they naturally occur in the plants and have minimum risk of side effects. Most common PSMs include, tannins, saponins and essential oils (EOs).

EOs are aromatic liquids, extracted from plants and have specific antimicrobial activity against a wide range of bacteria, yeasts and moulds due to their ability to modify cell permeability in microbes (Giordani *et al.* 2004). Also, EOs are classified as generally recognized as safe (GRAS) food/feed additives. *In vitro* antimethanogenic activity of essential oils (EOs) extracted from various plants is well established (Agarwal *et al.* 2009, Pawar *et al.* 2014, Mbiriri *et al.* 2016). But by inclusion of few of the EOs, methane inhibition is associated with the reduction in feed degradability (Pawar *et al*. 2014, Patra and Yu 2012). Therefore, the present experiment was conducted to test ajwain (*T. copticum*) oil (AjO), lemongrass (*C. citratus*) oil (LO), clove (*S. aromaticum*) oleoresin (CO) individually and a mixture of the 3 to minimize the level of individual oil, possibly to minimize the adverse effects of EOs.

MATERIALS AND METHODS

In vitro *gas production:* Three EOs, viz. ajwain (*T. copticum,*AjO), lemongrass (*C. citratus,*LO) and clove (*S.*

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aromaticum) oleoresin (CO) [supplied by PLANT LIPIDS (P) LIMITED, Cochin, India] individually and a blend (BEO) of the three were tested using *in vitro* gas production test (Menke and Steingass 1988). The blend was prepared by mixing of the three EOs in equal proportion. The inclusion level was 1µL/mL of incubation medium. The substrate used was concentrate mixture and wheat straw in 1:1 ratio. The concentrate mixture (maize, 320; solvent extracted soybean meal, 200; wheat bran, 450; mineral mixture, 20; and salt, 10 g per kg) had organic matter (OM) 924; crude protein (CP), 181; ether extract (EE), 36; neutral detergent fibre (NDF), 279; and acid detergent fibre (ADF), 106 g/kg on DM basis.

Rumen liquor samples collected at 0 h feeding from 2 fistulated buffaloes fed on the same diet were pooled in equal proportion and used as inoculum. Each set of syringes had 2 blank syringes (without substrate) along with each treatment in triplicate and 3 such sets were run to have n=3. The syringes were incubated for 24 h at 39°C with intermittent shaking. After 24 h total gas was measured by recording the displacement of piston in the syringe and subtracted from the gas produced in blank syringes to get net gas production. Methane was estimated by gas chromatograph, Nucon-5765 equipped with stainless steel column packed with Porapak-Q and fitted with flame ionization detector (Agarwal *et al.* 2008). Estimations of the fermented medium were done for NH_3-N (Weatherburn 1967) and volatile fatty acids (VFA) using gas chromatograph (Nucon-5765) fitted with a flame ionization detector and a Chromosorb 101 packed glass column (Cottyn and Boucque 1968, Agarwal *et al.* 2008). After 24 h incubation, the syringe contents were transferred quantitatively by repeated washings with neutral detergent solution (100 ml) in 500 mL spout less beaker and *in vitro* true digestibility (IVTD) was estimated as per the procedure of Van Soest and Robertson (1988).

Animals, diets and experimental design: Ten crossbred cattle calves (average body weight, 68±5 kg) were divided into 2 groups of 5 animals each by completely randomized design. The 2 groups were fed on a diet without any supplementation (control) and with BEO supplementation @ 0.1 mL/kg BW (treated). All the animals were fed on a diet containing concentrate mixture and wheat straw in 1 : 1 ratio. The concentrate mixture comprised wheat bran, 33.0; maize, 32.0; soybean meal, 32.0; mineral mixture, 2.0; and salt 1.0% with OM, 924; CP, 219; EE, 32; NDF, 278; ADF, 92; and total ash 76 g/kg on DM basis. After 21 d of BEO feeding, rumen liquor was collected at 0 h feeding through stomach tube on three consecutive days for estimation of rumen enzyme and microbial profiles..

Enzyme estimation: Whole rumen liquor (30 mL) was treated with carbon tetrachloride (0.2 mL/mL) and 0.4% of lysozyme solution (0.2 mL/mL) as described by Hirstov *et al*. (1999) and Agarwal *et al*. (2000). For the estimation of activities of α-glucosidase and β-glucosidase, p-nitrophenyl-α-D-glucopyranoside and p-nitrophenyl-β-D-glucopyranoside were used as substrates (Shewale and Sadana 1978) and the enzyme activity was expressed as µmol p-nitrophenol released/h/mL. Amylase, avicelase, xylanase and carboxymethylcellulase activities were estimated using starch, avicel, xylan and carboxymethyl cellulose as substrate, respectively, and enzyme activities were expressed as µmol of reducing sugars produced/h/mL (Miller 1959, Agarwal *et al.* 2000). Protease activity was measured using azocasein as the substrate (Iversen and Jorgensen 1995) and was expressed as µg protein hydrolysed/h/mL under assay conditions. The protein content of the enzyme samples was estimated following the method of Lowry *et al*. (1951). Specific activity of the enzymes was expressed as units/mg protein.

Rumen microbial profile

The absolute quantification of rumen microbes was done by real time PCR (qPCR). Extraction of total genomic DNA was done by Qiagen stool kit (Yu and Morrison 2004). To plot the standard curves, the PCR amplicons produced using respective primers of bacteria, fungi, *F. succinogens, R. flavefaciens* (Denman and McSweeney 2006), methanogens (Denman *et al.* 2007), protozoa (Sylvester *et al*. 2004) and *R. albus* (wang *et al.* 2007) were purified, cloned in pGEMT easy vector (Promega) and transformed in *Escherichia coli.* The plasmid with insert was extracted and copy number was calculated (Ritalahti *et al.* 2006). The plasmid was serially diluted to make the standard curve for the respective microbes. The qPCR reactions were performed in a total volume of 20 µL, containing 2 ng of template DNA, 10 µL of 2X kappa SYBR master mix, 0.6 µL of each primer (10 µM) and nuclease free water using a CFX96^{TM} Real-Time System machine (BIO-RAD). The amplification conditions were 95°C for 3 min of denaturation, 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for bacteria, fungi, *F. succinogens, R. flavefaciens*, methanogens, and *R. albus* and 55°C for protozoa for 20sec, and extension at 72°C for 20 sec.

Statistical analyses: The data were analyzed for main effects using independent t-test and means were compared assuming variances are equal as per methods of SPSS 16.0 (2007). For all statistical analyses, probability values less than 0.05 were considered as significant.

REULTS AND DISCUSSION

The gas production (mL/g DM) was significantly lower in all the 3 EOs and BEO as compared to control (Table 1) and the inhibition was maximum by inclusion of BEO (91.6%) followed by AjO (65.3%) and then LO and CO (about 50%). The per cent reduction in methane production (mL/g DM) was 52.9, 69.6, 66.0 and 97.2 and when expressed as mL/g DDM the per cent reduction was 36.9, 54.1, 50.5 and 96.0 by inclusion of LO, AjO, CO and BEO, respectively, as compared to control (Fig. 1). The synergistic effect of the 3 oils is visible remarkably as BEO induced maximum inhibition in *in vitro* methane production. Pawar *et al*. (2014) also tested 7 EOs and found reduction in *in vitro* gas and methane production by all the EOs even

Table 1. Effect of EOs and BEO on *in vitro* feed fermentation

Attribute	Control	LO	AjO	CO	BEO	SEM	P value
Gas (mL/g DM)	165.1^{d}	82.9^{c}	57.2^{b}	76.3^{c}	13.82^{a}	11.40	<0.001
Methane (mL/g DM)	31.2^{d}	14.7^{c}	9.5^{b}	10.6^{b}	0.87^{a}	2.31	<0.001
Methane (mL/g DDM)	49.7^{c}	31.4^{b}	22.8^{b}	24.6^{b}	1.97^{a}	3.62	<0.001
IVTD%	62.8^{b}	47.2^{a}	41.8^{a}	43.1^{a}	43.50^{a}	1.89	<0.001
NH_3-N (mg/dL)	12.19^{b}	10.87^{ab}	8.24^{a}	12.46^{b}	8.42^{a}	0.059	0.032
Volatile fatty acids (mmol/dL)							
TVFA	3.75^{b}	2.19^{a}	1.44^{a}	1.93^{a}	1.17^{a}	0.233	<0.001
Acetic acid	2.60^{c}	1.54^{b}	1.11^{ab}	1.12^{ab}	0.74^{a}	0.163	<0.001
Propionic acid	0.79^{c}	0.30^{ab}	0.20^{a}	0.54^{bc}	0.18^{a}	0.059	<0.001
Butyric acid	0.35	0.35	0.13	0.28	0.25	0.036	0.294
Ac:Pr ratio	3.34^{ab}	5.57^{c}	5.48^{c}	2.10^{a}	4.28^{bc}	0.351	<0.001

Ajwain oil (AjO), lemongrass oil (LO), clove oleoresin (CO) and their blend of essential oils (BEO) in 1:1:1 each included @ 1 µL/mL of inoculum. ^{abcd}Means bearing different supertscripts within a row differ significantly

at the lowest dose of 167 µL/L but percent reduction was variable with different EOs. Yatoo *et al*. (2014) found linear inhibition (P<0.001) in gas and methane production when a mixture of three EOs, ajwain (*T. copticum*), garlic (*A. sativum*) and cinnamon (*C. zeylanicum*) leaf oil mixed in equal proportions was included at graded levels of 0.0 to 2.0 µl/ml of incubation medium. Unlike present results, inclusion of peppermint oil at graded levels reduced *in vitro* methane production linearly but gas production was not affected (Agarwal *et al*. 2009). Patra and Yu (2012) examined 5 essential oils (EOs), namely, clove oil, eucalyptus oil, garlic oil, origanum oil, and peppermint oil, in *in vitro* at 3 different doses (0.25, 0.50, and 1.0 g/L) and observed linear depression in methane production. Kouazounde *et al*. (2016) screened 9 essential oils in *in vitro* batch culture system using *Andropogon gayanus* grass as substrate and found that methane production reduced more than 15% at dosages of 300–1,200 mg/L. Joch *et al.* (2016) examined 11 active compounds of EOs and reported that only 9 of them inhibited *in vitro* methane production. The discussion clearly revealed that the EOs do have antimethanogenic activity but the extent of methane

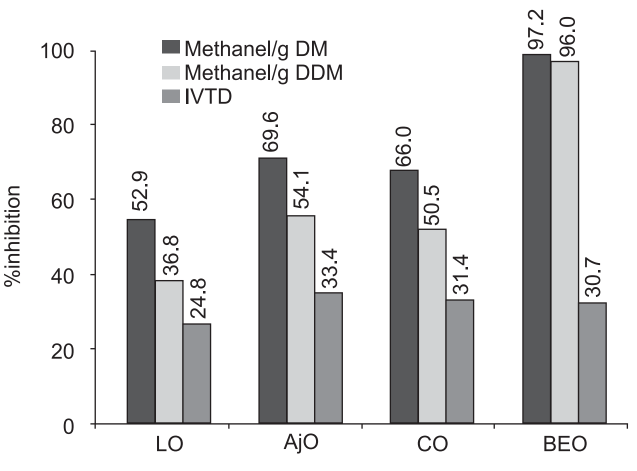

Fig. 1. Percent reduction in *in vitro* methane production and IVTD by inclusion of essential oils LO, lemongrass oil; AjO, ajwain oil; CO, clove oleoresin; BEO, blend of essential oils.

inhibition depends on the type active principles they have.

The IVTD of feed was significantly (P<0.001) reduced by inclusion of 3 individual EOs and BEO as compared to control but was similar among the 4 treatments (Table 1) indicating that though methane inhibition was significantly higher by inclusion of BEO as compared to individual EOs but feed IVTD reduced only up to certain extent irrespective of EOs. The methane inhibition by inclusion EOs was associated with depression in IVTD in most of the studies (Agarwal *et al*. 2009, Pawar *et al.* 2014, Yatoo *et al*. 2014, Patra and Yu 2012, Kouazounde *et al*. 2016)

The levels of total volatile fatty acids (TVFA), acetic acid and propionic acid in the fermented medium (P< 0.001) decreased significantly by the inclusion of EOs and BEO but butyric acid remained unchanged. Acetate to propionate ratio was similar in control, CO and BEO but was significantly higher with LO and AjO as compared to other 3 groups (Table 1). The concentration of NH_3-N was significantly low in the fermented medium by inclusion of AjO as compared to control but was comparable with LO which reflects some modification in nitrogen metabolism (Cardozo *et al*. 2004). Similar changes in TVFA were inclusion of EOs were observed by Agarwal *et al*. (2009), Pawar *et al.* (2014), Rira *et al.* (2015). But all these changes in feed digestibility reveled that feed fermentation was not only affected by type of EOs but also their doses. Like other fermentation characteristics, change in NH_3-N is also type and dose of EO dependent (Pawar *et al*. 2014).

In *in vitro* studies, inclusion of BEO resulted in highest reduction in methane emission with other fermentation parameters at par with individual EO therefore BEO was selected for the feeding trial to study the effect of BEO on rumen fermentation, enzyme and microbial profiles in young male crossbred calves. The feeding of BEO did not affect DMI. Samal *et al*. (2016) also found no change in intakes of DM and OM by dietary supplementation of AjO and LO mixed in equal proportion, at the rate of 0.05% of dry matter intake in fistulated adult buffaloes. DMI was not affected by eucalyptus (*E. camaldulensis*) crude oil supplementation in rice straw based diet of swamp buffaloes (Thao *et al.* 2014). However, the DMI was higher in cows fed a blend of EOs (1.2 g/cow/day) as compared to control (Kung *et al*. 2008). Above discussion indicated that DMI is more sensitive to the dose rather than type of EO.

BEO feeding to crossbred calves did not influence rumen environment as there was no change in pH, concentration of ammonia nitrogen volatile fatty acids, activities of major fibre degrading enzymes like avicelase, carboxymethyl cellulase, xylanase, glucosidases and amylase (Table 2). No change in pH by feeding of EO @ 1 g/day but increased pH at the dose of 2g/day was reported by Beauchemin and McGinn (2006) and Benchaar *et al*. (2006) in cattle. No changes in rumen pH, TVFA and NH_3-N by feeding EOs have been reported in majority of studies (Anantasook *et al*. 2013, Thao *et al*. 2014, Samal *et al*. 2016). Supplementation of BEO in the diet of calves had no effect on population density of total bacteria, fungi, *F. succinogenes,* and *R. flavefaciens,* whereas, the population of protozoa and *R. albus* decreased (P<0.05). Methanogen population was also tended to decrease (P=0.074) by BEO feeding (Table 3). The effects of PSM on fibre degrading microbes are not always consistent. Thao *et al*. (2014) reported that EuO supplementation did not affect the bacterial population in swamp buffaloes fed rice straw or urea treated rice straw. Samal *et al*. (2015) also observed similar type of variations among different rumen microbial groups. Difference in the sensitivity of methanogens, fungi and protozoa populations to a blend of EOs (CRINA) feeding was reported by Tomkins *et al*. (2015). Protozoa as H_2 producers have a prominent position in methanogenesis and their close physical association with methanogens, favours H_2 transfer from former to later (Morgavi *et al.* 2010). In the present study both protozoa and methanogens populations were decreased by BEO feeding which is an indicative of anti-methanogenic property of BEO.

Table 2. Effect of BEO supplementation on rumen fermentation pattern in crossbred calves

	Control	BEO	P value
DMI/100 kg BW	2.86±0.109	2.95±0.100	0.584
pH	6.85±0.064	6.81±0.065	0.638
NH_3-N (mg/dL)	8.43±0.217	8.88±0.269	0.211
Lactic acid (mg/dL)	1.38±0.040	1.46±0.057	0.231
Volatile fatty acids (mmol/dL)			
Acetic acid	5.43±0.727	5.29±0.587	0.882
Propionic acid	1.50±0.191	1.40±0.160	0.688
Butyric acid	0.819±0.155	0.739±0.136	0.706
TVFA	7.75±1.05	7.43±0.860	0.815
Ac/Pr	3.59±0.128	3.81±0.140	0.268
Enzyme specific activity (units/mg protein)			
Avicelase	4.61±0.535	3.52±1.13	0.394
CMCase	11.45±2.29	9.59±2.44	0.587
Xylanase	21.57±0.90	19.76±1.24	0.144
Amylase	79.26±8.24	55.97±8.57	0.070
α-glucosidase	4.59±0.869	4.33±0.724	0.821
β-glucosidase	1.16±0.158	0.917±0.294	0.478
Protease	5.12±1.27	4.59±2.75	0.864
Urease	2.47±0.370	2.52±0.642	0.942
Acetyl esterase	17.30±2.07	11.58±3.40	0.173

BEO: ajwain oil+ lemongrass oil+ clove oleoresin in 1:1:1 ratio; units: avicelase, CMCase and amylase: µmol glucose/h/mL; xylanase: µmol xylose/h/mL; á-glucosidase, â-glucosidase and acetyl esterase: µmol p-nitrophenol released/h/mL; protease: µg azocasein hydrolysed /h/mL; urease: µmol NH_3-N/h/mL

Table 3. Effect of BEO supplementation on rumen microbial profile in crossbred calves

Microbes	Control	Treatment	P value
Bacteria	10.89±0.054	10.77±0.212	0.602
Fungi	5.38±0.165	4.98±0.148	0.125
Methanogens	7.20±0.085	6.96±0.072	0.074
Protozoa	5.61±0.076	5.16±0.158	0.042
Fibrobacter succinogenes	7.75±0.173	7.69±0.102	0.803
Ruminococcus flavefaciens	6.81±0.178	6.48±0.145	0.207
Ruminococcus albus	5.91±0.099	5.44±0.063	0.008

BEO: ajwain oil+ lemongrass oil+ clove oleoresin in 1:1:1 ratio

Our results indicated that mixture of EOs (BEO) comprised of equal proportion of oils of *Trachyspermum copticum*, *Cymbopogon citrates* and *Syzygium aromaticum* oleoresin was more effective in suppressing *in vitro* methane production as compared to individual oils might be due to the synergistic effect of the three. BEO feeding did not affect rumen metabolites, enzymes and microbial profile except inhibition in protozoa and methanogen population which are the positive signs for an effective feed additive to mitigate methane production in buffaloes. Since the changes in rumen fermentation pattern are very much dependent on doses of BEO therefore, optimization of dose is utmost important to maximize beneficial effects and minimize adverse effects.

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