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Effect of combination of essential oils on *in vitro* methane production, volatile fatty acid fractions and feed digestibility with goat rumen flora

RAVINDRA KUMAR[1]✉, J K SINGH[1], K GURURAJ[1] and S GUPTA[1]

ICAR-Central Institute for Research on Goats, Makhdoom, Uttar Pradesh 281 122 India

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ABSTRACT

Different combination of garlic oil (GO from *Allium sativum L.*), clove oil (CO from *Eugenia* spp.) and peppermint oil (P from *Mentha piperita L.*) as GO+PO (1:1), GO+CO (1:1), CO+PO (1:1) and GO+PO+CO (1:1:1) was evaluated in *in vitro* gas production test for their effect on total gas production, methane production, dry matter organic matter digestibility and volatile fatty acid fractions. The addition level was low (D_1) at 0.25 g/L, medium (D_2) at 0.5 g/L, and high (D_3) at 1.0 g/L of fermentation medium. Concentrate mixture and bengal gram straw (*Cicer arietinum L.*) in 40:60 ratio was incubated with incubation medium containing goat rumen liquor for 24 h at 39°C in glass syringes. Three goats fed with concentrate pellet and Bengal gram straw served as donor of the rumen liquor. *In vitro* fluid *p*H varied between 6.50 to 6.59. Total gas production (mL/g DM) in control group was 154.07, at lower dose level (0.25 g/L) there was increase in gas production which significantly decreased at highest dose level. There was significant ($P<0.05$) decrease in the methane production (mL/g DM and mL/g DDM) on GO and PO supplementation together. At low dose level (D_1, 0.25g/L) there was no statistically ($P>0.05$) significant difference in *in vitro* dry matter and organic matter digestibility with the addition of different EO combinations evaluated as compared to control except PO+ CLO (1:1). At dose level (D_2 and D_3) GO+PO combination led to a moderate decrease in the in IVDMD and IVOMD along with decrease in *in vitro* methane production. Fraction of propionic acid was higher on addition of garlic and peppermint oil together. There was relative decrease in the methanogens population with bacteria as housekeeping gene as expressed by RT-PCR amplification with GO+PO addition. Present study concluded that among the EOs combinations *in vitro* tested, garlic and peppermint oil combination (GO+PO) has better potential in reducing *in vitro* methane production with lower effect on *in vitro* digestibility.

Keywords: Essential oils, Goat, Methane, Real time PCR, Volatile fatty acids

Essential oils are plant secondary metabolites, reported to affect the rumen fermentation and methane emission in ruminants (Permata *et al*. 2023). The term "essential" derives from "essence," which means smell or taste, and relates to the property of these substances of providing specific flavors and odours to many plants. The main active compounds of essential oils usually belong to two main groups—terpenoids and phenylpropanoids (Calsamiglia *et al*. 2007) but the composition of EO also includes, although in lower concentrations, substances such as alcohols, acids, acyclic esters, aldehydes, etc. (Benchaar *et al*. 2008). Garlic oil contains organo sulfur compounds (alliin and allicin), clove oil contains phenyl propanoid (eugenol) and peppermint oil contains monoterpinoid monocyclic non phenol (menthol). Because of their antimicrobial properties, essential oils affect ruminal microbial populations and have the potential to reduce methane emissions (Cobellis *et al*. 2016). Most of the EOs evaluated has found to be inhibiting the methanogenic archaea and methane production along with adverse effects on fiber digestion and fermentation (Calsamiglia *et al*. 2007, Patra and Saxena 2010). Essential oils produced by different plant species have different chemical structures, stereochemistry and their functional groups which affects their bioactive activities. Kumar *et al.* (2009) and Agarwal *et al*. (2009) studied effect of inclusion of Eucalyptus oil and peppermint (*Mentha piperita)* oil, respectively with buffalo rumen flora and reported reduction in *in vitro* methane production along with digestibility. Singh *et al*. (2023) comparatively evaluated the effect of different EOs (garlic, peppermint and clove) having methane reduction properties with goat rumen flora and reported good methane reducing effect of garlic oil. All these oils behave differently in manipulating the rumen microbial populations and fermentation. Combination of these essential oils might have different activity towards rumen fermentation and digestibility. Therefore present study was conducted to evaluate the combination of essential oils on total gas production, methane production, and volatile fatty acid production and feed degradability in *in vitro* gas production test.

Present address: [1]ICAR-Central Institute for Research on Goats, Mathura, Uttar Pradesh. ✉Corresponding author email: ravindra.srivastava@gmail.com

MATERIALS AND METHODS

Feed for IVGPT and rumen inoculum: Substrate (0.2 g) for *in vitro* gas production test was gram straw *(Cicer arietinum*) and concentrate mixture (60:40). They were fermented for 24 h with nutrient buffer (30 mL) in 100 mL capacity syringes for *in vitro* gas production test as per Menke and Steingass (1988). Rumen liquor was collected from the three adult male Barbari goats (Bwt. 36.53±1.4 kg and 1-2 years of age) by stomach tube method. These animals were fed with 400 g concentrate pellet feed and *ad lib.* gram straw. Water was available free choice. The rumen liquor was sampled just before feeding (0 h) from all three these animals and transported in insulated flasks under anaerobic conditions to the laboratory, pooled in equal proportions and used as a source of inoculum. Concentrate mixture was prepared by mixing Barley 60%, Wheat bran 15%, Alsi cake 22%, and Mineral mixture 2%, Salt 1%. These samples of individual feed ingredients were dried at 65°C for 48 h, ground in laboratory; Wiley mill, passed through 1 mm screen, and mixed evenly. Gram straw was similarly dried ground and passed through 1 mm screen before using in IVGPT. Substrates were analyzed for proximate composition as per AOAC (2006). The NDF and ADF contents were analyzed as per Van Soest *et al*. (1991).

In vitro gas production test: Accurately weighed 0.08 g concentrate mixture and 0.12 g straw (0.2 g substrate) was taken in the graduated 100 mL calibrated glass syringe with the help of weighing boat with removable stem, so that the sample was put at the bottom of the syringes without sticking to its wall. The piston was greased with paraffin soft while LR (Hi media laboratories Pvt. Ltd. 39-56°C) up to the mark on it and pushed into the barrel of the syringes. Each essential oil was tested at 0.0 (control), 0.25 g, 0.5 g and 1.0 g/L fermentation medium. For testing of each essential oil sixteen syringes (4 syringes for each dose level) were prepared was prepared along with two syringes of substrate blank (without substrate), two syringes as standard (Subabul leaves). Two set of syringes were prepared one was used for methane and fermentation metabolites and second one was used for estimation of digestibility. Syringes were incubated at 39°C for 24 h in water bath.

Essential oils: Essential oils (EOs), i.e. garlic oil (GO from *Allium sativum L.*), clove oil (CO from *Eugenia* spp.) and peppermint oil (PO from *Mentha piperita L.*) were purchased from Merck Sigma-Aldrich (Merck Sigma-Aldrich, Darmstadt, Germany).These oils were kept at 4°C till used. Control was without EOs (D0) and different combinations were GO+PO (1:1), GO+CO (1:1), CO+PO (1:1) and GO+PO+CO (1:1:1) and evaluated at 3 doses: low (D_1) at 0.25g/L, medium (D_2) at 0.5 g/L, and high (D_3) at 1.0g/L of fermentation medium, respectively.

Estimation of fermentation metabolites: After 24 h of incubation at 39°C in water bath, displacement of the syringe piston indicated gas production. The reading of blank was subtracted to calculate gas and methane production from the substrate. From the head space of each syringe 100 µl gas was collected by purging the silicon tube and injected in gas chromatograph (Clarus 580 Perkin Elmer) equipped with stainless steel column packed (porapak-Q) and Flame ionization detector for the estimation of methane. The standard calibration gas (Sigma gas and Services, New Delhi) consisted of 30.50% carbon dioxide, 31.16% methane and rest is hydrogen. The flow rates for nitrogen, hydrogen and air were 30, 30 and 320 mL/min. respectively. Temperatures of injector, oven and detector were 50°C, 40°C and 50°C, respectively. After that contents of the test syringes were used *p*H measurement, for sampling DNA extraction and VFA analyses. For volatile fatty acid 1 mL of the supernatant was mixed with 0.2 mL of 25% meta phosphoric acid and after 2 h, centrifuged at 5000×*g* for 10 min. From the clear supernatant 1 µL was injected into Clarus 580 Gas chromatograph from Perkin Elmer equipped with FID and capillary column. Temperature of injection port, column and detector was set at 200, 180 and 210°C, respectively. The flow rate of carrier gas (nitrogen) through the column was 40 mL/min; and the flow rate of hydrogen and air through FID was 30 and 300 mL/min, respectively. Different VFAs of the samples were identified on the basis of their retention time and their concentration (mM) was calculated by comparing the retention time as well as the peak area of standards (Cottyn and Boucque 1968).

Estimation of feed digestibility: The content of other set of syringes was transferred to spout fewer beakers by repeated washing with 100 mL neutral detergent solution. The flask contents were refluxed for 1 h and filtered through pre-weighed Gooch crucibles (Grade G1). The dry matter content of the residue was weighed and *in vitro* true digestibility of feed was calculated as follows (Van soest and Robertson 1988).True digestibility (TD) = (Initial DM of feed taken for incubation -NDF residue)/ (Initial DM of feed taken for incubation) × 100.The residue was ashed at 550°C in muffle furnace and *in vitro* organic matter digestibility was calculated.

Quantification of methanogens: DNA was extracted from the *in vitro* fluid as per CTAB DNA extraction method using Zirconium beads (CSIRO labs) and quantification of bacteria and methanogens was carried out using reeal time PCR with their specific primers. Primers designed for the detection of methanogens targeted the methyl coenzyme-M reductase *mcrA* gene (Denman *et al*. 2007) (*qmcr*, A-F: 5-TTCGGTGGATCDCARAGRGC-3; *qmcr*, A-R: 5-GBARGTCGWAWCCGTAGAATCC-3). Primers targeting the 16S rDNA gene (Maeda *et al*. 2003) were used to detect total bacteria (BF: 5-GTGSTGCAYGGYTGTCGTCA-3; BR: 5-ACGTCRTCCMCACCTTCCTC-3). The amplification reaction mixture (20 µL) comprised SYBR green mix (Luna-universal qPCR master mix from New England Biolabs Inc. United states, cat#M3003L) (10 µL), primers* (forward and reverse, 1 µL each), PCR grade water (7 µL)

and template (1 µL) conc. of minimum 100 ng/µL. The assay was conducted with the following cycle conditions: one cycle at 50°C for 2 min. and at 95°C for 5 min for initial denaturation; 40 cycles at 95°C for 15 s and at 60°C for 30 s for primer annealing and product elongation. A negative blank (without the DNA template) was also run for each primer pair. Changes in targeted populations were calculated using a relative quantification calculation and the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen 2001).

Statistical analysis: The data is presented in the form mean ±SE. The data of *in vitro* gas production test was analyzed using SPSS (1996). For comparison of essential oils Generalized Linear model multivariate ANOVA procedures were used and means were compared using Duncan's Multiple Range Test at a significance level of 95% as per Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Chemical composition of feed used in in vitro gas production test: Samples of gram straw and concentrate mixture used during *in vitro* study was analysed for their chemical composition. The per cent organic matter (OM), crude protein (CP) and ether extract (EE) in concentrate were 93.16, 17.32 and 4.51, respectively and while for gram straw it was 88.95, 6.29 and 0.94, respectively. The content of total ash (%) in concentrate and gram straw were 6.83 and 11.04, respectively. Neutral detergent fibre (NDF), acid detergent fibre (ADF), and cellulose per cent in concentrate were 26.96, 10.06, 1.82, respectively and that for gram straw were 55.89, 36.48 and 13.46, respectively.

Effect of essential oils combination on in vitro fermentation parameters: In vitro fluid *p*H varied between 6.50 to 6.59 after addition of different combinations of essential oils. The value is within the normal range of 6.5-7.0 of rumen fluid showing no adverse effect on the rumen environment with the addition of essential oils. However, with individual oil *in vitro* fluid *p*H was slightly higher with peppermint (6.77) and lower with garlic (6.64) garlic oil blend (6.59) and clove oil (6.62) as compared to control (6.72) (Singh *et al*. 2023). Combination of these oils might have modified the fermentation activities and no such effect was observed. Kumar *et al*. (2011) also reported no change in ruminal *p*H of buffalo fed with plant part mixture or peppermint oil. Kim *et al*. (2019) reported that *p*H was quadratically changed when essential oil mixture (EOM) was increasingly added (P=0.032) in *in vitro* study.

Total gas production (ml/g DM) during 24 h in *in vitro* trial with addition of different combination of essential oils, i.e. GO+PO, GO+CO, CO+PO and GO+PO+CO are presented in Table 1. Total gas production (mL/g DM) in control group was 154.07, at lower dose level (0.25 g/L) there was increase in gas production which significantly decreased at highest dose level. Maximum decrease was observed in total gas production (64.58) when GO+PO was used at concentration of 1.0 g/L as compared to control (154.07). The reduction in gas production might be due to reduced activity of microbes involved in *in vitro* fermentation of feeds. Dey *et al*. (2021) also reported that Garlic oil supplementation at 33.33 µl /L of buffered rumen fluid increased (P<0.05) total gas production in comparison with 166.66 µl/L; however, it remained comparable (P>0.05) with control and 83.33 µl/L buffered rumen fluid.

Effect of essential oils combination on in vitro methane production: Effect of addition of combination of essential oils on methane production (mL/kg DM) and methane (mL/kg DDM) is presented in Table 2. Methane production (ml/kg DM) and methane (ml/Kg DDM) was 33.22 and 55.37, respectively in control group. With addition of combination of essential oils there was overall (P<0.05) significant decrease in methane production. With increase in dose level there was reduction in methane production. There was significant (P<0.05) decrease in the methane production (mL/g DM and mL/g DDM) on GO and PO supplementation together. Methane production (mL/g DM) was 39.18, 19.74 and 1.24 for their respective doses of D_1, D_2 and D_3. GO and CO combined supplementation resulted in methane production (mL/g DM) 36.11, 19.93 and 1.86 for doses D_1, D_2 and D_3, respectively. The combination of GO+PO at highest dose level (0.1g/dL) produced least methane in comparison to all the combination of essential oil tested. Overall it was found that the combination having garlic oil as a component has better methane reducing effect (Table 2). Dey *et al*. (2021) studied effect of Garlic oil *in vitro* with buffalo rumen liquor and reported that graded doses of garlic oil inclusions reduced (P<0.001) methane concentration (%) in total gas and total methane production (mL/g DM), irrespective of concentrations. Pawar *et al*. (2014) evaluated seven essential oils (EOs): Garlic (*Allium*

Table 1. Effect of combination of essential oils on *in vitro* total gas production (mL/g DM)

Oil combination	Doses of oil (g/L)				
	D_0(0.0)	D_1(0.25)	D_2(0.5)	D_3(1.0)	Gr mean
Control (no oil)	154.07±8.87^{cd}				154.07±8.87
GO+PO (1:1)		180.46±6.72^{ab}	129.51±4.63^{d}	64.58±9.37^{f}	124.43±16.51
GO+CLO(1:1)		181.94±8.72^{ab}	141.86±4.53^{cd}	93.64±4.60^{e}	138.90±12.40
PO+ CLO(1:1)		150.18±7.87^{cd}	185.73±8.34^{ab}	81.84±3.66^{ef}	139.25±14.06
GO+PO+CLO (1:1:1)		191.57±6.34^{a}	160.90±6.07^{bc}	100.31±5.39^{e}	147.23±12.24
Mean	154.07±8.87^{A}	175.00±5.36^{A}	157.19±6.56^{A}	85.09±5.34^{B}	

^{a,b,c,d} Means with different superscript differ significantly (P<0.05), ^{A,B,C} Means of dose with different super script in a row differ significantly (P<0.05).

Table 2. Effect of combination of essential oils on *in vitro* methane production (mL/g DM)

Oil combination	Doses of oil (g/L)				
	D_0 (0.0)	D_1(0.25)	D_2 (0.5)	D_3(1.0)	Gr mean
In vitro methane production (mL/g DM)					
Control (No oil)	33.22±2.40 ^{b}				33.22±2.40
GO+PO (1:1)		39.18±2.16^{ab}	19.74±5.04 ^{cd}	1.24±0.36 ^{e}	20.08±5.30
GO+CLO(1:1)		36.11±5.20^{ab}	19.93±0.64 ^{cd}	1.86±0.93 ^{e}	19.24±4.94
PO+ CLO(1:1)		33.00±1.86 ^{b}	40.43±2.11^{ab}	15.41±2.40 ^{d}	29.61±3.35
GO+PO+CLO (1:1:1)		41.28±1.31^{a}	25.66±0.76 ^{c}	3.85±1.47 ^{e}	21.99±4.79
Mean	33.22±2.40^{AB}	37.13±1.65 ^{A}	27.38±2.62 ^{B}	5.59±1.62 ^{C}	
In vitro methane production (mL/g DDM)					
Control (No oil)	55.37±4.00 ^{b}				55.37±4.00
GO+PO (1:1)		65.3±3.60^{ab}	32.90±8.41^{cd}	2.07±0.60^{e}	33.48±8.83
GO+CLO(1:1)		60.19±8.67^{ab}	33.22±1.07^{cd}	3.10±1.55^{e}	32.07±8.23
PO+ CLO(1:1)		55.01±3.11 ^{b}	67.38±3.53^{ab}	25.68±4.00^{d}	49.36±5.59
GO+PO+CLO (1:1:1)		68.79±2.19^{a}	42.78±1.28^{c}	6.41±2.46^{e}	36.65±7.99
Mean		55.37±4.00^{AB}	61.89±2.75^{A}	45.64±4.36^{B}	9.32±2.71^{C}

^{a,b,c,d} Means with different superscript differ significantly (P<0.05), ^{A,B,C} Means of dose with different super script in a row differ significantly (P<0.05).

sativum), clove (*Syzygium aromaticum*), lemongrass (*Cymbopogon citratus*), wild turmeric (*Curcuma aromatica*), turmeric (*Curcuma longa*) and cinnamon (*Cinnamomum zeylanicum*) for methane reduction and reported that at the level of 167 µL l^{-1} of incubation medium, garlic oil caused maximum methane inhibition with minimum adverse effect on feed digestibility. Patra and Yu (2012) reported that EOs significantly reduced methane production with increasing doses, with reductions by 34.4%, 17.6%, 42.3%, 87%, and 25.7% for clove oil, eucalyptus oil, garlic oil, origanum oil and peppermint oil, respectively at 1.0 g/L compared with the control. Along with garlic peppermint oil individually has shown methane reducing effect in *in vitro* gas production system (Singh *et al*. 2023 and Agarwal *et al*. 2009). Kumar *et al*. (2009) also reported reduction in *in vitro* methane production (mL/g DM and mL/g DDM) after inclusion of eucalyptus oil. Patra *et al*. (2011) reported lower methane energy loss as per cent of digestible energy intake tended to be in *T. chebula* (P=0.09) and Mix (*T. chebula* and garlic) (P=0.08) supplemented groups as measured in open circuit respiration chamber.

Effect of essential oil addition on in vitro digestibility: Effect of addition of different essential oil combination on per cent *in vitro* true dry matter (IVDMD) and organic matter digestibility (IVOMD) are presented in Table 3. At low dose level (D1, 0.25 g/L) there was no statistically (P>0.05) significant difference in *in vitro* dry matter and organic matter digestibility with the addition of different EO combinations evaluated as compared to control except PO+

Table 3. Effect of combination of essential oils on *in vitro* feed digestibility (%)

Oil combination	Doses of oil (g/L)				
	D_0 (0.0)	D_1(0.25)	D_2(0.5)	D_3(1.0)	Gr mean
In vitro true dry matter digestibility (%)					
Control (No oil)	60.00±0.52 ^{cd}				60.00±0.52^{ZY}
GO+PO (1:1)		60.35±0.52^{cd}	53.98±1.48^{e}	53.65±1.96^{e}	56.34±0.97^{YX}
GO+CLO(1:1)		59.06±0.62^{d}	55.82±1.00^{e}	42.92±0.95^{f}	52.60±1.92^{X}
PO+ CLO(1:1)		63.38±2.07^{ab}	64.58±1.59^{a}	59.13±1.37^{d}	62.58±0.88^{Z}
GO+PO+CLO (1:1:1)		61.99±0.64^{bc}	53.40±0.50^{e}	42.18±0.47^{f}	51.86±2.41^{X}
Mean		60.00±0.52^{A}	61.32±0.56^{A}	56.98±1.13^{A}	49.23±1.87^{B}
In vitro organic matter digestibility (%)					
Control (No oil)	61.43±0.78^{ab}				61.43±0.78 ^{ZY}
GO+PO (1:1)		61.91±0.30^{ab}	56.35±1.02^{c}	54.70±1.79^{c}	57.30±1.14^{YX}
GO+CLO(1:1)		60.76±0.51^{b}	55.82±1.00^{c}	45.64±0.75^{d}	53.59±1.69^{X}
PO+ CLO(1:1)		64.86±1.30^{a}	65.74±0.22^{a}	60.82±1.43^{b}	62.92±1.01^{Z}
GO+PO+CLO (1:1:1)		64.51±1.26^{a}	57.06±1.13^{e}	44.26±0.28^{d}	55.28±2.27^{YX}
Mean		61.43±0.87^{AB}	62.64±0.60^{A}	58.09±1.03^{B}	51.09±1.63^{C}

^{a,b,c,d}Means with different superscript differ significantly (P<0.05). ^{A,B,C}Means of dose with different super script in a row differ significantly (P<0.05). ^{z,y,x}means of EO with different super script in a column differ significantly (P<0.05).

CLO (1:1) combination in which IVDMD was (P<0.05) significantly higher (63.38) as compared to control (60.00). At dose level (D_2 and D_3) there was significant decrease in the IVDMD and IVOMD after addition of EO combinations (GO+PO, GO+CO and GO+PO+CO) while no significant difference was observed with PO+CO as compared to control. At dose level (D_2 and D_3) GO+PO combination led to a moderate decrease in the IVDMD and IVOMD along with decrease in *in vitro* methane production. With GO+ PO supplementation, IVDMD (%) was 53.98 at D_2 which as similar to 53.65 at D_3. Similar pattern was observed with IVOMD after GO+ PO supplementation. The effect of essential oil supplementation was quite variable depending upon the type and dose of essential oils. Kumar *et al*. (2009) and Agarwal *et al*. (2009) reported a decrease in IVTD on addition of Eucalyptus oil and peppermint oil, respectively. Kim *et al*. (2019) reported an increased IVDMD and IVNDFD after addition of EOM (essential oil mixture containing eugenol, thymol and cinnamaldehyde) in *in vitro* batch culture system after 24 h incubation.

Benchaar *et al.* (2007) observed a significant decrease in IVDMD when diets were supplemented with carvacrol (400 mg/L) and eugenol (800 mg/L) (P<0.05), but other EO (cinnamon leaf oil, clove leaf oil, sweet orange oil, oregano oil, and thyme oil) and EOC (cinnamaldehyde and thymol) had no effect on IVDMD in an *in vitro* batch culture system after 24 h incubation. Castillejos *et al*. (2005) reported that IVDMD was not changed by supplementation of EOM containing thymol, limonene, and guaiacol in an *in vitro* continuous culture system. Benchaar *et al.* (2007), Castillejos *et al*. (2006), and Fraser *et al*. (2007) observed a decrease in IVNDFD. Benchaar *et al*. (2007) explained that fibrolytic bacteria might be sensitive to high concentrations of phenolic compounds in EO thereby fiber digestibility might be impaired by EO supplementation.

Effect of essential oil addition on volatile fatty acid and its fractions: Effect of addition of combination of essential oils (EOs) on the total volatile fatty acid production and its fractions was evaluated and presented in Table 4. Total VFA (mmol/100 mL) production was 4.60 for control group while 4.29, 3.65 and 2.49 in D_1, D_2 and D_3 group on GO and PO combination supplementation being statistically similar at D_1 while significantly (P<0.001) decreased at D_2 and D_3 level. Propionic acid (%) increased and acetic acid (%) decreased significantly as compared to control at D_2 (0.5 g/L) dose. But at D_3 dose level acetic acid proportion increased and propionic acid proportion decreased. With individual oil we observed increased propionate with garlic oil and decreased propionate with peppermint oil (Singh *et al*. 2023).On *in vitro* addition of GO+CO total VFA (mmol/100 mL) was statistically similar in D_0, D_1 and D_2 group while reduced significantly (P<0.01) in D_3 group. Butyric acid (%) increased (P<0.05) significantly on addition of combination of clove and garlic oil being highest at D_3 level. In PO+CO combination supplementation at D_1 and D_2 dose level there was no statistically significant difference in total VFA and its fractions was observed. But at D_3 dose level acetic acid and butyric acid proportion increased and propionic acid proportion decreased. Among the three volatile fatty acids, acetic acid and butyric acid synthesizing pathways are hydrogen producers and propionate pathways is hydrogen

Table 4. Effect of combination of essential oils on total volatile fatty acids and its fraction

Attribute	Doses of oil (g/L)				
	D_0 (0.0)	D_1(0.25)	D_2(0.5)	D_3(1.0)	P-value
GO+PO (1:1)					
TVFA(mmol/100 mL)	4.60±0.20^a	4.29±0.11^a	3.65±0.07^b	2.49±0.07^c	0.001
Acetic acid %	49.35±0.68^b	47.49±0.27^c	42.25±0.20^d	55.20±0.66^a	0.000
Propionic acid %	24.72±0.39^b	25.33±0.18^b	30.58±0.16^a	17.77±0.26^c	0.000
Butyric acid %	25.92±0.34^b	27.17±0.13^a	27.15±0.10^a	27.02±0.41^a	0.035
GO+CLO (1:1)					
TVFA (mmol/100mL)	3.3±0.18^a	3.58±0.015^a	3.30±0.087^a	2.43±0.15^b	0.00
Acetic acid %	49.98±0.48^a	43.92±0.30^b	41.89±1.01^b	39.29±0.65^c	0.000
Propionic acid %	29.43±0.47^a	29.57±0.28^a	29.49±0.57^a	16.69±0.20^b	0.000
Butyric acid %	20.57±0.12^c	26.50±0.37^b	28.60±1.58^b	44.01±0.47^a	0.000
PO+ CLO (1:1)					
TVFA (mmol/100ml)	4.36±0.25^a	4.35±0.21^a	4.62±0.28^a	2.95±0.15^b	0.00
Acetic acid %	48.82±0.53^b	47.78±0.22^b	47.94±0.21^b	51.77±0.60^a	0.003
Propionic acid %	25.68±0.56^a	25.95±0.43^a	25.98±0.16^a	15.40±0.41^b	0.000
Butyric acid %	25.48±0.31^b	26.25±0.58^b	26.06±0.20^b	32.81±1.00^a	0.025
GO+PO+CLO (1:1:1)					
TVFA (mmol/100ml)	4.40±0.15^b	5.51±0.22^a	5.50±0.37^a	3.41±0.09^c	0.00
Acetic acid %	64.11±0.53^a	61.17±0.21^b	53.73±0.86^d	56.07±0.81^c	0.000
Propionic acid %	20.31±0.71^b	21.01±0.31^b	26.42±1.39^a	18.85±0.99^c	0.001
Butyric acid %	15.57±0.20^c	17.81±0.20^b	19.84±1.22^b	25.06±1.60^a	0.000

^{a,b,c,d}Means with different superscript differ significantly (P<0.05).

consumer and therefore methane reduction is usually associated with increase in propionate production and reduction in acetate/butyrate production Butyric acid (%) increased ($P<0.05$) significantly on addition of combination of (garlic + peppermint + clove) being highest at D_3 level. The effect may be modified due to interaction of different metabolites present in essential oils.

This VFA pattern might be responsible for the accumulation of molecular hydrogen at the higher levels of peppermint oil inclusion resulting in a lower fermentation of feed as indicated by lower gas production and a decrease in *in vitro* digestibility of feed. Patra and Yu (2012) also reported increase in the propionic acid proportion with the addition of garlic oil and increase in the acetic acid proportion with the addition of clove oil and peppermint oil. Agarwal *et al*. (2009) reported that addition of peppermint EO lead to increased acetate and A/P ratio with linearly (0 to 2 µL/mL). Beyazi (2020) reported that ruminal acetate production was increased, and propionate and butyrate production were decreased with 3.0 µL/mL peppermint EO supplementation in IVGPT with buffalo rumen liquor. Effect of individual essential oil and their combinations varied depending upon their interaction and dose level. Kim *et al*. (2019) reported that addition of commercial EOM containing eugenol, thymol, and cinnamaldehyde did not significantly affect total VFA concentration, proportion of each VFA, and acetate to propionate ratio.

Relative quantification of methanogen population: Effect of addition of combination of essential oils (EOs), on relative methanogen population was studied using $2^{-\Delta\Delta}$Ct analysis with bacteria as housekeeping gene as expressed by RT-PCR amplification. In case of GO+PO addition the value was 1.08, 0.43 and 0.09 for D_1, D_2 and D_3 dose, respectively. With supplementation of all three EOs (GO+PO+CO) there was increased value of 1.09, 1.31 and 1.05 at same dose level (Table 5). Essential oils produced by different plant species can vary in chemical structures and stereochemistry as well as bioactive activities which determine their antimicrobial activity and their action on different microbes. Relative expression of methanogens was minimum at the highest dose level of garlic oil and peppermint oil addition led to decrease in the expression of methanogens linearly with increase in the dose level (Singh *et al*. 2023). Patra and Yu (2012) studied the effect of essential oils on rumen microbial population using quantitative real-time PCR, and reported that all the EOs decreased the abundance of archaea, protozoa, and major cellulolytic bacteria (i.e. *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *R. albus*) linearly with increasing EO doses. On the basis of denaturing gradient gel electrophoresis analysis, different EOs changed the composition of both archaeal and bacterial communities to different extents. Kim *et al*. (2019) reported that EOM addition in batch culture system significantly improved relative protozoa abundance and fiber degrading bacteria *Selenomonas ruminantium* and *Ruminococcus albus* as compared to control.

Table 5. Relative expression of *mcrA* gene of methanogens

Oil combinations	Doses of oil (g/L)			
	D_0 (0.0)	D_1(0.25)	D_2 (0.5)	D_3 (1.0)
GO+PO (1:1)	1	1.08	0.435	0.097
GO+CLO (1:1)	1	0.687	0.664	0.646
PO+ CLO (1:1)	1	0.521	0.692	0.697
GO+PO+CLO (1:1:1)	1	1.094	1.310	1.049

This study concluded that garlic oil (GO) and peppermint oil(PO) in 1:1 combination has better potential in reducing *in vitro* methane production with lower adverse effect on *in vitro* feed digestibility. So this combination can be evaluated in *vivo* feeding trial for further definite conclusion.

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