

Effect of Essential Oils of Eucalyptus (*Eucalyptus globulus* Labill) and Angelica (*Heracleum persicum* Desf. ex Fischer) on *In vitro* Ruminal Fermentation, Protozoal Population and Methane Emission Using Afshari Sheep Inoculum

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ABSTRACT

The effect of eucalyptus (*Eucalyptus Globulus* Labill; EGEO) and Angelica (*Heracleum Persicum* Desf. ex Fischer; HPEO) Essential Oils was assessed at the levels of 0, 3, 30, 300 or 3000 ($\mu\text{l } 30 \text{ ml}^{-1}$) on *in vitro* fermentation of buffered rumen fluid from 3 castrated male sheep. The fermentation kinetics were estimated after 54 hours incubation. The fermentation kinetic values, *In Vitro* Gas Production (IVGP), Methane Production (MP), ammonia-N ($\text{NH}_3\text{-N}$) concentration, Organic Matter Degradability (IVOMDe), Partitioning Factor (PF), Microbial Mass (MM), Volatile Fatty Acids (VFA) concentrations and protozoa population were evaluated. The results showed that EGEO supplementation at 300 μl increased the insoluble fraction (b) ($P= 0.027$). Cumulative IVGP at 54 hours was the lowest for EGEO ($P= 0.014$) and HPEO ($P= 0.001$) at 3,000 μl . The HPEO supplementation at 30 and 3,000 μl decreased ($P= 0.036$) the constant rate (c) of gas production during incubation 54 hours. The EGEO inclusion improved GP in 24 hours at 3 μl , but inhibited fermentation at 3,000 μl ($P = 0.004$), whereas addition of HPEO inhibited fermentation at 3,000 μl ($P= 0.000$) only. The addition of EGEO and HPEO ($P= 0.000$) inhibited MP at all levels. HPEO treatments reduced ($P= 0.005$) the $\text{NH}_3\text{-N}$ concentration at 3 and 3,000 μl levels. The IVOMDe was increased at dose rates of 3, 30 or 300 μl EGEO, but decreased at 3000 μl of HPEO. At the inclusion of 3,000 μl of EGEO and HPEO, PF, MM and Efficiency of Microbial Mass (EMM) were enhanced ($P= 0.001$). Apart from the inclusion level of 300 μl , total VFA concentrations were decreased by EGEO ($P= 0.002$) and HPEO ($P= 0.001$). The EGEO and HPEO treatments showed antiprotozoal activity. It is suggested that EGEO and HPEO could be added at the level of 300 $\mu\text{l } 30 \text{ ml}^{-1}$ to improve ruminal fermentation (*i.e.* increasing EMM and decreasing MP and protozoa population), which may lead to better nutrient utilization and animal growth.

Keywords: *Eucalyptus globulus*, Essential oils, *Heracleum persicum*, Methane, Protozoa, Rumen fermentation.

INTRODUCTION

Microbial degradation of feed in the rumen is characterized by losses of energy and nitrogen in the form of methane (CH_4) and urea respectively (Blümmel *et al.*, 2005). Global enteric CH_4 emissions from livestock were estimated/predicted at 2,079 and 2,344 million tonnes CO_2 -equivalent/year for 2010 and 2020,

respectively (Gerber *et al.*, 2013). Production of CH_4 represents a loss of 2–12% of the gross energy consumed by ruminants depending on the type of diet (Johnson and Johnson, 1995). Methane is a greenhouse gas with a large potential for increasing global warming (Eckard *et al.*, 2010). In the year 2010, 34, 24 and 15 percent of the global CH_4 emissions from ruminant livestock came from Asia, Latin America

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and Africa, respectively (Smith *et al.*, 2012) (total world emissions are estimated at 100 Tera gram per year (Tera gram= 1 million tonne)).

Plant extracts and Essential Oils (EOs) have been widely used to improve the efficiency of protein metabolism in the rumen by reducing amino acid deamination (Nooriyan Soroor *et al.*, 2013), a reduction in the acetate to propionate ratio in the rumen (Nooriyan Soroor *et al.*, 2013) and a decrease in methane production (Nooriyan Soroor and Rouzbehan, 2012). Modification of rumen microbial fermentation has great potential for both the environment and ruminant nutrition (*i.e.*, enhance ruminal microbial protein and lower methane production) (Beauchemin *et al.*, 2008).

Numerous studies have shown that a variety of compounds and substances were able to reduce methane production in the rumen (Beauchemin *et al.*, 2008; Buddle *et al.*, 2011; Patra, 2012), including plant extracts, essential oils and secondary metabolites (Calsamiglia *et al.*, 2007; Torabi Sagvand *et al.*, 2011; Nooriyan Soroor *et al.*, 2013). For example, 1, 8-Cineole (79.3%), α -pinene (9.3%), trans pinocarveol (3.2 %), *p*-cymene (1%) and pinocarvone (1%) were identified as the major secondary metabolites of *Eucalyptus Globulus* (EG) leaf EO (Torabi Sagvand *et al.*, 2011). The major active constituents of *Heracleum persicum* essential oil (HPEO) were identified as viridiflorol (23.05%), elemol (3.63%), β -maliene (3.07%), spathulenol (3.34 %) and 2-tetradecanol (3.38%) (Mojab *et al.*, 2003).

There are a few experimental data on effects of the *Eucalyptus globulus* (Kumar *et al.*, 2009, Sallam *et al.*, 2009, Patra and Yu, 2012) and *Heracleum persicum* (Jahani-Azizabadi *et al.*, 2011) EO on rumen digestion and fermentation patterns, including rumen methanogenesis. Therefore, this study was conducted to evaluate the influence of two EO on *in vitro* gas production, methane production, protozoa population, partitioning factor, NH_3 -N and Volatile Fatty Acid (VFA) concentrations.

MATERIALS AND METHODS

Preparation of Essential Oil (EO)

The EO was obtained using a Clevenger apparatus by the method described by Mohamed *et al.* (2006). In brief, shade-dried samples of EC and HP were finely ground and stored at room temperature until extraction. For EO preparation, 100 g of dried ground sample was suspended in 700 ml of distilled water, and the EO extracted by a distillation process, (100°C for 3 hours).

Animals

The rumen fluid was obtained from three fistulated castrated male Afshari sheep (43.8±1.9 kg) before the morning feed (Menke and Steingass, 1988). The animals were fed twice daily (08:30 and 16:30) at a maintenance energy level with a basal diet containing 700 g kg⁻¹ alfalfa and 300 g kg⁻¹ barley (DM basis). Fresh water and mineral blocks (Fe= 1,232 mg; Cu= 150 mg; Co= 25 mg; Zn= 500 mg; I= 50 mg; Se= 15 mg and Na= 382 mg kg⁻¹) were freely available at all times (NRC, 2007).

Experimental Design and Fermentation Method

This study was conducted using an *in vitro* gas production method at various incubation time intervals. The experiment was of a Complete Randomized Design (CRD). Incubation was done in three separate *in vitro* runs each with four replicates. The four doses of EGEO and HPEO (0 (control), 3, 30, 300, or 3,000 μl 30 ml⁻¹ culture medium) were added to the buffered rumen fluid and 200 mg substrate in 120 ml Wheaton bottles. The substrates were a 60:40 ratio of Roughage (R, alfalfa hay) and Concentrate (C, barley grain). The chemical composition of the substrate was DM 929 g kg⁻¹ fresh weight, CP 150, NDF 397, ADF, 187 and Ash 139 g kg⁻¹ DM).

In vitro Fermentation

To assess the CH₄ production, twenty-four hour incubation was carried out using a batch system (Theodorou *et al.*, 1994). One set of Wheaton bottles (containing four replicates for each treatment in each set) were incubated at 39°C for 24 hours. The rumen fluid, which was pooled to use the mixture of the three animals, had been collected into a pre-warmed (39°C) vacuum flask and filtered through four layers of cheesecloth under continuous flushing with CO₂. The mineral buffer solution was prepared (Menke and Steingass, 1988) and prior to the addition of rumen fluid, had been warmed to 39°C and continuously flushed with CO₂. Rumen fluid and buffer were mixed in the ratio of 1:2 (volume/volume). A settlement time of 5 minutes was allowed after filling the bottles then the gas pressure was equilibrated by passing a needle through the stoppers to release the gas and the time recorded to mark the beginning of incubation.

In another set of incubation using 100 mL glass syringes to assess the effect of the treatments on IVGP and fermentation kinetics, the volume of produced gas was recorded at times of 0, 2, 4, 6, 8, 10, 12, 18, 24, 36, 48 and 54 hours (*i.e.*, asymptote gas) (Menke *et al.*, 1979). The constants of fermentation kinetics (a, b and c) were calculated by Fitcurve 6.0 software. The kinetic parameters were estimated using simplified model of Ørskov and McDonald (1979) as follows:

$$P = b(1 - e^{-ct})$$

Where: P is the gas production at time t , b is the gas production from insoluble fraction (ml g⁻¹ OM), c is the gas production rate for b and t is the incubation time (h).

Fermentation Parameters

After 24 hours of incubation, the IVGP accumulating in the headspace of each bottle was measured. Volume of CH₄ production was recorded by inserting a graduate syringe

(which contains a needle) from the top of the Wheaton bottle, and gas volume was corrected using the following equation:

$$P = P_o \times e^{-\gamma_o \times g \times h / P_o}$$

Where, P = Actual pressure in a defined altitude; P_o = Pressure at sea-level (9.81×10⁴ N m⁻²); γ_o = gas density at sea-level (1.2 kg m⁻³); g = gravity (9.81 m s⁻²); h = Altitude above sea-level (1,189 m). The gas produced by the fermentation of the substrate was calculated by subtracting the gas produced in a blank bottle (containing no substrate, only the inoculum and buffer) from the total gas produced in the bottle containing substrate and inoculum (Makkar, 2010). The bottles were then swirled on ice to stop fermentation and opened to take samples of incubation medium for NH₃-N, protozoa enumeration and a supernatant (0.8 ml) for VFA analysis. The Partitioning Factor (PF) was determined according to Makkar (2010) using the remaining bottle contents.

Methane content was determined using a Shimadzu GC-14 B Gas Chromatography (GC) (Shimadzu, Tokyo, Japan) which was equipped with a Carboxen TM 1000, 45/60, 2m×1/8 column (Supelco, St. Louis, MO, USA) and a flame ionization detector. Methane content in samples was calculated by external calibration, using serial concentrations: 40, 50, 100, 200, 300, 400, 500 and 600 µl (purity of the standard was 99.99%, Air Products and Chemicals, Inc., Allentown, PA, USA).

The VFA (acetate-C₂ (A), propionate-C₃ (P), *n*-butyrate and C₂:C₃ ratio) were analyzed using HPLC (Samuel *et al.*, 1997). 1 ml of the supernatant was collected in a microfuge tube containing 0.20 ml metaphosphoric acid (25 ml100 ml⁻¹). The mixture was allowed to stand for 3 hours at room temperature then centrifuged at 15,000× g at 4°C for 15 minutes and the supernatant was collected and stored at -20°C until being analyzed.

The NH₃-N concentration in the samples was determined by the phenol-hypochlorite method using spectrophotometric determination as described by Broderick and Kang (1980).



Rumen ciliates on the basis of three subfamilies, *Entodiniinae*, *Ophryoscolecine*, *Diplodiniinae* and the *Isotrichidae* family were identified according to the method described by Dehority (2003). All measurements were corrected using a suitable blank.

Determination of PF and Microbial Mass

For determining PF; OMDe (Organic Matter Degradability) and IVGP values are required. The PF= mg OMDe ml⁻¹ IVGP (Makkar, 2010). The mass difference of original residue and the detergent extracted residue was taken as a rough estimate of Microbial Mass (MM) (Makkar, 2010). The ratio of MM (mg) to gas volume (ml) at 24 hours of incubation was used as an index of Efficiency of Microbial Mass (EMM).

Proximate Analyses and Organic Matter Disappearance (OMD)

The substrate was analyzed for dry matter (ID number 930.15), as [Equation (1)] was estimated according to Menke *et al.* (1979):

$$\text{OMD}\% = \frac{14.88}{(0.889 \times \text{GP}_{24}) + (0.045 \times \text{XP}) + (0.065 \times \text{XA})} \quad (1)$$

Where OMD is OM disappearance, GP₂₄ is the net gas production (ml) after 24 hours, XP crude protein (g Kg⁻¹ DM) and XA ash (g Kg⁻¹ DM).

Statistical Analysis

The data of the *in vitro* gas production test (IVGP, methane emission, VFA, C2:C3, NH₃-N concentration, PF, OMDe and IVOMD) and subfamily protozoa counts were analyzed by one-way Analysis of Variance (ANOVA) using the Statistical Package for Social Science (SPSS 18.5). The Completely Randomized Design (CRD) with three separate *in vitro* runs with four replicates and treatment means were

compared using Duncan's test. Polynomial linear and quadratic contrasts were used to test the effect of treatments.

The protozoa population counts were checked for normal distribution by the Kolmogorov-Smirnov test before statistical analysis. The results were analyzed according to the following statistical model:

$$Y_{ij} = \mu + T_i + e_{ijk}$$

Where: Y_{ij} represents the value of each individual observation, μ the average, T_i the effect (treatment) of the i th dose of additive (i = Four level of EO) and e_{ijk} represents the residual error.

RESULTS

Effect on the Kinetics of Gas Production

The gas production (54 hours) was decreased by EGEO supplementation at 3,000 μ l (C; P= 0.014) and gas production from the insoluble fraction (b) was affected by EGEO at 300 μ l (C; P= 0.027) (Table 1). HPEO supplementation decreased the gas production rate at 30 μ l and above (c) (C; P= 0.036) and gas production (54 hours) at 3,000 μ l ml⁻¹ (Q; P= 0.013) (Table 2).

Effect of EO on Fermentation Characteristics

The total gas (24 hours) was decreased at 3,000 μ l of EGEO and HPEO (Q; P= 0.000) (Tables 1 and 2). Methane production after 24 hours incubation was reduced (C; P= 0.000) with the addition of EGEO (Table 1), HPEO (Table 2). The dose rate of 3000 μ l had a more noticeable effect on methane production than the other levels of EGEO and HPEO. The NH₃ concentration (mg l⁻¹) reduced at all HPEO doses except 300 μ l (C; P= 0.042) (Table 2) whereas EGEO showed no difference between treatments apart from a slight increase at 3 μ l (Table1). The IVOMDe was increased at 3, 30 or 300 μ l by EGEO (C; P= 0.035) but decreased at 3,000 μ l (Table1), whereas HPEO increased

Table 1. Effect of different levels of *Eucalyptus globulus* essential oil (EGEO) on kinetic of gas production and fermentation parameters from *in vitro* fermentation using buffered sheep rumen fluid.

Rumen Parameters ^a	EGEO levels ($\mu\text{l } 30 \text{ ml}^{-1}$)						SEM	L	Q	C
	Control	3	30	300	3000					
Fermentation kinetic values										
<i>b</i>	59.3 ^b	55.0 ^b	58.6 ^b	68.3 ^a	51.7 ^b	2.06	0.740	0.238	0.027	0.027
<i>c</i>	0.067	0.064	0.073	0.067	0.064	0.007	0.006	0.447	0.104	0.104
Gas (54 h, ml)	67.0 ^a	73.7 ^a	69.0 ^a	72.5 ^a	41.9 ^b	3.32	0.012	0.001	0.235	0.235
Fermentation Parameters										
Total Gas (24 h, ml 200 mg ⁻¹ DM)	48.8 ^b	55.5 ^a	51.4 ^{ab}	53.8 ^{ab}	24.1 ^c	3.15	0.001	0.001	0.003	0.003
MP ($\mu\text{mol } 200 \text{ mg}^{-1} \text{ DM}$)	460.8 ^a	158.3 ^b	180.7 ^b	207.3 ^b	40.5 ^c	37.71	0.001	0.006	0.001	0.001
MP ($\mu\text{mol } \text{mg}^{-1} \text{ OMD}$)	302.1 ^a	114.8 ^b	123.5 ^b	145.2 ^b	16.5 ^c	25.14	0.001	0.027	0.001	0.001
Ammonia-N ($\text{mg } \text{l}^{-1}$)	119.9 ^b	138.6 ^a	113.8 ^b	126.2 ^b	124.5 ^b	2.70	0.049	0.320	0.002	0.002
IVOMDe ($\text{mg } 200 \text{ mg}^{-1}$)	131.2 ^b	144.8 ^a	136.6 ^a	141.3 ^a	80.7 ^c	6.45	0.001	0.001	0.004	0.004
PF ($\text{mg } \text{ml}^{-1}$)	2.34 ^b	2.27 ^b	2.31 ^b	2.28 ^b	2.92 ^a	0.06	0.002	0.003	0.231	0.231
MM	33.6 ^d	46.2 ^c	54.3 ^b	62.7 ^b	113.7 ^a	7.67	0.001	0.002	0.131	0.131
EMM (%)	19.0 ^d	25.9 ^c	30.4 ^c	35.2 ^b	63.8 ^a	4.07	0.001	0.002	0.136	0.136
Total VFA ($\text{mmol } 200 \text{ mg}^{-1} \text{ DM}$)	55.4 ^a	26.6 ^c	38.9 ^b	68.1 ^a	20.3 ^c	5.26	0.002	0.263	0.144	0.144
VFAs ($\text{mol } 100 \text{ mol}^{-1}$)										
Acetate	68.6 ^a	50.5 ^b	53.4 ^b	47.8 ^b	51.2 ^b	2.19	0.001	0.002	0.108	0.108
Propionate	24.1 ^b	30.0 ^a	29.4 ^a	30.0 ^a	17.7 ^c	1.44	0.001	0.001	0.001	0.001
Butyrate	18.8 ^b	19.5 ^b	20.1 ^b	19.5 ^b	30.8 ^a	1.30	0.006	0.055	0.002	0.002
Acetate: Propionate	2.85 ^a	1.68 ^b	1.72 ^b	1.62 ^b	2.89 ^a	0.168	0.835	0.001	0.732	0.732

^a *b*= The gas production from the insoluble fraction; *c*= The gas production rate constant for the insoluble fraction (b); IVGP₅₄= *In Vitro* Gas Production after 54 hours; IVGP₂₄= *In Vitro* Gas Production after 24 hours; C= Cubic effect; EEO= Eucalyptus Essential Oil; EMM= Efficiency of Microbial Mass; IVOMDe= *In Vitro* Organic Matter Degradability; L= Linear effect; MM= Microbial Mass; MP= Methane Production; NH₃-N= Ammonia-N; PF= Partitioning Factor; Q= Quadratic effect.

Table 2. Effect of level of Angelica (*Heracleum persicum*) essential oil (HPEO) on kinetic of gas production and fermentation parameters from *in vitro* fermentation using buffered sheep rumen fluid.

	Rumen parameters ^a				HPEO levels ($\mu\text{l } 30 \text{ ml}^{-1}$)				P-Value		
	Control	3	30	300	3000	SEM	L	Q	C		
Fermentation kinetic values											
<i>b</i>	59.3	57.5	63.5	65.1	61.7	8.54	0.249	0.171	0.423		
<i>c</i>	0.067 ^a	0.102 ^a	0.039 ^c	0.057 ^b	0.034 ^c	0.034	0.848	0.012	0.036		
Gas (54 h, ml)	67.0 ^a	57.5 ^b	66.3 ^a	66.7 ^a	46.7 ^c	2.35	0.025	0.001	0.340		
Fermentation parameters											
Total gas (24 h, ml 200 mg ⁻¹ DM)	48.8 ^a	43.9 ^a	49.1 ^a	49.4 ^a	27.6 ^b	2.32	0.001	0.001	0.001		
MP ($\mu\text{mol } 200 \text{ mg}^{-1} \text{ DM}$)	460.8 ^a	167.5 ^b	185.0 ^b	182.3 ^b	37.4 ^c	37.38	0.001	0.001	0.001		
MP ($\mu\text{mol } \text{mg}^{-1} \text{ OMD}$)	302.1 ^a	101.6 ^b	121.7 ^b	121.3 ^b	16.1 ^c	25.19	0.001	0.001	0.001		
Ammonia-N($\text{mg } \text{l}^{-1}$)	119.9 ^d	93.1 ^b	116.5 ^a	108.8 ^a	78.6 ^c	1.48	0.457	0.084	0.005		
IVOMDe (mg 200 mg ⁻¹)	131.2 ^a	121.2 ^a	131.8 ^a	132.3 ^a	87.8 ^b	2.2	0.997	0.001	0.998		
PF (mg ml ⁻¹)	2.34 ^b	2.4 ^b	2.33 ^b	2.33 ^b	2.77 ^a	0.149	0.002	0.008	0.121		
MM	33.6 ^c	57.9 ^b	58.7 ^b	57.3 ^b	87.7 ^a	4.83	0.003	0.012	0.134		
EMM	19.0 ^c	32.5 ^b	33.0 ^b	32.1 ^b	49.2 ^a	2.66	0.001	0.006	0.097		
Total VFA (mmol 200 mg⁻¹ DM)											
VFA	55.4 ^a	45.4 ^e	51.9 ^b	60.2 ^a	38.8 ^d	2.11	0.177	0.001	0.004		
Acetate	68.6	61.0	56.0	59.2	54.8	1.80	0.466	0.936	0.466		
Propionate	24.1 ^b	26.2 ^a	29.0 ^a	26.4 ^a	9.0 ^c	1.96	0.001	0.001	0.177		
Butyrate	18.8 ^b	19.5 ^b	20.1 ^b	19.5 ^b	30.9 ^a	1.86	0.001	0.001	0.853		
Acetate: Propionate	2.7 ^b	2.3 ^b	1.9 ^b	2.2 ^b	6.2 ^c	0.511	0.007	0.016	0.393		

^a *b*= The gas production from the insoluble fraction; *c*= The gas production rate constant for the insoluble fraction (b); IVGP₅₄= *In Vitro* Gas Production after 54 hours; IVGP₂₄= *In Vitro* Gas Production after 24 hours; C= Cubic effect; EMM= Efficiency of Microbial Mass; GEO=Eucalyptus Essential Oil; IVOMDe= *In Vitro* Organic Matter Degradability; L= Linear effect; MM= Microbial Mass; MP= Methane Production; NH₃-N= Ammonia-N; PF= Partitioning Factor, Q= Quadratic effect.

IVOMDe at 300 μl but decreased it at 3,000 μl (Table 2). The partitioning factor (PF) (Efficiency of microbial protein synthesis was improved with all doses of EGEO (C; $P=0.000$) (Table 1) and HPEO (C; $P=0.005$) (Table 2) when compared with the control group and as the dose rate increased from 0 to 3,000 μl , a progressive increase of PF from 2.8 to 5.8 and 2.8 to 4.4 occurred from EGEO and HPEO respectively. The MM and EMM were increased as the EGEO (C; $P=0.001$) (Table 1) and HPEO (C; $P=0.002$) (Table 2) dose rates increased.

The effect of EGEO on VFA production showed that the concentration of total VFA (mmol l^{-1}) decreased at 3, 30 and 3,000 μl dose rates but showed no difference to the control at 300 μl (C; $P=0.000$) (Table 1). The molar proportion of acetate decreased at all dose rates (Q; $P=0.030$) and propionate increased (Q; $P=0.000$) at 3, 30 and 300 μl dose rates but at 3,000 μl showed no difference to the control. The concentration of butyrate was markedly increased at a dose rate of 3,000 μl . The $C_2:C_3$ ratio was lowest (Q; $P=0.000$) at the 300 μl dose rate (Table 1) but the ratio was higher at 3,000 μl and lower at 3 and 30 μl .

HPEO at dose rates of 3 or 3,000 μl lowered total VFA (Table 2) (C; $P=0.000$). Compared to the control diet, the addition of HPEO decreased the molar proportion of acetate at all dose rates but only increased the propionate at 3,000 μl compared to control and there was an increase in butyrate

at the 3,000 μl rate (Table 2). The $C_2:C_3$ ratio was lowered at all dose rates but more at 3,000 μl (Table 2).

Effects on Rumen Protozoa

The effect of EGEO showed that numbers of total protozoa (L; $P=0.000$) decreased at 30, 300 and 3,000 μl dose rates and the *Ophryoscolecine* subfamily (Q; $P=0.000$) and the *Isotrichidae* family (L; $P=0.035$) decreased at all dose rates, *Diplodininane* (Q; $P=0.00$) decreased at 30, 300 and 3,000 μl dose rates (Table 3) but *Entodininnae* (C; $P=0.038$) showed higher numbers at dose rates of 3 and 300 μl . HPEO showed a decrease in the numbers of total protozoa (Q; $P=0.000$), subfamilies *Diplodininane* (Q; $P=0.000$) and *Ophryoscolecine* and *Isotrichidae* family (Q; $P=0.000$) at all dose rates (Table 3) but not in *Entodininnae*.

DISCUSSION

Effect on the Kinetics of Gas Production

The results of *in vitro* fermentation kinetics confirm the known fact that the addition of EGEO (at 300 μl) in the fermentation medium can increase gas production from the insoluble fraction (b) (Kongmun *et al.*, 2010, Nooriyan Soroor and Rouzbehan, 2012). Also, the inhibition of b

Table 3. Protozoa population ($\times 10^5 \text{ ml}^{-1}$ RF) subfamily from *in vitro* fermentation using buffered sheep rumen fluid containing different levels of Eucalyptus *globulus* essential oil (EGEO).^a

Protozoa population ($\times 10^5 \text{ ml}^{-1}$ RF)	EGEO levels ($\mu\text{l 30 ml}^{-1}$)					SEM	P-Value Contrasts	
	Control	3	30	300	3000		L	Q
Total protozoa	2.83 ^a	2.61 ^{ab}	1.77 ^{bc}	2.00 ^{abc}	1.16 ^c	0.163	0.000	0.889
Subfamily								
<i>Entodininnae</i>	1.16 ^b	1.89 ^a	1.50 ^b	1.67 ^a	1.11 ^b	0.115	0.670	0.038
<i>Ophryoscolecine</i>	0.50 ^a	0.16 ^b	0.22 ^b	0.00 ^c	0.00 ^c	0.035	0.000	0.045
<i>Diplodininane</i>	0.63 ^a	0.55 ^a	0.33 ^{ab}	0.05 ^b	0.05 ^b	0.063	0.001	0.434
<i>Isotrichidae</i> (Family)	0.54 ^a	0.00 ^b	0.00 ^b	0.00 ^b	0.00 ^b	0.035	0.000	0.000

^a EGEO= Eucalyptus Essential Oil; L= Linear effect; Q= Quadratic effect; RF= Rumen Fluid; R^2 = Coefficient of determination, r= Correlation coefficient.



fraction and cumulative gas production at 54 hours at the 3,000 μl level suggests that the activity of rumen microbes was decreased (Kumar *et al.*, 2009, Sallam *et al.*, 2009, Patra and Yu, 2012). Burt (2004) illustrated that phenolic compounds in these essential oils have high antimicrobial activity due to the presence of a hydroxyl group within the phenolic structure. These researchers noted a linear decrease in the protozoa and major cellulolytic bacteria (*i.e.*, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *R. albus*) with increasing EGEO doses. However, the inclusion of HPEO at all dose rates showed decreases in *c* value but no effect on *b* suggesting that major changes in rumen microbial population had not occurred (Newbold *et al.*, 2004).

Effects of EO on Fermentation Characteristics

Studies have shown that EOs have markedly inhibited the activity of ruminal bacteria *in vitro* (Benchaar *et al.*, 2008). In the current experiment, the inhibition of total gas production (24 hours) can be attributed to the inhibitory potential of both EGEO and

HPEO at 3,000 μl dose rate on ruminal bacteria and protozoa. Similar findings were obtained when EGEO and HPEO were used (at higher levels) in *in vitro* gas production (Sallam *et al.*, 2009, Jahani-Azizabadi *et al.*, 2011).

Methane production ($\mu\text{mol mg}^{-1}$ OMD) was inhibited cubically with increasing levels of EGEO and HPEO. This result was in agreement with several previous studies on inhibitory properties of EO both *in vitro* and *in vivo*. For example, EO of Eucalyptus (Kumar *et al.*, 2009, Sallam *et al.*, 2009, Patra and Yu, 2012) has been reported to have strong inhibitory effects on methanogenesis *in vitro* study. Furthermore, the *in vivo* study of Thao *et al.* (2014) showed that inclusion of EO from eucalyptus plants in the diet could reduce CH_4 production in Swamp Buffaloes. Additionally, results in the current work illustrated that the total count of protozoa population (Tables 3 and 4) in treatments with inclusion of EGEO and HPEO was lower than that in treatments without EO supplementation and this could be the explanation for the decreasing CH_4 production since ruminal protozoa provide a habitat for methanogens that live on and

Table 4. Protozoa population ($\times 10^5 \text{ ml}^{-1}$ RF) subfamily from *in vitro* fermentation using buffered sheep rumen fluid containing different levels of Angelica (*Heracleum persicum*) essential oil (HPEO).^A

Protozoa population ($\times 10^5 \text{ ml}^{-1}$ RF)	HPEO levels ($\mu\text{l 30 ml}^{-1}$)					SEM	P-Value Contrasts	
	Control	3	30	300	3000		L	Q
Total protozoa	3.46 ^a	2.44 ^b	2.27 ^{bc}	2.00 ^{bc}	1.50 ^c	0.207	0.000	0.349
Subfamily								
<i>Entodininae</i>	1.29 ^a	1.27 ^a	1.22 ^a	1.25 ^a	0.92 ^a	0.068	0.171	0.384
<i>Ophryoscolecine</i>	0.50	0.16	0.22	0.16	0.16	0.637	0.136	0.288
<i>Diplodininae</i>	1.13 ^a	0.66 ^b	0.66 ^b	0.72 ^b	0.33 ^c	0.077	0.000	0.593
<i>Isotrichidae</i>	0.54 ^a	0.33 ^{ab}	0.167 ^{bc}	0.00 ^c	0.00 ^c	0.064	0.000	0.205
(Family)								

^A HPEO= Angelica Essential Oil; L= Linear effect; Q= Quadratic effect; RF= Rumen Fluid; R^2 = Coefficient of determination, *r*= Correlation coefficient.

within them (Newbold *et al.*, 1995). A similar relationship between gas production and methane was observed in other *in vitro* experiments (Patra and Yu, 2012).

The notable decrease in $\text{NH}_3\text{-N}$ concentration in the presence of HPEO may be due to a decrease in amino acid deamination by ruminal bacteria (McIntosh *et al.*, 2003; Kongmun *et al.*, 2010; Benchaar *et al.*, 2008) or a reduction in protozoa numbers (Newbold *et al.*, 2004). Phenolic compounds in these essential oils have high antimicrobial activity due to the presence of a hydroxyl group within the phenolic structure (Burt, 2004). Protozoa also possess proteolytic and deaminating activities (Williams and Coleman, 1992) so defaunation of the rumen may prevent recycling of N between bacteria and protozoa, resulting in a decrease of $\text{NH}_3\text{-N}$ in rumen fluid. However, in contrast with the current study, $\text{NH}_3\text{-N}$ concentration increased with ginger (*Zingiber officinale*) essential oil at dose rates of 300 mg/l (Busquet *et al.*, 2006) and 2.0 mg l⁻¹ of ginger extract (Alexander *et al.*, 2008) but unchanged by dose rates of 3, 30 and 3,000 mg l⁻¹ (Busquet *et al.*, 2006). Similar to our findings, several *in vitro* studies have shown that the addition of EO resulted in a decrease in $\text{NH}_3\text{-N}$ concentration which was attributed to the inhibition of amino acid deamination by ruminal protein degrading and hyperammonia producing bacteria (McIntosh *et al.*, 2003; Newbold *et al.*, 2004; Patra and Yu (2014). However, the inability of EO from eucalyptus to reduce ammonia concentrations in *in vitro* suggests that this EO had no significant potency to inhibit proteolysis and aminogenesis, although it reduced protozoa population (Patra and Yu, 2012). This discrepancy suggests that EO has different inhibitory effects on proteolytic bacteria and producing hyperammonia, resulting in varying reduction in ammonia production depending upon type and dose of EO.

An increase or decrease in IVOMDe by EGEO and HPEO depending on the dose rate may be due to the enhancement or

inhibition of these essential oils on rumen cellulolytic bacterial populations (Patra *et al.*, 2009) as is also evidenced from lower gas production and enzyme activity. Similarly, Alexander *et al.* (2008) noted that the addition of *Plumbago zeylanica* and *Zingiber officinale* extracts at 2.0 mg/ml of incubation medium, increased IVOMDe compared to control. In contrast, other research has shown that essential oil extract at 0.25 ml decreased IVOMDe (Patra *et al.*, 2009). Differences in studies may be due to the fact that these effects may vary with diet, chemical composition, and the dose used (Busquet *et al.*, 2006).

Since the EO treatment affected the PF and MM, the EMM was increased, which might be attributed to the synchronization of energy and N sources (Bach *et al.*, 2005). Methane production in the rumen represents a significant feed energy loss (2 to 12%), depending upon types of diets (Johnson and Johnson, 1995), and in the current study EGEO and HPEO treatments produced a notable reduction in methane production and $\text{NH}_3\text{-N}$. Therefore, as expected, MM has increased with EO addition. Similarly, there is evidence that *Zingiber officinale* (2 mg l⁻¹) did not improve the EMM (Alexander *et al.*, 2008).

Methane production in ruminal fermentation is usually associated with an increased propionate percentage and a reduced acetate percentage and C₂:C₃ ratio (Russell, 1998). The mechanism in which the shifting occurs is considered to be connected to the anti-protozoal effect of EO (Sallam and Abdalla, 2011). When the protozoa population is reduced in the presence of EO, acetate is concomitantly reduced since it is a product of protozoa metabolism from the fermentation of sugar (Van Soest, 1994). In the current study, the decrease of acetate may be due to a decrease in protozoa population and this leads to a reduction in the C₂:C₃ ratio with both EGEO and HPEO. Other studies have observed that when methane production decreased, acetate decreased (García-González *et al.*, 2008, Hu *et al.*, 2005). In the current study the C₂:C₃



ratio decreased and this result is in agreement with Kim *et al.* (2012) when garlic and ginger extracts were used, and García-González *et al.* (2008) when secondary metabolites of *Rheum officinale* and *Frangula alnus* were used. Although in other studies, ginger extract had no influence on C₂:C₃ ratio at any level (Patra *et al.*, 2006). Methane emission from the rumen medium is closely related to the individual VFA, and the decreased methane emission may have led to a higher molar proportion of propionate and a lower C₂:C₃ ratio (Wolin, 1960).

Effect on Rumen Protozoa

Not all protozoan genera have the same role in methanogenesis (Morgavi *et al.*, 2010) so the role of the various subfamilies of protozoa on fermentation parameters was evaluated in our study. Decreasing the number of H₂ producers such as protozoa in the rumen is an important way to reduce methane emissions (Benchaar *et al.*, 2008; Morgavi *et al.*, 2010). In our study, the decrease in the total number of protozoa, *Ophryoscolecine* subfamily and *Isotrichidae* (family) by EGEO and total protozoa *Diplodininane* subfamily and *Isotrichidae* (family) by HPEO was probably due to the antiprotozoal activities of these EO secondary compounds (Agarwal *et al.*, 2009; Benchaar *et al.*, 2008). The decrease in protozoa counts and decline in methane production might be due to the reduction in the hydrogen supply to methanogenic bacteria. Many possible mechanisms have been given for the effect of essential oil on protozoa, leading to the loss of cell contents and cell lysis (Benchaar *et al.*, 2008). Decreased rumen protozoa counts with some diets containing essential oil rich plants (Williams and Coleman 1992; Nooriyan Soroor *et al.*, 2013) have been reported. In contrast, protozoa counts increased with ginger extract (Patra *et al.*, 2006) and some blends of essential oils (Newbold *et al.*, 2004).

The effects of defaunation on methane production is less clear; (Table 5). Morgavi *et*

al. (2010) reported that defaunation resulted in a 10.5% decrease in methane emissions. In contrast, results obtained from another study (Goel *et al.*, 2008) show no relationship between methane and protozoa. To further the explanation of the relationship between methane production and rumen protozoa this study has defined regression equations. With the exception of two equations, the regression equations show that the relationship between these two variables is positive. In other words, reducing protozoa results in less H₂ which in turn is a substrate for methane production (Newbold *et al.*, 1995). The evaluation of the regression equations shows that the *Ophryoscolecine* and *Isotrichidae* (Family) have the greatest impact on the production of methane. Whereas, a previous study indicated that rumen ciliates were apparently responsible for 17 percent (between 9 and 25) of methanogenesis in rumen fluid (Newbold *et al.*, 1995), the current trial suggests that rumen ciliates are responsible for 34 percent of methanogenesis in rumen fluid. High regression between the two variables was reported for *E. amoneum* (Nooriyan Soroor, 2012) and tea saponin (Zhou *et al.*, 2011) previously.

CONCLUSIONS

The addition of EGEO and HPEO at the level of 300 µl 30⁻¹ ml could improve rumen fermentation due to the reduction of MP and protozoal population and an increase in EMM. The *Entodininnae* and *Diplodininane* protozoa subfamilies have the greatest impact on the production of methane and correlation between the two variables of methane and protozoa is high. However, more research, especially on animal responses, is needed to confirm the generally positive nutritional characteristics of both EO.

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چکیده

تأثیر اسانس های اکالیپتوس و گلپر در سطوح صفر، ۳، ۳۰، ۳۰۰ و ۳۰۰۰ (میکرولیتر در ۳۰ میلی لیتر) بر تخمیر شکمبه با استفاده از مایع شکمبه بافری سه راس گوسفند نر و به روش برون تنی ارزیابی شد. کیتیک تخمیر در زمان ۵۴ ساعت گرمخانه گذاری برآورد شد. فراسنجه های کیتیک تخمیر، تولید گاز، متان، غلظت نیتروژن آمونیاکی، تجزیه پذیری ماده آلی، ضریب تفکیک پذیری، تولید توده میکروبی، غلظت اسیدهای چرب فرار و جمعیت پروتوزوایی مورد ارزیابی قرار گرفتند. نتایج نشان داد که افزودن ۳۰۰ میکرولیتر اسانس اکالیپتوس سبب افزایش گاز تولیدی از بخش دیر تخمیر (b) شد ($p=0/027$). کل گاز تولیدی در ۵۴ ساعت تخمیر در سطح ۳۰۰۰ میکرولیتر اسانس اکالیپتوس و گلپر کمترین مقدار را نشان داد. افزودن ۳۰۰ میکرولیتر اسانس گلپر سرعت گاز تولیدی (c) را در طی ۵۴ ساعت تخمی کاهش داد ($p=0/036$). سطح ۳ میکرولیتر اسانس اکالیپتوس مقدار گاز تولیدی ۲۴ ساعت را بهبود ($p=0/004$) و در سطح ۳۰۰۰ میکرولیتر مهار ($p=0/001$) کرد، در حالی که اسانس گلپر تخمیر را فقط در سطح ۳۰۰۰ میکرولیتر (۰/۰۰۰) مهار کرد. استفاده از اسانس های اکالیپتوس و گلپر در تمام سطوح تولید پروتئین میکروبی را مهار کرد ($p=0/001$). تیمارهای اسانس گلپر در سطوح ۳ و ۳۰۰۰ میکرولیتر غلظت نیتروژن آمونیاکی را کاهش داد ($p=0/042$). ماده آلی تجزیه شده در سطوح ۳، ۳۰ و ۳۰۰ میکرولیتر اسانس اکالیپتوس بهبود و در سطح ۳۰۰۰ گلپر کاهش یافت. افزودن ۳۰۰۰ میکرولیتر اسانس اکالیپتوس و گلپر سبب افزایش ضریب تفکیک پذیری، تولید توده میکروبی و راندمان تولید توده میکروبی شد ($p=0/001$). غلظت اسیدهای چرب فرار تحت تأثیر اسانس های اکالیپتوس و گلپر در سطح ۳۰۰ میکرولیتر کاهش یافت ($p=0/001$). اسانس های اکالیپتوس و

گلپر خاصیت ضد پروتوزوایی از خود نشان دادند. نتایج کلی نشان داد اسانس اکالیپتوس و گلپر در سطح ۳۰۰ میکرولیتر در هر ۳۰ میلی لیتر مایع شکمبه می تواند تخمیر شکمبه ای را بهبود (یعنی افزایش راندمان پروتئین میکروبی، کاهش پروتئین میکروبی و جمعیت پروتوزوایی) را دارند، که ممکن است منجر به مصرف بهتر ماده مغذی و در نهایت رشد دام شوند.