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Effect of Essential Oils of *Zataria multiflora*, *Eucalyptus globolus* and Their Combination on Fermentation Parameters Using Merghoz Goat Rumen Liquor

Research Article

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ABSTRACT

In this experiment, different doses of *Zataria multiflora* (ZM) and *Eucalyptus globolus* (EG) essential oils (EOs) (0, 100, 250, 500, 750 and 1000 µg/mL) and a mixture of them were added to alfalfa hay incubated with buffered rumen liquor of Merghoz goat to assess *in vitro* gas production, rumen fermentation and protozoa population. In all treatments the asymptotic gas volume (B) and the rate constant (c) were decreased while the lag phase was increased. There were no effects of ZM and EG on pH, but in high doses of ZE pH was higher than that of control (P<0.001). The ammonia-N concentration was decreased due to addition of combination of EOs (P<0.01) and volatile fatty acid concentration was reduced (P<0.01) following incorporation of EOs. Gas production and organic matter digestibility were decreased (P<0.01) 24 h after incubation, whereas the partitioning factor was increased. Metabolizable energy was decreased, (P<0.05). By inclusion of EOs, total protozoa population and individual genera reduced (P<0.001). The results revealed that EOs of ZM and EG could be potentially used to modulate rumen fermentation, but using them at high level doses have anti-protozoal effects.

KEY WORDS methane production, organic matter digestibility, partitioning factor, protozoa population.

INTRODUCTION

Improving the protein and energy efficiency in ruminant nutrition is a major concern. Essential oils (EOs) have strong antimicrobial properties and can modulate ruminal fermentation to improve nutrient utilization in ruminants by decreasing deamination, methanogenesis activity and methane production in the rumen (Benchaar *et al*. 2008). *Zataria multiflora* (ZM) is a medicinal plant which belongs to the family Labiatae. The essential oils of ZM have strong inhibitory effects against some bacteria. Carvacrol, a monoterpenoid phenol, is the main constituent of ZM essential oil (Talebzadeh *et al*. 2012). The other major constituents were p-cymene, thymol, p-pinene and carvacrol methyl ether. Eucalyptus globolus (EG) is a tall evergreen tree and produce a wide variety of oils. The main active ingredient of EG essential oil is 1, 8- cineole. Pinene, o-cymene and limonene are the other components of EG (Maciel *et al*. 2010). Based upon these characteristics, an *in vitro* experiment was conducted to study the addition effect of ZM and ZM essential oils, and their combination to alfalfa hay incubated with buffered rumen liquor of Merghoz goat on *in vitro* gas production, rumen fermentation and protozoa population.

MATERIALS AND METHODS

Essential oils

Air-dried aerial parts of ZM at full flowering stage (collected from Shiraz Province, Iran) and Eucalyptus leaves (collected from, Kermanshah Province, Iran), were hydro-distillated for 2.5 h, using Clevenger-type apparatus, according to the method described by the British Pharmacopoeia (1988). The amount of oil that was obtained from ZM and EG were 2.24% and 2.60%, respectively. Essential oils were dried over anhydrous sodium sulphate and stored in sealed glass vials at 4 ˚C. Stock solutions were prepared by dissolving the essential oils in absolute ethanol (mg/mol). For control bottles also equal volumes of ethanol (1% vol/vol) were added as a positive control.

Rumen inoculum

Rumen inoculums were collected from six Merghoz goats using esophageal tube before morning feeding of a diet containing alfalfa hay. The chemical composition of the diet was organic matter (OM), 930 g/kg; crude protein (CP), 138.6 g/kg; neutral detergent fiber (NDF), 544 g/kg; ether extract, 15.6 g/k on dry matter (DM) basis. Rumen contents were strained through four layers of cheese cloth and were continuously purged with CO_2 to stabilize anaerobic condition and kept at 39 ˚C in a water bath before use.

In vitro gas production (IVGP)

For measuring the kinetics of gas production, 200 mg of alfalfa hay was weighed into a 120 mL Wheaton vial. The vials were subsequently filled with 30 mL of inoculation medium consisting of 10 mL of rumen fluid and 20 mL of buffer solution as described by Menke and Steingass (1988). ZM or EG essential oils (0, 150, 300, 450 and 600 µg/mL) and a combination of them (0, 250, 500, 750 and 1000 µg/mL) were added to the vials, subsequently. Three bottles as blanks containing 30 mL of inoculation medium were also included. The vials were sealed (under CO_2) and placed in a rotor inside incubator (39 ˚C). The gas pressure was recorded at 0, 2, 4, 6, 8, 10, 12, 24, 32, 48, 72, 80 and 96 h after incubation. The pressure of gas produced in each vial was recorded using a pressure transducer (Testo 512; Testo Inc. Germany). These recorded pressures were used to estimate the generated gas volumes (Lopez *et al*. 2010). After subtraction of gas production from blank bottles, data were fitted to exponential model (Ørskov and McDonald, 1979):

$$y= B [1 − \exp −c × (t−lag)]$$

Where:

y: cumulative volume of gas produced at time t (h).
B: asymptotic gas volume.
c: rate constant.
lag: time (h) between inoculation and commencement of gas production.

Chemical analysis

Alfalfa sample was oven-dried and ground through a 1 mm screen mill (Foss, model CyclotecTM 1093). 500 mg of the substrate and 40 mL of buffered rumen fluid were added to the bottles (Makkar, 2010) and different doses of EOs were included, subsequently. After 24 h incubation, the pressure of gas produced in the headspace of each bottle was recorded using a pressure transducer (Testo 512; Testo Inc. Germany). Then bottles were respectively transferred to an ice bath to stop fermentation and then opened to measure medium pH using a pH meter (Inolab level 2, Germany). Supernatants were collected and frozen at −20 ˚C until ammonia and total volatile fatty acids (TVFA) analysis. NH_3-N concentration of the bottle content was determined by spectrophotometer (CARY100, VARIAN) according to Broderick and Kang (1980). Total VFAs concentration was measured by Markham apparatus according to the method described by Barnett and Reid (1957) and methane content of the produced gas was determined according to Demeyer *et al*. (1988) and Fievez *et al*. (2005). The metabolizable energy (ME) of substrate was calculated on the basis of the formula proposed by Menke and Steingass (1988), as follows:

$$ME (MJ/kg DM)= 2.20 + 0.136 × GP + 0.0057 × CP + 0.00029 × EE^2$$

Where:
ME: metabolizable energy (MJ/kg DM).
EE: ether extract.
GP: cumulative gas production after 24 h incubation.

In a separate run, *in vitro* organic matter digestibility (OMD) after 24 h incubation was calculated using method described by Makkar (2010). The ratio of substrate truly degraded (mg) to gas volume (mL) at different incubation times was expressed as the partitioning factor (PF) which was determined according to Blümmel *et al*. (1997). Also microbial mass was calculated as mg substrate truly degraded − (mL gas volume×stoichiometrical factor) as described by Blümmel *et al*. (1997).

Protozoa enumeration

For counting protozoa population, whole contents of vials were sustained by diluting with an equal volume formalin solution.

Total numbers and three subfamilies of Entodiniinae, Ophryscolecinae, Diplodiniinae and family Isotrichdae of ciliate protozoa were identified according to the procedures described by Dehority (1993).

Statistical analyses

The observations of experiment were subjected to statistical analysis of variance using the following model to examine the effects of different doses of ZM, EG or their combination on all parameters in three replicates:

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

Where:
Y_{ij}: observation.
μ: overall mean for each parameter.
T_i: effect of doses.
e_{ij}: residual error.

Data were analyzed using the procedure of SPSS 23.0 software (SPSS, 2015). For all analyses, specific orthogonal contrasts were used to test 1) control *vs*. the average of EOs doses and 2) linear (L), quadratic (Q) and cubic (C) effects of EOs doses on parameters. For protozoal count data, normality assumptions of residuals were tested using Proc Univariate (SPSS 23.0) with the Kolmogorov–Smirnov test. For all statistical analyses, significance was declared at ($P<0.05$) and trends at ($P<0.1$). The data for kinetics were processed with the $y= B [1 - \exp -c \times (t-lag)]$ using the Prism 3.0 software. The results were subjected to one-way variance analysis and compared by using the Duncan test with 5% probability.

RESULTS AND DISCUSSION

Effects of essential oils on gas production

Results showed that control group had the higher ($P<0.001$) 'B' and 'c' values and the lower ($P<0.001$) lag time (L) than those of other treatments (Table 1). A large increase in lag time was observed in high doses of essential oils for all treatments.

Similar to the present study, Taghavi-Nezhad *et al.* (2014) found that asymptotic gas production and rate of gas production decreased with the addition of *Zataria multiflora* essential oil to a concentrate-based substrate and Talebzadeh *et al.* (2012) reported comparable results with the incorporation of 150-600 mg/mL of *Zataria multiflora* essential oil to the incubation medium. This reduction can be due to decreased fermentation activity of microorganisms. Gallucci *et al.* (2009) reported that carvacrol and thymol (the main constituents of ZM) are known to have bactericidal or bacteriostatic effects.

The eucalyptus is also a rich source of an antiseptic component (cineole) and contains substances with strong antibacterial properties (Sallam *et al.* 2009). Results showed that ZM and EG were more effective than their combination in reducing gas production.

A noticeable increase in the 'L' value was observed at high doses of EOs. This is due to the fact that essential oils decrease colonization and digestion of readily fermentable substrates without effect on fibrous substrates (Wallace *et al.* 2002). Others have also shown that phenolic compounds inhibit digestion of soluble fractions of feeds as well as the attachment of bacteria to insoluble components of feeds (McAllister *et al.* 1994).

The gas production after 24 h (GP_{24}) was decreased by different levels of essential oils of ZM, EgG ($P<0.001$) and their combination ($P<0.05$). This finding is in agreement with observations of Macheboeuf *et al.* (2008) reporting decrease in gas production up to 83% after addition of oregano to the incubation media. Carvacerol and thymol caused a reduction in gas production (Benchaar *et al.* 2007). Reduction in gas production may due to decline in TVFA (Table 2), methane productions (Table 1) and fermentable organic matter (Table 2).

Methane production decreased ($P<0.001$) with increased level of essential oils in all the treatments and it might be due to decreased gas production which represent reduction in fermentation of incubated material. Sallam *et al.* (2009) also observed the linear reduction in methane emission due to the Eucalyptus essential oil supplementation. They emphasized that the reduction in methane production was attributed to a decrease in the fermentable substrate rather than to a direct effect on methanogenesis.

Garcia-González *et al.* (2008) in their study also showed that plant active compounds can reduce methane production by affecting protozoa population. Methane production decreased in batch culture when essential oils were added at 1 µL/mL or at 70, 140 and 280 ppm (Jahani-Azizabadi *et al.* 2014).

Fermentation parameters and digestibility

Treatment with Zataria and Eucalyptus did not affect pH of media and it was in normal range but the combination of EOs increased the value of pH ($P<0.05$). Supplementation of diet with cinnamaldehyde (the active compound of cinnamon) in dairy cows (Benchaar *et al.* 2008) and beef cattle (Yang *et al.* 2010) did not alter ruminal pH. The ammonia nitrogen (NH_3-N) was not affected by Eucalyptus or low doses of Zataria but it decreased due to incorporation of the combination of EOs ($P<0.05$) and high doses of Zataria ($P<0.001$). At all doses of Eucalyptus and low doses of Zataria the concentration of NH_3-N remained unchanged. At high doses of Zataria decline in NH_3-N was observed.

Table 1 Effect of different doses of essential oils on kinetics of gas production

Parameters	Essential oil (EO, µg/mL)						SEM	Contrasts[2]			
	0	100	250	500	750	1000		Control *vs.* EO	L	Q	C
Zataria multiflora											
B	281.56^{d}	330.73^{e}	290.06^{d}	213.63^{c}	124.26^{b}	52.09^{a}	23.96	***	***	***	***
C	0.051^{c}	0.029^{abc}	0.017^{ab}	0.017^{ab}	0.041^{bc}	0.009^{a}	0.004	**	*	NS	*
L	1.30^{a}	4.24^{ab}	10.67^{bc}	15.89^{c}	10.61^{bc}	13.84^{c}	1.44	***	***	*	NS
GP_{24} mL/g OMD	374.43^{e}	325.40^{d}	249.46^{c}	178.86^{b}	156.06^{b}	64.13^{a}	25.61	***	***	NS	NS
Methane % of GP_{24}	29.97^{d}	26.95^{d}	18.41^{c}	17.38^{c}	11.96^{b}	2.47^{a}	2.24	***	***	NS	NS
Methane mL/g OMD	112.36^{f}	87.66^{e}	45.93^{d}	31.06^{c}	18.60^{b}	1.80^{a}	9.45	***	***	***	NS
Eucalyptus globolus											
B	281.56^{c}	298.36^{cd}	311.50^{d}	272.23^{c}	191.46^{b}	100.99^{a}	18.1	***	***	***	NS
C	0.051^{b}	0.031^{a}	0.025^{a}	0.016^{a}	0.018^{a}	0.032^{b}	0.003	**	*	**	NS
L	1.30^{a}	3.23^{ab}	3.22^{ab}	10.24^{bc}	10.56^{bc}	13.84^{c}	1.36	*	***	NS	NS
GP_{24} mL/g OMD	374.43^{d}	369.2^{d}	355.7^{d}	306.16^{c}	262.8^{b}	193.86^{a}	16.10	***	***	***	NS
Methane % of GP_{24}	29.97^{e}	26.60^{cd}	23.96^{cd}	20.48^{bc}	16.58^{b}	3.42^{a}	2.22	***	***	*	NS
Methane mL/g OMD	112.36^{d}	94.56^{cd}	88.26^{c}	63.23^{b}	43.66^{b}	7.16^{a}	8.85	***	***	*	NS
Zataria and Eucalyptus combination											
B	281.56^{c}	-	320.16^{d}	274.56^{c}	176.66^{b}	113.90^{a}	20.63	***	***	***	**
C	0.051^{b}	-	0.020^{a}	0.013^{a}	0.013^{a}	0.023^{a}	0.004	***	**	**	NS
L	1.30^{a}	-	10.13^{ab}	17.04^{bc}	23.08^{c}	17.30^{bc}	2.33	**	**	*	NS
GP_{24} mL/g OMD	374.43^{c}	-	348.2^{bc}	308.46^{b}	257.06^{a}	230.03^{a}	15.66	*	***	NS	NS
Methane % of GP_{24}	29.97^{c}	-	25.27^{c}	18.57^{b}	11.24^{a}	11.03^{a}	2.13	***	***	NS	NS
Methane mL/g OMD	112.36^{d}	-	88.03^{c}	57.13^{b}	29.36^{a}	25.23^{a}	9.32	**	***	NS	NS

[1] B: the asymptotic gas volume; c: the rate constant and L: lag time.
[2] L: linear; Q: quadratic and C: cubic.
GP: gas production and OMD: organic matter digestibility.
The means within the same column with at least one common letter, do not have significant difference (P>0.05; P>0.01 and P>0.001).
SEM: standard error of means.
NS: non significant.
* (P<0.05); ** (P<0.01) and *** (P<0.001).

However, in this study the NH_3-N in all levels of essential oils was in normal rang (85-300 mg/L, McDonald *et al*. 2010). A reduction in ammonia concentration reflects an inhibitory effect of EOs on proteolytic activity of rumen microorganisms. As reported essential oils inhibit amino acid deamination by ruminal microbes (Mcintosh *et al*. 2003) and lead to a reduction in protozoal population (Newbold *et al*. 2004).

The levels of 750 and 1000 µg/mL of EOs, decreased the concentration of TVFA. It might be a result of inhibited protozoa activity in the rumen (Williams and Coleman, 1992; Table 3).

The inconsistency of VFAs concentration because of essential oils was observed in the literatures. With the use of plant secondary metabolites, Spanghero *et al*. (2008) found decrease in VFAs, but Newbold *et al*. (2004) reported that essential oils tended to stimulate VFA production and Talebzadeh *et al*. (2012) observed an increase in TVFA by adding low level (150 µg/mL) of ZM to the fermentation media. In contrast, Beauchemin and McGinn (2006) reported no changes in VFAs production, and Castillejos *et al*. (2007) observed different responses to EOs concerning VFAs production depending on the type and dose of EOs and experimental conditions.

These differences may be due to the synergistic effects of cineole, carvacrol and other secondary metabolites in eucalyptus and ZM essential oil (Joch *et al*. 2016). Organic matter digestibility was influenced by incorporation of EOs and the effect was more significant in EG than other treatments. Reduction in OMD might be a consequence of decrease in fermentation of substrate due to EOs as can been seen from reduced gas production (Table 1). Same to this result, cinnamon oil (Fraser *et al*. 2007) and thymol (Castillejos *et al*. 2007) caused decline in dry matter digestibility, however, addition of eugenol had no significant effect (Castillejos *et al*. 2007). Higher values for PF were obtained at levels > 500 µg/mL of EOs. Microbial biomass (MB) and efficiency of microbial biomass by adding ZM (at all levels) or EG and their combination (at high levels) were increased (Table 2). Similar to these results, other investigators reported an increase in PF and MB by supplementation of *Thymus kotschyanus* (Mirzaei *et al*. 2016) and *Zingiber multifloria* (Talebzadeh *et al*. 2012) essential oils. As partitioning factor (PF) represents the efficiency of fermentation and microbial protein production (Blümmel *et al*. 1997), probably digested organic matter by addition of EOs resulted in greater microbial biomass growth rather than VFA production (Taghavi-Nezhad *et al*. 2011).

Table 2 Effect of different doses of essential oils on *in vitro* fermentation parameters

Parameters	Essential oil (EO, µg/mL)						SEM	Contrasts[2]			
	0	100	250	500	750	1000		Control *vs*. EO	L	Q	C
Zataria multiflora											
pH	6.65	6.61	6.67	6.74	6.75	6.72	0.231	NS	NS	NS	NS
NH_3-N (mg/L)	162.29^{bc}	171.83^{c}	182.50^{c}	147.96^{bc}	126.87^{ab}	94.5^{a}	11.1	NS	**	*	NS
TVFA (mmol/L)	51.66^{bc}	61.66^{c}	53.33^{bc}	47.58^{b}	22.00^{a}	21.01^{a}	3.98	*	***	**	*
OMD (mg)	179.43^{cd}	189.43^{d}	187.56^{d}	170.06^{c}	139.43^{b}	127.03^{a}	5.94	**	***	***	**
PF	3.05^{a}	3.23^{ab}	3.35^{ab}	3.46^{b}	3.34^{c}	6.49^{d}	0.202	***	***	***	***
MB (mg)	50.13^{a}	60.38^{ab}	64.36^{b}	61.90^{ab}	68.71^{b}	84.01^{c}	2.85	**	***	NS	*
EMB (%)	27.77^{a}	31.82^{ab}	34.26^{b}	36.31^{b}	49.28^{c}	66.07^{d}	3.21	***	***	***	*
ME (MJ/kg DM)	10.27^{e}	10.26^{e}	9.94^{d}	9.05^{c}	6.74^{b}	6.94^{a}	0.488	***	***	***	*
Eucalyptus globolus											
pH	6.65	6.71	6.64	6.73	6.80	6.76	0.215	NS	NS	NS	NS
NH_3-N (mg/L)	162.29^{a}	146.37^{a}	156.63^{a}	164.25^{a}	165.50^{a}	165.67^{a}	0.854	NS	NS	NS	NS
TVFA (mmol/L)	51.66^{b}	43.33^{ab}	65.00^{c}	68.33^{c}	30.83^{a}	31.66^{a}	3.82	NS	**	***	NS
OMD (mg)	179.43^{b}	153.90^{a}	146.8^{a}	159.7^{a}	160.66^{a}	159.4^{a}	2.77	***	NS	**	**
PF	3.05^{c}	2.64^{ab}	2.53^{a}	2.90^{bc}	3.38^{d}	5.08^{e}	0.210	*	***	***	*
MB (mg)	50.13^{c}	25.99^{ab}	19.25^{a}	38.69^{bc}	56.17^{c}	90.42^{d}	5.18	NS	***	***	NS
EMB (%)	27.77^{b}	16.83^{a}	13.03^{a}	24.15^{b}	34.78^{c}	56.71^{d}	3.52	NS	***	***	NS
ME (MJ/kg DM)	10.27^{d}	10.19^{d}	10.17^{d}	9.76^{c}	8.47^{b}	6.65^{a}	0.339	***	***	***	***
Zataria and Eucalyptus combination											
pH	6.65^{a}	-	6.59^{a}	6.73^{ab}	6.86^{bc}	6.92^{c}	0.037	*	***	NS	NS
NH_3-N (mg/L)	162.29^{b}	-	124.39^{a}	115.65^{a}	120.99^{a}	109.33^{a}	5.56	***	***	*	NS
TVFA (mmol/L)	51.66^{b}	-	42.66^{ab}	42.65^{ab}	33.33^{a}	36.00^{a}	2.03	**	**	NS	NS
OMD (mg)	179.43^{d}	-	176.78^{d}	150.70^{c}	130.43^{b}	117.41^{a}	6.66	***	***	NS	*
PF	3.05^{a}	-	3.02^{a}	3.61^{b}	4.20^{c}	4.96^{d}	0.20	***	***	**	NS
MB (mg)	50.13^{a}	-	48.13^{a}	58.81^{ab}	61.97^{b}	65.28^{b}	2.21	**	***	*	*
EMB (%)	27.77^{a}	-	27.22^{a}	39.01^{b}	47.46^{c}	55.57^{d}	3.02	***	***	*	*
ME (MJ/kg DM)	10.27^{c}	-	10.23^{c}	7.96^{b}	6.06^{a}	5.50^{a}	0.544	***	***	NS	**

[1] NH_3-N: ammonia nitrogen; TVFA: total volatile fatty acids; OMD: organic matter digestibility; PF: partitioning factor; MB: microbial biomass; EMB: efficiency of microbial biomass and ME: metabolizable energy.
[2] L: linear; Q: quadratic and C: cubic.
The means within the same column with at least one common letter, do not have significant difference (P>0.05; P>0.01 and P>0.001).
SEM: standard error of means.
NS: non significant.
* (P<0.05); ** (P<0.01) and *** (P<0.001).

Table 3 Effect of different doses of essential oils on protozoa population ($\times 10^4$/mL)

Parameters	Essential oil (EO, µg/mL)						SEM	Contrasts			
	0	100	250	500	750	1000		Control *vs*. EO	L	Q	C
Zataria multiflora											
Total protozoa	15.00^{c}	9.44^{b}	8.05^{ab}	4.72^{a}	5.27^{a}	5.83^{ab}	0.93	***	***	**	NS
Entodinium spp.	11.39^{c}	8.33^{bc}	7.50^{ab}	4.44^{a}	5.27^{ab}	5.83^{ab}	0.65	***	***	*	NS
Isotricha spp.	0.833^{b}	0.28^{ab}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.11	*	*	NS	NS
Diplodiniinae	0.833^{b}	0.28^{ab}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.11	*	*	NS	NS
Ophryoscolecinae	1.94^{b}	0.56^{a}	0.28^{a}	0.28^{a}	0.00^{a}	0.00^{a}	0.22	**	**	NS	NS
Eucalyptus globolus											
Total protozoa	15.00^{c}	9.16^{b}	6.11^{ab}	3.05^{a}	4.45^{a}	3.05^{ab}	1.11	***	***	**	NS
Entodinium spp.	11.39^{c}	6.66^{b}	5.55^{ab}	3.05^{a}	3.89^{ab}	3.05^{a}	0.77	***	***	**	NS
Isotricha spp.	0.833^{a}	0.56^{a}	0.28^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.13	NS	NS	NS	NS
Diplodiniinae	0.833^{a}	0.833^{a}	0.28^{a}	0.00^{a}	0.56^{a}	0.00^{a}	0.18	NS	NS	NS	NS
phryoscolecinae	1.94^{b}	1.11^{a}	0.56^{a}	0.00^{a}	0.55^{a}	0.00^{a}	0.26	NS	NS	NS	NS
Zataria and Eucalyptus combination											
Total protozoa	15.00^{b}	-	3.88^{a}	4.72^{a}	3.05^{a}	2.77^{a}	1.30	***	***	**	*
Entodinium spp.	11.39^{b}	-	3.05^{a}	4.16^{a}	3.05^{a}	2.77^{a}	0.94	***	***	**	*
Isotricha spp.	0.833^{b}	-	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.12	**	*	NS	NS
Diplodiniinae	0.833^{a}	-	0.28^{a}	0.28^{a}	0.00^{a}	0.00^{a}	0.13	*	*	NS	NS
Phryoscolecinae	1.94^{b}	-	0.56^{ab}	0.28^{ab}	0.00^{a}	0.00^{a}	0.25	*	*	NS	NS

[1] L: linear; Q: quadratic and C: cubic.
The means within the same column with at least one common letter, do not have significant difference (P>0.05; P>0.01 and P>0.001).
SEM: standard error of means.
NS: non significant.
* (P<0.05); ** (P<0.01) and *** (P<0.001).

Addition of essential oils into substrate caused a reduction in metabolizeable energy (ME). This result may be related to reduction in gas production, VFA concentration and OMD in the fermentation medium especially in high doses.

Effects of essential oils on protozoa concentration

The results showed that essential oils decreased total protozoal count (P<0.001). The concentration of *Entodinium* spp. (P<0.01), *Isotricha* spp., *Diplodiniinae* and *Ophryoscolecinae* also reduced (P<0.001). The antiprotozoal effect of EOs was most likely due to the phenolic structure of its main active compounds (Talebzadeh *et al.* 2012). Such a structure can lead to demolition of cell membrane, inhibition of enzymes and lack of substrates which are essential for cell metabolism (Goel *et al*. 2005) and it may be related to the lipophilic nature of compounds such as anethol which facilitates permeation of EO across the protozoal membrane (Cardozo *et al*. 2006). It appeared that population of protozoa was more sensitive to combination of Zataria and Eucalyptus essential oils than each of them (Table 3).

CONCLUSION

The results of this experiment indicate that EOs of *Zataria multiflora*, *Eucalyptus globolus* have a potential to manipulate rumen fermentation favorably with antimethanogenic and defaunating properties. As regards to the essential oils combination, there is a need to identify the suitable doses without adverse effect on feed digestibility.

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