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Research Article

The Effects of *Anise* (*Pimpinella anisum*) Essential Oil and Extract on *In Vitro* Rumen Fermentation Parameters and Protozoa Population of Sheep

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ABSTRACT

The aim of this study was to evaluate the effects of different levels of Anise (*Pimpinella anisum L.*) essential oil (AEO) and extract (AEX) on rumen fermentation parameters and protozoa population of Sanjabi sheep. Levels of 0, 250, 500, 750 and 1000 µL of AEO and AEX were added into 30 mL rumen buffered fluid. Gas production test was performed in a completely randomized design with five replicates for each level. All levels of AEO reduced gas production compare with control (P=0.001), but this value increased as AEX level increased. All AEO levels decreased organic matter digestibility rate (10.3 to 3.3%), but AEX increased this parameter amount (8 to 24.9%; P=0.001). Different levels of both additives resulted in reduction of the nitrogen ammonia concentration (P=0.001). Inclusion of AEO and AEX increased and decreased partitioning factor (PF), respectively. However, total volatile fatty acids were increased by addition of AEX (P=0.001). The metabolisable and net energy lactation were improved by AEO, but AEX had contrary effect (P=0.001). Addition of AEO and AEX increased total protozoa numbers by increasing the *Entodinium* spp. population. It is concluded that AEO has the ability to improve partitioning factor (PF) and reduce ammonia concentration but AEX was more effective in this regard. It seems that AEO and AEX has no antiprotozoal effect.

Key words: In vitro, Total gas, Essential oil, Extract, Partitioning factor, Ammonia-N, Sheep

INTRODUCTION

Manipulating the rumen fermentation with the aim of reducing the ammonia (NH_3-N) production, increasing volatile fatty acids (VFA) concentration and organic matter digestibility (OMD) is the goal for improving the animal production efficiency (Busquet *et al.*, 2006) Chemical feed additives such as antibiotics, ionophores and antiprotozoal substances are among suitable candidates in improving rumen fermentation (Patra and Yu, 2012). As these additives may have adverse effects on animals and their product consumers, researchers have been trying to replace them with naturally plant-derived additives (Patra and Yu, 2012). Having active component and antibacterial effects make plant extract and essential oils as useful additives in ruminant nutrition (Castillejos *et al.*, 2006; Castillejos *et al.*, 2008).

Anethol (1-methoxy-4-propenylbenzene) is the main constituent of anise essential oil (AEO) ranging from 86% (Cardozo *et al.*, 2005) to 96.3% (Gende *et al.*, 2009) of active component. *Anethol* is a cyclic phenylpropanoid compound (Calsamiglia *et al.*, 2007). Other constituents of Anise essential oil are fatty acids such as palmitate acid and oleic acid (8-11%), carbohydrates (4%) and protein (18%) (Besharati-Seidani *et al.*, 2005). An *in vitro* report by (Busquet *et al.*, 2005) showed that *anethol* and AEO (2.2 mg/L) decreased TVFAs and acetate to propionate ratio, increased butyrate concentration, and had no effect on NH_3-N concentration (Busquet *et al.*, 2006). Another *in vitro* study with steer rumen fluid receiving 90% concentrate and 10% forage diet revealed that AEO caused a reduction in NH_3-N and acetate concentrations and an increase in propionate, although total fatty acids concentration did not change (Cardozo *et al.*, 2005). Also, (Gunal *et al.*, 2014) determined that addition of AEO (125, 250 or 500 mg/L) to *in vitro* culture did not affect the TVFA and all dose of its increased ammonia-N concentration. Nevertheless, adding 0.22 mg/L AEO into continuous culture resulted in higher NH_3-N concentration without any change in TVFA concentration (Cardozo *et*

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al., 2005). Dry matter intake of heifer by adding AEO into their diet (90% concentrate and 10% forage) was increased. The TVFA concentration didn't changed, but acetate reduced and propionate increased. It showed an antiprotozoal effect (Cardozo *et al.*, 2005).

The supposed to be that anise essential oil and extract have the ability to improve rumen fermentation, therefore, purpose of present study was to investigate and compare the *in vitro* effects of AEO and anise extract (AEX) on rumen fermentation parameters (gas production, digestibility, nitrogen ammonia and energy) and protozoa population.

MATERIALS AND METHODS

Preparation of Essential Oil (AEO) and Extract (AEX)

Anise essential oil (AEO) was obtained from its air-dried grain using Clevenger-type apparatus and then oil was dried over anhydrous sodium sulfate. The extract of anise (AEX) was collected according to Hohenheim method by using pure ethanol as solvent (Goel *et al.*, 2008).

Animals

The ruminal fluid was obtained from three fistulated castrated male Sanjabi sheep (42.0 ±1.5 kg) before the morning feed (Menke *et al.*, 1979). The animals were fed twice daily (08:00 and 16:00) with a basal diet containing alfalfa hay and barley (40 and 60 percent DM basis). Fresh water and mineral blocks (Fe =1232 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 (Council, 2007).

Experimental design and fermentation method

AEO and AEX at 5 levels (0, 250, 500, 750 and 1000 µL/30 mL rumen buffered fluid) and 5 replications (for each level) were added to fermentation medium. The total 25 wheaten bottle120 ml were used. The 200 mg of substrate (60% barley and 40% alfalfa hay) was weighed into a 120 mL Wheaton vial, and then 30 mL rumen buffered fluid (buffer solution and rumen fluid in 2 to 1 ratio) was added (Menke *et al.*, 1979). They were incubated at 39°C for 24 hours. Then after, total gas production in each bottle was recorded using a scaled syringe.

Fermentation parameters

After incubation, samples of incubated rumen fluid were prepared in order to measure organic matter digestibility (OMD), ammonia nitrogen (NH_3-N) and total volatile fatty acids (TVFAs) concentration. The NH_3-N concentration was analyzed by spectrophotometer at 630 nm wavelength according to (Broderick and Kang, 1980) method. Partitioning factor (mg OMD/mL gas production) was determined by using Eq. 1 (Vercoe *et al.*, 2010).

Eq. 1: $PF = c - (a-b)/ IVGP$

Where *c* is mg OMD in each bottle, *a* is undigested matter (mg), *b* is ash of undigested matter (mg), and IVGP is in vitro gas production.

After measuring produced gas during 24 h incubation, bottle containing was transferred into a beaker and boiled in neutral detergent solution for 1 h. Then the containing was filtered by Whatman paper and the rest was dried in oven at 100°C for 10 h. By subtracting the empty crucible from the containing weight after oven, the indigested matter in each bottle (*a*) was calculated. Then, the crucible and its containing were transferred into furnaces at 550◦C and the ash (*b*) was recorded. By subtracting *b* from *a*, the indigested organic matter was computed.

Total fatty acids (mmol/L) were investigated by 2 previously described methods of Markham apparatus (Barnett, 1957) and Eq. 3 (Jiménez-Peralta *et al.*, 2011):

Eq. 3: $SCFA_{mmol/L}=[(0.0222\times GP)- 0.00425)\times] \ 100$

By using equation 4, metabolizable energy was estimated (Menke *et al.*, 1979) .

Eq. 4: $ME_{mj/KgDM}= [(2.2) + (0.136\times GP) + (0.0057+CP) + (0.00029\times EE^2)]$

Where ME is metabolisble energy (MJ/kg DM), EE is ether extract and GP is cumulative gas production after 24 h incubation. Also according to Eq. 5, net energy lactation of treatments was calculated (Abaş *et al.*, 2005).

Eq. 5: $NE_{L\ mj/KDM} = [(0.115\times GP)+(0.0054+CP)+ (0.014\times EE)-(0.0054\times CA)- 0.36]$

Where NE_L is net energy lactation (Mj/ kg), GP is gas production (mL), CP is crud protein, EE is crud fat and CA is ash.

Proximate analyses and organic matter disappearance (OMD)

The substrate was analyzed for dry matter (ID number 930.15), ash (ID number 924.05), and total N (ID number 984.13) (AOAC, 2000). The OMD percentage (Eq. 7) was estimated according to (Menke *et al.*, 1979):

Eq. 7: $OMD\ \% = 14.88 + [0.889 \times GP_{24}] + [0.045\times XP] + [0.065 \times XA]$

Where OMD is OM disappearance, GP_{24} is the net gas production (mL) after 24 hours, XP crude protein (g Kg^{-1} DM) and XA ash (g Kg^{-1} DM).

Protozoa enumeration

After fermentation process, rumen fluid was mixed with formalin solution with 1:5 ratio (8/1g sodium chloride in 900 mL distillated water and 100 mL formalin 36%) and stored in 4°C. Population of ciliate protozoa of three subfamilies *Entodiniinae*, *Ophryscolecinae*, *Diplodiniinae* and family *Isotrichdae* were determined by a Hemocytometer chamber and light microscope (model Nikon, YS 100) with 9 repetitions in each treatment according to below equation.

Eq 6: $$NP\ mL = \frac{N}{[area\ mm\ .\ Dmm.\frac{1}{n}]} \times 1000$$

Where NP is counted protozoa per each mL, N is protozoa number in each chamber counting, $area_{mm}$ is area of each any square ($1mm^2$), D_{mm} is the depth of each square of chamber (0.1 mm), and $\frac{1}{n}$ is dilution coefficient ($\frac{1}{5}$).

Statistical analysis

Collected data from gas production test and protozoa population during incubation period were compared by the SPSS 24 software (SPSS, 2016) for 5 levels of essential oil and extract according to equation 10, and means compared by Duncan test with 5% and 1% probability.

Eq. 10: $Y_{ijk}=\mu+T_i+\varepsilon_{ij}$

Data of protozoa population first examined by Kolmogorov–Smirnov test and then multifactor data was analyzed by equation 9:

Eq. 9: $Y_{ijk}=\mu+T_i+S_j+\varepsilon_{ij}$

Where Y_{ij} is the amount of each observation, μ is the overall mean for each parameter, T_i is the effect of treatment, Sj is effect of each observation and e_{ij} is the residual error. AEO and AEX were compared by independent t test.

RESULTS

Effect of AEO and AEX on fermentation characteristics

Results presented in Table 1 showed that gas production in all levels of anise essential oil (AEO) and anise extract (AEX0 after 24 h. All levels of AEO reduced (P=0.001) while AEX showed an increasing manner in gas production (P=0.001) in compered with control group. Rising in AEX level resulted in higher gas production as the highest gas volume was produced at 1000 µL level of AEX. In addition, compared between gas production resulted AEO and AEX showed that there was a significant difference between of AEO and AEX supplementations.

The AEO reduced the organic matter digestibility (OMD) compared with control (P=0.001); but no significant differences were found between levels. While AEX caused an increase in OMD (P=0.001). A large increase in OMD was observed in high doses of extract. Both additives (AEO and AEX) decreased NH_3-N concentration where the least value was observed at 1000µL of AEO level. The AEO increased partitioning factor whereas AEX decreased it (P=0.001), although in both treatments PF was lower than normal range (2.74-4.65). Partitioning factor in AEO was higher than AEX (P=0.001).

Based on the Table 2 data, AEO and AEX levels increased TVFA concentration (P=0.001). No significant differences were found between levels of AEO and AEX except 500 µL level (Table 2). Adding AEO into fermentation medium resulted in a reduction in metabolisable energy (Mj/kg DM; P=0.001), although AEX increased ME (P=0.001); as it was more obvious at high levels. However, the effect of AEX on metabolisable energy was more than AEO (P=0.001). The effect of AEO on net lactating energy was negative (P=0.001) but AEX had positive effect on this parameter. All levels of AEX had higher effect in this regard (P=0.001).

Table 1: Effect of level of Anise essential oil (AEO) and extract (AEX) on fermentation parameters from *in vitro* fermentation using buffered sheep rumen fluid.

Rumen Parameters	Additives		Additives levels (µL/30 mL) Control	250	500	750	1000	SEM	P-Value
Total Gas (24 h, mL/ 200 mg DM)		AEO	47.1^{a}	35.6^{b}	35.9^{b}	36.9^{b}	37.2^{b}	1.244	0.001
		AEX	47.1^{e}	56.3^{d}	63.2^{c}	66.8^{b}	75.2^{a}	1.500	0.001
	Sig		*1.00*	*0.001*	*0.001*	*0.001*	*0.001*		
IVOMDe (mg/200 mg)		AEO	108.0^{a}	88.5^{b}	89.1^{b}	90.8^{b}	82.8^{b}	2.104	0.001
		AEX	108.0^{d}	123.6^{c}	135.2^{b}	141.3^{b}	155.4^{a}	3.468	0.001
	Sig		*1.00*	*0.001*	*0.001*	*0.001*	*0.001*		
IVOMDe (%)		AEO	56.8^{a}	46.5^{b}	46.8^{b}	47.7^{b}	43.5^{b}	1.105	0.001
		AEX	56.8^{a}	64.9^{b}	71.1^{b}	74.3^{b}	81.7^{b}	1.823	0.001
	Sig		*1.00*	*0.001*	*0.001*	*0.001*	*0.001*		
Ammonia-N (mg/L)		AEO	155.2^{a}	140.1^{b}	135.2^{bc}	127.1^{c}	119.5^{d}	2.995	0.001
		AEX	155.2^{a}	137.8^{bc}	142.2^{bc}	144.1^{b}	137.9^{c}	1.483	0.001
	Sig		*1.00*	*0.001*	*0.001*	*0.001*	*0.001*		
PF (mg/mL)		AEO	2.30^{c}	2.48^{ab}	2.48^{ab}	2.45^{b}	2.57^{a}	0.217	0.001
		AEX	2.30^{a}	2.19^{b}	2.14^{bc}	2.11^{cd}	2.07^{d}	0.187	0.001
	Sig		1.00	0.001	0.001	0.001	0.001		

Table 2: Effect of level of Anise essential oil (AEO) and extract (AEX) on total volatile fatty acids and energy.

Rumen Parameters	Additives		Additives levels (µL/30 mL) Control	250	500	750	1000	SEM	P-Value
Total VFA (mmol/L)		AEO	104.2^{a}	78.6^{b}	79.3^{b}	81.6^{b}	71.1^{b}	2.761	0.001
Equation		AEX	104.2^{d}	124.6^{c}	139.9^{b}	147.9^{b}	166.3^{a}	4.552	0.001
	Sig		*1.00*	*0.001*	*0.001*	*0.001*	*0.001*		
Total VFA (mmol/L)		AEO	136.0^{b}	180.0^{a}	234.0^{b}	240.0^{a}	252.0^{b}	9.713	0.001
Barnett and Reid		AEX	136.0^{b}	196.8^{a}	192.0^{b}	237.6^{a}	244.8^{b}	9.334	0.001
	Sig		1.00	0.264	0.003	0.267	0.739		
ME(MJ/KgDM)		AEO	8.6^{a}	7.0^{b}	7.1^{b}	7.2^{b}	6.6^{b}	0.169	0.001
		AEX	8.6^{a}	9.9^{a}	10.8^{ab}	11.3^{c}	12.4^{d}	0.279	0.001
	Sig		*1.00*	*0.001*	*0.001*	*0.001*	*0.001*		
NE (MJ/KgDM)		AEO	5.0^{a}	3.7^{b}	3.8^{b}	3.9^{b}	3.3^{b}	0.142	0.001
		AEX	5.0^{d}	6.1^{c}	6.9^{b}	7.3^{b}	8.3^{a}	0.235	0.001
	Sig		*1.00*	*0.001*	*0.001*	*0.001*	*0.001*		

Table 3: Protozoa population (N×10^5/mL RF) subfamily from *in vitro* fermentation using buffered sheep rumen fluid containing different levels of AEO and AEX.

Protozoa Population	Additives	Additives levels (µL/30 mL)						P-Value
		Control	250	500	750	1000	SEM	
Total Protozoa	AEO	1.244^{cd}	1.100^{d}	1.533^{bc}	1.800^{b}	2.022^{a}	0.052	0.001
	AEX	1.244^{e}	1.966^{d}	2.722^{c}	4.511^{b}	5.188^{a}	0.116	0.001
Sig		*1.00*	*0.001*	*0.001*	*0.001*	*0.001*		
Subfamily								
Entodininnae	AEO	1.166^{c}	1.044^{c}	1.477^{b}	1.744^{a}	1.955^{a}	0.049	0.001
	AEX	1.166^{e}	1.900^{d}	2.622^{c}	4.400^{b}	5.111^{a}	0.115	0.001
Sig		*1.00*	*0.001*	*0.001*	*0.001*	*0.001*		
Diplodininane	AEO	0.0222	0.0333	0.0222	0.0222	0.0333	0.007	0965
	AEX	0.0222^{b}	0.0333^{abc}	0.0555^{ab}	0.0777^{a}	0.0000^{c}	0.008	0.026
Sig		*1.00*	*1.00*	*0.243*	*0.081*	*0.083*		
Ophryoscolecine	AEO	0.000	0.000	0.000	0.000	0.000	0.000	1.000
	AEX	0.000	0.000	0.0111	0.000	0.0222	0.004	0.242
Sig		*1.00*	*1.00*	*0.323*	*1.00*	*0.160*		
Isotrichidae (Family)	AEO	0.0555	0.0222	0.0333	0.0333	0.0333	0.0085	0.812
	AEX	0.0555	0.0333	0.0333	0.0333	0.0555	0.0630	0.841
Sig		*1.00*	*0.650*	*1.00*	*1.00*	*0.465*		

Effects on rumen protozoa

Table 3 was set out the effect of different levels of AEO and AEX on rumen protozoa population. Total protozoa count numerically increased by adding both additives (P=0.001) where high levels of AEO and AEX showed more protozoa numbers than low levels.

Furthermore, both additives increased *Entodinium* spp. population (P=0.001). The population of *Diplodiniinae* subfamily in all levels of AEO was the same as control (P=0.956), but in levels 250, 500 and 750 of AEX showed an increasing manner (P=0.026). Treatments had no effect on *Ophryscolecinae* subfamily (P=1.00). Also, comparison between tow additives didn't revealed any difference on *Ophryscolecinae* subfamily (P=0.242). The *Isotrichdae* family numbers were not affected in any levels of AEO and AEX (P=0.812 and P=0.841). In studied family and subfamilies population, *Entodinium* spp., *Diplodiniinae*, *Isotrichdae* and *Ophrys-colecinae* had more population number, respectively.

DISCUSSION

Effects of AEO and AEX on fermentation characteristics

Analysis of essential oils of anise Mill seeds, by GC/MS shows the presence of trans-anethole as major compound (82.7%), followed by pseudoisoeugenyl-2-methyl butyrate, cadinène and eustragole to the respective levels of 6.68, 2.80, and 1.22% (sidi moctar Yacoub *et al.*, 2015)

Unlike the AEO, addition of the AEX into fermentation medium resulted in higher gas production. Gas production is an indicator of digestibility of soluble and insoluble carbohydrates (Menke *et al.*, 1979) as well as fatty acids production. The main constituent of anise (anithol) is a phenyl propanoeid that have antibacterial effect (Calsamiglia *et al.*, 2007). Reduction in gas production by AEO may be due to anise antimicrobial effect suppressing the microorganism's activity. In the other hand, variation in gas production in AEO and AEX were as the same as variations in OMD. Hence, the adverse impact of effective constituent of anise essential oil on OMD can be the other reason for reducing gas production.

Based on our results, the AEX insert a positive stimulating effect on fermentation process resulting in higher gas production. As anise is containing 8-11% fatty acids rich in palmitate and oleic, 4% carbohydrates and 18% protein (Besharati-Seidani *et al.*, 2005), it seems that organic constituents of these ingredients may be used as carbon source by microorganisms and therefore increase gas production (Yadeghari *et al.*, 2013). In agreement to the results of present study, (Gunal *et al.*, 2014) reported that supplementation of AEO (125, 250 or 500 mg/L) decreased gas production under *in vitro* condition and by Holstein dairy cow rumen fluid.(Kilic *et al.*, 2011) have indicated that *in vitro* gas production was decreased by essential oils of oregano (*Origanum vulgare*), garlic (*Allium sativum*) and anise (*Pimpinella anisum*), unaffected by black seed (*Nigella sativa*), laurel (*Laurus nobilis*) and cinnamon (*Cinnamomum verum*) and increased by cumin (*Cumminum cyminum*).

In agreement to the results of present study, others reported that the main constituent of *Origanum majorana* essential oil (Macheboeuf *et al.*, 2008), as well as thymol and, carvacrol (Benchaar *et al.*, 2007) reduced *in vitro* gas production. Similar to current results, supplementation of *Echium Amoneum* extract in the *in vitro* sheep rumen fermentation increased gas production when compared with control (Soroor *et al.*, 2013). They attributed this increase in gas production to the effect of carbohydrates in *Echium Amoneum* extract.

In this study, changing in OMD was similar to gas production in both AEO and AEX. The AEX could have increased the gas production after 24h at level 1000µL up to 59% compare with control. This increase probably was due to a rise in OMD and VFAs productions which were also increased by AEX supplementation. Similarly, (Busquet *et al.*, 2006); (Agarwal *et al.*, 2009a; Agarwal *et al.*, 2009^{b}) and (Macheboeuf *et al.*, 2008) noted that the essential oils were caused reduction in OMD. In an *in vitro* experiment, (Benchaar *et al.*, 2007) showed that thymol decreased the carbohydrates digestibility. This is probably duo to the inhibitory effect of thymol on rumen cellulolytic bacteria and fungi. (Castillejos *et al.*, 2006) also indicated that adding 5, 50 and 500 mg/L of eugenol didn't have any effect on OMD.

In current study, the NH_3-N concentration in all levels of AEO and AEX was in normal range (85-300 mg/L,(McDonald *et al.*, 2011)). Phenyl propanoeids compounds (*Anethol*) by disturbing cell membrane may cause cell death. It seems that the anethol reduced NH_3-N by decreasing urease enzyme activity (Hussain and Cheeke, 1995), reducing the number of ammonia producer and proteolytic bacteria (McIntosh *et al.*, 2003); (Sivakumaran *et al.*, 2004), inhibiting the amino acids deamination (Busquet *et al.*, 2006) and/or reducing protozoa population (Benchaar *et al.*, 2008a; Benchaar *et al.*, 2008b). One of the reasons for reduced NH_3-N might be due to reduced protozoa number in this experiment where. Due to proteolytic and deamination activity of protozoa (Benchaar *et al.*, 2008a; Benchaar *et al.*, 2008b), lower number of them may lowered their engulfment by bacteria. and therefore, reduced NH_3-N. Similar to the current results, addition of 300 and 3000 mg/L cinamaldehyde and eugenol (Busquet *et al.*, 2006), 3000 mg cinnamon oil and clove bud oil (containing phenylpropanoid compound; (Busquet *et al.*, 2006), and 5, 50, 500 and 5000 mg/L eugenol (Castillejos *et al.*, 2006) had reduced the *in vitro* NH_3-N concentration. Also, supplementation of AEX and cinamaldehyde in 4 levels (0.3, 3, 30 and 300 mg/L) reduced NH_3-N concentration in calves (Cardozo *et al.*, 2005).On the other hand, in contrast to the current results, supplementation of 125 and 250 mg/L AEO *in vitro* culture media increased NH3-H as compared with control group (Gunal *et al.*, 2014). However, adding 3, 30, 300 and 3000 mg/L AEO (Busquet *et al.*, 2006) and 2.2 cinnamon extract did not have any effect on this parameter. Furthermore, (Yang *et al.*, 2010) reported that supplementation of 400, 800 and 1600 mg/d of cinnamaldehyde in the diet of growing beef cattle had no effect on the NH_3-N concentration.

As shown in Table 1, partitioning factor (PF) increased by AEO and decreased by AEX as the level of additive into fermentation medium increased. (Vercoe *et al.*, 2010) came to this conclusion that judgment about fermentation process just by amount of produced gas or OMD is incorrect. Therefore, partitioning factor looks more convenient parameter for explaining the fermentation process. PF is calculated by mg OMD to gas 24 h ratio and the normal range of this parameter is 2.25 to 4.41 (Blümmel *et al.*, 1997). Selection of plants based on higher partitioning factor means that those plants with high *in vitro* digestibility and low gas production must be selected (Blümmel *et al.*, 1997). Thus, according to (Blümmel *et al.*, 1997) higher PF value represents the fermentation improvement.

In some studies contradictory results have been reported, for instance, (Sallam *et al.*, 2009) observed that thyme and fennel essential oil reduced PF mean while Ginger (*Zingiber officinal*e) and *Nigella sative* increased it although gas production decreased. *Echium amoneum* extract decreased PF (Soroor *et al.*, 2013) and levels 500, 750 and 1000 of oregano essential oil increased it (Yadeghari *et al.*, 2013).

Variation in volatile fatty acids (VFAs) usually is reflected by altering in gas production and also microbial protein amounts (Vercoe *et al.*, 2010). In the current study, changing in total VFA (in equation method) in both essential oil and extract groups was in agreement with changing in total gas production where total short-chain VFA concentration decreased by anise essential oil and increased by its extract similar to the total 24 h gas production. Although using (Barnett, 1957) method, VFA concentration showed increased amount in all levels of both treatments, there wasn't any difference between essential oil and extract. Acetate is the end product of protozoa metabolism (Hess *et al.*, 2003) and therefore, reducing protozoa population causes reduced acetate production (Patra *et al.*, 2006). It seems that due to increased number of protozoa, total VFAs concentration has been affected by increased acetate production. Hydrophobic property of essential oils make them able to enter the bacterial bilateral cell membrane (Calsamiglia *et al.*, 2007), cause structural changes in cell membrane, which increase the fluidity and membrane penetration, and therefore dissipating the ion equilibrium in the membrane sides and decreasing the ion gradient (Newbold *et al.*, 2004). In some cases, cells amends this imbalance by ion pomp and prevent the cell death. This action requires a large amount of energy and reduces the bacteria growth and so cause some changes in fermentation and VFAs profile (Calsamiglia *et al.*, 2007). In agreement to the results of this study, supplementation of 500 mg/L anise oil (Gunal *et al.*, 2014), 3000 mg/L of anise oil and levels 300 and 3000 mg of clove oil (Busquet *et al.*, 2006) and also using anise extract and cinamaldehyde at level 300 mg/L, declined total VFA concentration (Cardozo *et al.*, 2005). Likewise, levels 5, 50, 500 and 5000 mg/L of eugenol decreased total VFA concentration (Calsamiglia *et al.*, 2007). On the other hand, Cinamaldehyde oil (containing phenylpropanoid) and Cinanamon extract didn't have any effect on VFA production (Busquet *et al.*, 2005). Similarly, VFA concentration didn't affect by *in vitro* using of 400, 800 and 1600 mg/d Cinamaldehyde in beef cattle (Yang *et al.*, 2010).

The VFA produced in the fermentation medium provides about 65-75% of animal energy requirement (Benchaar *et al.*, 1998). Reduction of metabolisable energy (ME) by adding essential oil may be related to the reduction in gas production, VFA concentration and OMD in the fermentation medium especially in high doses while ME increases simultaneously with increase in above mentioned parameters when extract is added. In this study, metabolisable energy and net lactating energy were shown decreasing and increasing pattern respectively when VFAs concentration declined by addition of anise essential oil and increased by anise extract.

Effect on rumen protozoa

As presented in Table 3, total protozoa numbers increased by adding AEO and extract (table 3). In all levels of AEX, total protozoa numbers were higher than AEO. The increasing effect of AEO and AEX in this research could be due to the lacking effect of anise active constituent (Anethol) on protozoa molecular structure. Also, it seems that some component of anethol were effective on protozoa cells cleavage. Comparison between AEO and AEX shows no difference in their effects. In a report by (Cardozo *et al.*, 2006), addition of cinamaldehyde (0.6 g/d) and eugenol (0.3 g/d) increased holotrich population in cow.

However, (Patra *et al.*, 2010) reported that clove extract decreased total protozoa, small entodiniums and holotrich numbers but it had no effect on large entodiniums population. Similarly, using 400, 800 and 1600 mg cinamaldehyde didn't have any effect on total protozoa, isotrichia, *dasytrichia* and *entodinium* numbers (Yang *et al.*, 2010).

Conclusion

The results of present study showed that anise alcoholic extract was more effective than its essential oil on fermentation and improved fermentation by increasing gas production, organic matter digestibility and energy. Both essential oil and extract had no antiprotozoal effect and increased protozoa population. Supplementation of anise essential oil and extract *in vivo* may improve acceptable outcomes.

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