In vitro evaluation of the effects of *Lavandula officinalis* and *Origanum vulgare* essential oils on ruminal fermentation using concentrate and roughage type substrates

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Abstract The aim of this research was to study the *in vitro* effect of *Lavandula officinalis* (LEO) and *Origanum vulgare* (OEO) essential oils on rumen fermentation using a concentrate type substrate (CTS) and roughage type substrate (RTS). Six Mehraban ewes were divided into 2 groups and fed a concentrate type or roughage type diet, and used as rumen fluid donors. Each essential oil (EO) was evaluated separately at different doses using a completely randomized design with a 5 × 2 factorial arrangement (EO dose × substrate type). In a third 5 × 2 factorial design experiment, the potential of LEO to inhibit rumen methanogenesis was tested. The gas produced after 24-h of incubation (GP_{24}) was stimulated and inhibited ($P < 0.01$) by LEO in CTS and RTS groups, respectively. The *in vitro* true dry matter (IVTDMD) and organic matter (IVTOMD) degradability were lowered significantly by LEO using CTS and RTS. A more pronounced fall was observed for total volatile fatty acids (total VFA) by LEO using RTS compared to CTS. The partitioning factor (PF) and NH_3 were decreased and increased ($P < 0.01$) in CTS and RTS groups, respectively, but microbial biomass (MB) was linearly decreased by LEO in both CTS and RTS groups. An interaction effect between OEO dose and substrate type were observed for all parameters except the total VFA and MB which decreased linearly ($P < 0.01$) by OEO. The GP_{24}, IVTDMD, IVTOMD and NH_3 were decreased linearly ($P < 0.01$) by OEO using both substrate types. The PF was enhanced with OEO dose, but only in RTS group. Methane production was reduced linearly by LEO dose ($P < 0.01$), but the CH_4/TG and CH_4/CO_2 showed linear and quadratic trends with LEO dose. An interaction effect between LEO dose and substrate type was also recorded for TG and CO_2 ($P < 0.01$), as their production was stimulated and inhibited by LEO in CTS and RTS groups, respectively. Collectively, this study demonstrated that LEO and OEO affected ruminal fermentation differently depending on their doses and the type of substrate.

Keywords: essential oil, rumen fermentation, lavender, oregano, substrate type

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Introduction

Essential oils (EOs) are one class of plant secondary metabolites, mainly constituted of terpenoids and phenyl propanoids (Calsamiglia et al., 2007), which are extracted principally by steam distillation from different parts of plants. These compounds are not involved in plant primary metabolism, but play an important role in plant-plant and plant-insects communications, ensuring their reproductive functions (Cox et al., 2001). They also defend plants against a variety of aggressive agents such bacterial, fungal, insect or herbivores invasions (Patra and Saxena, 2009). This role has principally been attributed to their antimicrobial property (Dorman et al., 2000; Cox et al., 2001) which has appreciated the animal nutritionists in recent years to consider EOs as a potential natural alternative to chemical feed additives such as antibiotics. The use of antibiotics as growth promoters is increasingly limited because of their residues in animal products and being harmful to human health.

Several studies have attempted in recent years to assess the efficacy of EOs to reduce the rumen methane production and protein degradation, enhance the degradability of DM especially that of fibre and modify ruminal volatile fatty acid (VFA) profile in favor of propionate (Calsamiglia et al., 2007; Patra and Saxena, 2009). However, inconsistent results have been obtained

in this respect, as for example, total VFA was decreased (Spanghero et al., 2008; Kumar et al., 2009), increased (Cardozo et al., 2005; Castillejos et al., 2005) or remained unchanged (Malecky et al., 2009a; Meyer et al., 2009) by EOs. Ruminal digestibility has been affected rarely by EOs (Wallace et al., 2002; Duval et al., 2007). However, there are more consistent data about EOs impact on rumen methane production which generally indicates a depressive or no effect of EOs on ruminal methanogenesis (Tatsouka et al., 2008; Jahani-Azizabadi et al., 2011; Patra, 2011). This inconsistency is related to differences in the chemical structures and dosage of EOs, chemical composition of diets, rumen conditions and geographical locations (Patra and Saxena, 2009). As mentioned above, EOs dose-effect and diet type (e.g. forage to concentrate ratio) are two major factors altering EOs impacts on rumen fermentation, however, comparative studies, taking into account the interaction among these factors, are scanty.

The purpose of the present study was to evaluate the *in vitro* effect of EOs of *Origanum vulgare* and *Lavandula officinalis* on rumen fermentation using two different fermentation substrates (roughage vs. concentrate) and also to assess the potential of LEO to reduce ruminal methane production.

Materials and methods

Essential oils

Steam-distilled essential oils of Oregano and Lavender were procured from Golkaran Company (Isfahan, Iran). Major components of LEO and OEO were 1, 8 cineol (29%) or carvacrol (49.7%), respectively (Baritch Essence Medicinal Laboratory, Kashan). The essential oils were first dissolved in absolute ethanol (1:4, v/v) and stored at 4 °C as stock solution; these were diluted further in deionized water to obtain the working solution with appropriate concentrations used in the incubations.

Experiments

In the first experiment the effect of different doses of LEO or OEO on ruminal fermentation was studied using two types of fermentation substrates (CTS vs. RTS). Another experiment evaluated the impact of LEO on ruminal methanogenesis in the presence of CTS and RTS.

Animals, diets and ruminal fluid

Ruminal fluid was collected from ewes fed the same diets used as the fermentation substrate in incubations.

Six Mehraban ewes (50 ± 4.5 kg BW) were allotted into two groups receiving *ad libitum* one of the roughage (RTS) or concentrate (CTS) type substrates (diet) during 2 weeks as the adaptation period. The RTS was entirely of alfalfa hay, and CTS was composed of 650 g/kg concentrate mix (containing 600 g barely grain and 50 g soybean meal) and 350 g/kg forage (alfalfa hay), based on dry matter. Chemical composition (per kg DM) of RTS was 908.1 g organic matter (OM), 136.7 g crude protein (CP), 385.2 g neutral detergent fiber (NDF), 330.8 g acid detergent fiber (ADF) and 21.4 g ether extract (EE), and that of CTS was 945.5 g OM, 123.3 g CP, 271.8 g NDF, 167.7 g ADF and 24.7 g EE. Ruminal fluid were collected at the end of the adaptation period, before the morning feeding, through an esophageal tube, pooled and strained through 4-layer cheese clothes into a pre-warmed (38-39°C) insulated flask and immediately transported to the laboratory.

In vitro gas production

Gas production was measured according to Menke and Steingass (1988) with some modifications as described by Makkar et al. (1995). A representative air-dried sample of each substrate was ground to pass a 1-mm sieve, and sub-samples of 500 mg (DM basis) were weighed into 100-ml glass syringes. Substrates were incubated in triplicate with 40 ml of buffered ruminal fluid and different doses of EOs (0 as control, 250, 500, 750 and 1000 µl/L of incubation medium) under continuous flow of CO_2. Three syringes containing 40 ml of buffered ruminal fluid without substrate were considered as blanks. Incubation was carried out in a water-bath at 39° C for 24 h. At the end of incubation, the contents were transferred into centrifuge tubes and immediately placed in cold water at 4°C to stop fermentation. The tubes were then centrifuged at 15000 × *g* for 20 min at 4°C, and 4-mL aliquots of the supernatant were mixed with 1 ml of 25% metaphosphoric acid and frozen at -20°C until analyzed for VFA and ammonia concentration. The remaining residues in the tubes were oven-dried at 60°C for 48 h. The *in vitro* true dry matter degradability (IVTDMD) was determined by refluxing the oven-dried residues with neutral detergent solution at 100 °C for 1 h and the recovered residues were subsequently incinerated in sintered glass crucibles at 600 °C to quantify the *in vitro* true organic matter degradability (IVTOMD). The ratio of the mass of truly degraded organic matter (mg) to the volume of gas produced (mL) after 24-h incubation was considered as the partitioning factor (PF) (Blümmel et al., 1997). The mass difference between the remai-

ning DM at the end of incubation and that recovered after neutral detergent extraction was considered as microbial biomass (MB).

Chemical analyses

Dry matter, total ash, ether extract (EE) and crude protein (CP) were measured according to the standard methods described by AOAC (2000). The NDF and ADF were determined as described by Van Soest et al. (1991) and are expressed inclusive of residual ash. Ammonia concentration in the supernatant was determined as illustrated by Broderick and Kang (1980). Total VFA concentration of the samples was quantified by the steam distillation method (Markham, 1942).

Measurement of methane production

In vitro determination of the methane produced during the incubation was according to the method illustrated by Fievez et al. (2005). Briefly, because of a limited volume of the syringes (100 ml), 15 ml of buffered rumen fluid was transferred into the syringes containing 100 mg of either RTS or CTS substrate. After addition of 0, 250, 500, 750 and 1000 µl/ l of LEO, the syringes were incubated in water-bath at 39° C for 24-h. At the end of incubation, all syringes were immediately cooled at 4°C to stop the fermentation. After initial recording of the gas produced as total gas (TG), 4 ml of NaOH (10 M) was added into the syringes and the volume of gas remaining (as the amount of methane), was recorded only after the decline (caused by NaOH) in the initial gas volume was stopped.

Statistical analysis

All experiments were repeated three times and the data (means within each run) were subjected to analysis of variance using the GLM procedure (SAS, 2002) as a completely randomized design with a 5 × 2 factorial arrangement (5 doses of EO and 2 substrate types). The means were compared using Duncan's multiple range test. Orthogonal contrasts were used to test the linear and quadratic relationships of the studied parameters with EOs dose.

Results

The main effects are presented in tables, and when a significant effect was detected for a particular paramet-

Table 1. Effect of Lavender essential oil (LEO) and substrate type (ST) on ruminal fermentation (main effects)

Treatments	Parameters [a]						
	GP_{24}	IVTDMD	IVTOMD	PF	MB	total VFA	NH_3
ST [b]							
CTS	129.2[a]	79.7 [a]	79.6[a]	2.95 [b]	161.8[a]	81.8 [a]	13.1 [a]
RTS	95.8[b]	62.8[b]	61.2 [b]	3.11[a]	153.2[b]	54.1 [b]	10.5 [b]
SEM	2.64	0.52	0.51	0.051	2.72	1.69	0.15
LEO dose [c]							
0	112	74	74.1	3.09	165.2	80.1	12.1
250	118.9	73.7	72.7	2.85	160.5	77.5	11.5
500	122.4	72.1	71.3	2.72	153.3	74	12.0
750	106.4	71.5	70.4	2.98	156.2	55.8	11.7
1000	102.8	69.1	65.8	3.49	152.3	52.1	11.7
SEM	4.7	0.89	0.93	0.080	4.30	2.67	2.53
P values							
LEO	0.02	<0.001	<0.001	<0.001	0.230	<0.001	0.48
Contrasts [d]							
L	0.03	<0.001	<0.001	0.001	0.039	<0.001	0.52
Q	0.017	0.14	0.32	<0.001	0.47	0.11	0.80
ST	<0.001	<0.001	<0.001	0.039	0.037	<0.001	<0.001
(LEO × ST)	<0.001	0.011	<0.001	<0.001	0.195	0.02	<0.001

[a]. GP_{24} (ml/500 mg): gas produced after 24-h of incubation, IVTDMD (%): *in vitro* true dry matter degradability, IVTOMD (%): *in vitro* true organic matter degradability, PF: partitioning factor, total VFA (mM): total volatile fatty acids, NH_4 (mM).
[b]. Substrate type, CTS: concentrate type substrate, RTS: roughage type substrate.
[c] LEO dose: lavender essential oil dose, control (0 µl/L) vs. treatments.
[d]. L: linear, Q: quadratic.

Table 2. Effect of Lavender essential oil (LEO) and substrate type (ST) on ruminal fermentation (interaction effects)

Treatments		Parameters [a]					
ST [b]	LEO dose [c]	GP_{24}	IVTDMD	IVTOMD	PF	Total VFA	NH_3
	0	107.4	81.0	80.8	3.55	92.7	14.2
	250	127.9	81.3	81.1	3.03	92.0	13.6
CTS	500	142.1	79.2	79.5	2.64	82.8	12.9
	750	131.1	79.8	79.6	2.87	66.8	12.9
	1000	137.4	77.4	77.4	2.67	74.4	12.0
SEM		5.12	0.54	0.51	0.13	4.37	0.39
P values[d]							
T		<0.001	0.029	0.029	<0.001	0.019	0.011
L		0.003	<0.001	<0.001	0.001	0.001	0.002
Q		0.019	0.189	0.132	0.027	0.568	0.906
	0	116.5	67.1	67.4	2.63	67.5	9.9
	250	109.8	66.0	64.4	2.67	63.0	9.5
RTS	500	102.7	65.0	63.2	2.79	65.2	11.2
	750	81.7	59.1	56.7	3.16	44.8	10.5
	1000	68.2	56.7	54.3	4.31	29.8	11.4
SEM		6.59	1.89	1.87	0.14	3.08	0.27
P values							
T		0.006	0.026	0.004	0.003	<0.001	0.028
L		<0.001	0.002	<0.001	<0.001	<0.001	<0.001
Q		0.291	0.319	0.581	0.001	0.004	0.781

[a] GP_{24} (ml/500 mg): gas produced after 24-h of incubation, IVTDMD (%): *in vitro* true dry matter degradability, IVTOMD (%): *in vitro* true organic matter degradability, PF: partitioning factor, TVFA (mM): total volatile fatty acids, NH_4 (mM).
[b] Substrate type, CTS: concentrate type substrate, RTS: roughage type substrate.
[c] LEO dose: lavender essential oil dose (µl/L).
[d] T: control (o µl/L) vs. treatments, L: linear, Q: quadratic.

er; it was shown in a separate table.

Effect of LEO and substrate type on ruminal fermentation

The volume of gas produced after 24-h of incubation, IVTDMD, IVTOMD, total VFA, MB and NH_4 were higher ($P < 0.05$) in CTS than RTS (Table 1). However, PF showed a higher value with CTS ($P < 0.05$). Most ruminal fermentation parameters were also affected by LEO (Table 1). The GP_{24} showed a linear and quadratic trend ($P < 0.05$) with LEO, with the highest volume of gas production at 500 µl/L. The IVTDMD exhibited a linear decrease with LEO dosage ($P <0.01$), although it was remained unchanged up to 750 µl/l of LEO, it was reduced ($P < 0.01$) at 1000 µl/l. The same trend was approximately observed for IVTOMD; it reduced linearly with LEO dose ($P < 0.01$). In contrast to GP_{24}, a reverse effect of LEO was on PF; a s its lowest value was recorded at 500 µl/l wich was different ($P < 0.01$)from that at 0 and 1000 µl/l of LEO. The MB was reduced linearly by LEO but the differences among different doses were not significant. Total VFA production decreased linearly ($P < 0.01$) as LEO dosage increased, however NH_3 concentration was not affected by LEO ($P > 0.05$).

With the exception of MB, a significant interaction effect was observed for other parameters (Table 1). The GP_{24} was changed differently by LEO, depending on the type of the substrate (Table 2). As with CTS, a positive linear and quadratic trend was observed for this parameter ($P < 0.05$), with the highest value recorded at 500 µl/L of LEO. In contrast to CTS, RTS resulted in a linear reduction in GP_{24} ($P < 0.001$). The IVTDMD and IVTOMD were decreased linearly by LEO in both CTS and RTS groups ($P < 0.01$); however, their fall was more pronounced with RTS. The PF was increased linearly with increasing dose of LEO in CTS group, but a reverse linear trend was observed for

Table 3. Effect of Oregano essential oil (OEO) and substrate type (ST) on ruminal fermentation (main effects)

Treatments	Parameters [a]						
	GP_{24}	IVTDMD	IVTOMD	PF	MB	total VFA	NH_3
ST [b]							
CTS	151.5[a]	78.5 [a]	78.1 [a]	2.61 [b]	135.4 [b]	86.4 [a]	15.4 [a]
RTS	82.8[b]	65.9 [b]	63.6 [b]	4.66 [a]	161.4 [a]	50.4 [b]	12.1 [b]
SEM	1.25	0.34	0.37	0.087	4.74	1.82	0.28
OEO dose [c]							
0	137.0	76.8	75.9	2.90	162.8	87.3	14.7
250	136.8	75.5	74.3	2.86	159.7	87.4	14.9
500	124.5	71.9	70.5	3.06	159.1	78.8	14.6
750	99.9	69.7	68.2	4.17	140.2	52.9	14.2
1000	87.7	67.0	65.4	5.20	120.3	35.4	10.4
SEM	1.97	0.55	0.58	0.137	7.49	2.89	0.48
P values							
OEO	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	<0.001
Contrasts [d]							
L	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Q	<0.001	0.55	0.64	<0.001	0.080	<0.001	<0.001
ST	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
(OEO × ST)	<0.001	<0.001	<0.001	<0.001	0.099	0.26	<0.001

[a.] GP_{24} (ml/500 mg): gas produced after 24-h of incubation, IVTDMD (%): *in vitro* true dry matter degradability, IVTOMD (%): *in vitro* true organic matter degradability, PF: partitioning factor, TVFA (mM): total volatile fatty acids, NH_4 (mM).
[b.] Substrate type, CTS: concentrate type substrate, RTS: roughage type substrate.
[c] OEO dose: oregano essential oil dose, control (0 µl/L) vs. treatments.
[d.] L: linear, Q: quadratic.

this parameter in RTS group ($P < 0.001$). There was also a quadratic trend for this parameter for both CTS and RTS ($P < 0.05$).Total VFA was reduced linearly by LEO using either CTS or RTS ($P < 0.01$). However, a quadratic trend was observed for total VFA ($P = 0.004$) with LEO using RTS as the substrate. The effect of LEO on NH_3, in contrast to total VFA, was different depending on the type of the substrate, as linear decrease and increase were observed with CTS and RTS, respectively ($P < 0.01$), when LEO dosage increased.

Effect of OEO and substrate type on ruminal fermentation

Except for the PF and MB, the other parameters (including GP_{24}, IVTDMD, IVTOMD, total VFA and NH_4) were higher ($P < 0.01$) in the group using CTS as the fermentation substrate when compared with RTS containing media (Table 3). Conversely, PF and MB showed higher values in RTS group. All parameters, except NH_3 and PF, were negatively affected ($P <0.01$) by OEO (Table 3). The GP_{24} was reduced with a linear and quadratic trends ($P < 0.01$) as OEO dosage increased. The IVTDMD and IVTOMD decreased linearly ($P < 0.01$) with OEO dosage. A linear and quadratic trend was recorded for PF ($P < 0.01$) with OEO dosage; PF remained unchanged up to 500 µl/L OEO but increased at higher doses. Compared to PF, a reverse trend was observed for MB ($P < 0.01$); it showed an increasing trend up to 500 µl/L OEO but decreased at higher doses. Similar to GP_{24}, total VFA was decreased in a linear and quadratic manner ($P < 0.01$) by OEO. The same trend was observed for NH_3 when OEO dosage was increased, but it was reduced only at the highest dose of OEO. An interaction effect between OEO dose and substrate type was observed ($P < 0.01$) for GP_{24}, IVTDMD, IVTOMD, PF and NH_3 (Table 4). The OEO had a depressive effect on GP_{24} using both substrates which was greater with RTS than CTS (Table 4); a linear ($P < 0.001$) and quadratic ($P < 0.05$) trend was observed for this parameter in both CTS and RTS groups. Similar trend was observed for IVTDMD, IVTOMD using both the CTS and RTS, though IVTOMD tended to vary quadratically ($P = 0.086$) in response to OEO dosage when CTS was used as the fermentation substr-

Table 4. Effect of Oregano essential oil (OEO) and substrate type (ST) on ruminal fermentation (interaction effects)

Treatments		Parameters [a]				
ST [b]	OEO dose [c]	GP_{24}	IVTDMD	IVTOMD	PF	NH_3
	0	160.8	80.84	80.61	2.54	16.03
	250	161.6	79.97	79.68	2.52	18.77
CTS	500	156.6	78.97	78.75	2.54	16.42
	750	142.9	77.67	77.17	2.77	15.99
	1000	135.6	74.97	74.20	2.70	9.78
SEM		2.49	0.63	0.66	0.032	0.343
P values[d]						
T		0.002	0.003	0.002	0.024	0.067
L		<0.001	<0.001	<0.001	<0.001	<0.001
Q		0.023	0.124	0.086	0.469	<0.001
	0	113.2	72.73	71.12	3.26	13.37
	250	112	71.93	68.84	3.20	11.05
RTS	500	92.3	64.80	62.30	3.58	12.69
	750	56.8	61.73	59.28	5.56	12.40
	1000	39.8	59.13	56.59	7.69	11.02
SEM		3.06	0.889	0.951	0.273	0.800
P value						
T		<0.001	<0.001	<0.001	<0.001	0.107
L		<0.001	<0.001	<0.001	<0.001	0.215
Q		0.002	0.669	0.466	<0.001	0.985

[a.] GP_{24} (ml/500 mg): gas produced after 24-h of incubation, IVTDMD (%): *in vitro* true dry matter degradability, IVTOMD (%): *in vitro* true organic matter degradability, PF: partitioning factor, NH_4 (mM).
[b.] Substrate type, CTS: concentrate type substrate, RTS: roughage type substrate.
[c] OEO dose: oregano essential oil dose (µl/L).
[d] T: control (o µl/L) vs. treatments, L: linear, Q: quadratic.

ate. The OEO had an increasing effect on PF in both CTS and RTS groups, though it was linear ($P < 0.001$) and linear and quadratic ($P < 0.001$) in CTS and RTS groups, respectively. A linear and quadratic response ($P < 0.001$) was observed for NH_3 in response to OEO using the CTS. However, no significant effect of OEO was recorded on NH_3 when RTS was the fermentation substrate.

Effect of LEO and substrate type on ruminal methane production

Using CTS as the fermentation substrate resulted in a higher TG and CO_2 (Table 5) than using RTS ($P < 0.01$). The volume of methane produced after 24-h of incubation did not differ between the substrates; however, the ratio of methane to total gas, as well as the ratio of methane to CO_2, was lower in CTS group than in RTS group ($P < 0.01$). The LEO affected TG linearly and quadratically ($P < 0.05$); an increasing trend was observed for CO_2. Despite a stimulatory effect of LEO observed for GP between 0 up to 750 µl/L, but it was reduced at the highest LEO dosage. Similar trend was on TG and CO_2, CH_4 was negatively affected by LEO, as it was reduced linearly ($P < 0.01$) with increasing LEO dosage. The ratios of CH_4 to TG and CH_4 to CO_2 showed a linear and quadratic relationship ($P < 0.05$) with LEO dosage; these decreased when LEO dose was increased to 750 µl/L, but at the highest dose, both were increased. A significant interaction effect between LEO doses and type of substrate was observed for TG and CO_2. The TG varied quadratically ($P < 0.05$) with LEO dose in both CTS and RTS groups (Table 6), however, the highest value for this parameter was at the dose of 250 and 500 µl/L in RTS and CTS groups, respectively. The TG showed a linear decrease ($P = 0.012$) with increasing LEO dosage in the media containing RTS. Approximately, the same trend was observed for CO_2 by LEO, as it changed quadratically

Table 5. Effect of Lavender essential oil (LEO) and substrate type (ST) on ruminal methanogenesis (main effects)

Treatments	Parameters [a]				
	TG	CO_2	CH_4	CH_4/TG	CH_4/CO_2
ST [b]					
CTS	58.0[a]	48.2 [a]	9.87	0.170 [b]	0.206 [b]
RTS	51.4[b]	41.5 [b]	9.87	0.192 [a]	0.239 [a]
SEM	0.71	0.64	0.183	0.0029	0.0043
LEO dose [c]					
0	53.8	43.3	10.5	0.195	0.243
250	57.2	46.7	10.5	0.184	0.226
500	57.0	47.2	9.8	0.173	0.210
750	55.0	45.7	9.3	0.170	0.206
1000	50.5	41.3	9.2	0.184	0.225
SEM	1.14	1.01	0.29	0.0046	0.0067
P values					
LEO	0.003	0.003	0.008	0.009	0.008
Contrasts [d]					
L	0.023	0.134	<0.001	0.020	0.020
Q	<0.001	<0.001	0.88	0.004	0.003
ST	<0.001	<0.001	0.99	<0.001	<0.001
(LEO × ST)	0.008	0.007	0.43	0.47	0.49

[a.] TG (ml/100 mg substrate): Total gas produced after 24-h of incubation.
[b.] Substrate type, CTS: concentrate type substrate, RTS: roughage type substrate.
[c] LEO dose (µl/L): lavender essential oil dose, control (0 µl/L) vs. treatments.
[d.] L: linear, Q: quadratic.

in both substrates; the highest value was observed at 250 and 500 µl/L of LEO in RTS and CTS groups, respectively. In addition, a linear effect was observed for CO_2; it increased or decreased with LEO dosage using CTS and RTS, respectively as the fermentation substrate.

Discussion

The impact of diet on ruminal fermentation, especially the modifications typically caused by concentrate and roughage type diets are well known. Generally, concentrates with higher ruminal degradation rate compared to roughages, produce a higher amount of ruminal fermentation end products, such as gas, VFA and ammonia (Mould et al., 2005). The present results were consistent with previous findings in this regard, thus the discussion was principally focused on the effect of LEO and OEO on ruminal fermentation and in the case of interaction effect, it is limited to these effects.

Effects of LEO and substrate type on ruminnal fermentation

Among the measured parameters, only the MB was not modified differentially by LEO using different types of substrates; it was higher in CTS as expected, because of higher degradation of organic matter in this group. A linear numerical decrease in MB with increased LEO dosage is in accordance with the reduction in IVTOMD, thus a lower availability of digested organic matter for microorganisms is probably the cause of lower MB with LEO.

Regarding the changes in GP_{24}, it seems that LEO had a stimulatory effect on rumen fermentation when CTS was used as the fermentation substrate. However, this stimulatory effect was not observed for IVTOMD and total VFA. Little data exist in the literature about Lavender, especially on its oil form; similar to our results, in an experiment conducted by Broudiscou et al. (2000), lavender dry extract had a stimulatory effect on in vitro gas production. Broudiscou et al. (2002) also reported a stimulatory effect of Lavender dry extract on rumen fermentation (gas and VFA production). In another experiment, Lavender essential oil had no effect on rumen fermentation at the doses up to 500 mg/L (Castillejos et al., 2008).

Several hypotheses can be suggested in the present

Table 6. Effect of Lavender essential oil (LEO) and substrate type (ST) on ruminal methanogenesis (interaction effects)

Treatments		Parameters [a]	
ST [b]	LEO dose [c]	TG	CO_2
CTS	0	55.5	45.2
	250	57.5	47.2
	500	60.5	50.8
	750	59.5	50.2
	1000	57.2	47.5
SEM		1.19	1.07
P values[d]			
T		0.039	0.011
L		0.188	0.048
Q		0.018	0.007
RTS	0	52.2	41.5
	250	56.8	46.2
	500	53.5	43.5
	750	50.5	41.2
	1000	43.8	35.2
SEM		1.93	1.71
P values			
T		0.653	0.998
L		0.004	0.009
Q		0.012	0.008

[a.] TG (ml/100 mg substrate): Total gas produced after 24-h of incubation.
[b.] CTS: concentrate type substrate, RTS: roughage type substrate.
[c] LEO dose (µl/L): lavender essential oil dose.
[d] T: control (0 µl/L) vs. treatments, L: linear, Q: quadratic.

study for the higher GP_{24} production despite a decrease in total VFA production as LEO dosage increased. This increase in GP_{24} does not appear to be a result of higher organic matter degradability. Furthermore, there is no evidence on the redirection of degraded organic matter into the microbial protein synthesis pathway, because, despite the decrease in total VFA, MB remained unchanged with LEO dosage (data not shown). In general, a portion of the gas produced during ruminal fermentation is originated from the buffering of VFA (Blummel and Orskov, 1993). Therefore, one hypothesis is that LEO stimulated gas producion from VFA buffering. Some recent studies reported that rumen microflora is capable of degrading terpenoids as the major constituents of Eos (Malecky and Broudiscou, 2009; Malecky et al., 2009b; Malecky et al., 2012). Thus, another possibility is that some constituents of LEO have been used as carbon source and degraded by rumen microorganisms, resulting in high amount of GP_{24}. The other possibility may be related to lower organic matter degradation at higher doses of LEO, though this decrease in organic matter was not of significance up to 750 µl/L of LEO; therefore, degradation of some components of LEO to CO_2 remains the most probable explanation for higher GP_{24} with LEO.

Decreased production of total VFA was in accordance with that observed for IVTDMD and IVTOMD (Table 2) in both CTS and RTS groups. However, a more pronounced decrease in total VFA at higher doses of LEO may be explained by a modification in VFA profile to a higher propionate to acetate ratio which, as mentioned earlier, favors buffering of VFA to gas. A decreasing value for PF with LEO dosage in CTS group may be due to a higher amount of GP_{24} than decreased IVTOMD. In general, a higher PF could refer to the orientation of degraded OM to synthesis of microbial biomass (Blümmel et al., 1997). However, contribution of organic matter to microbial biomass in decreasing PF at higher doses of LEO, seems to be of less importance, because changes in MB followed the same

trend as for IVTOMD. Nevertheless, increased PF with LEO in RTS group is probably due to the consumption of digested organic matter for microbial biomass, because despite the substantial decrease in GP at the doses ≥ 750 µl/L of LEO, the MB followed the IVTOMD changes (data not shown). Another explanation may be related to the fact that a part of OM dissolved in the matrices, were not used by microorganisms whereupon their population are restricted at these doses. Yet, a beneficial effect of LEO in CTS group was its depressive effect on NH_3 concentration. As noted earlier, a large number of essential oils have exhibited an inhibitory effect on ruminal ammonia production (Busquet et al., 2006; Calsamiglia et al., 2006; Benchaar et al., 2008). This decrease in NH_3 may be attributed to an initial decrease in protein degradability (Yang et al., 2007) or related to a direct negative effect of EO on ruminal bacteria involved in ammonia production (McIntosh et al., 2003; Benchaar et al., 2008). But increased production of NH_3 with LEO in RTS-containing media, may be attributed to the inhibition of its main consumers (i.e. cellulolytic bacteria) which are dominant in such media (Allison and Bryant, 1961).

Overall, it can be concluded that LEO had a different effect on ruminal microbial ecosystem depending on the type of the substrate used, as with CTS, it had no negative effect or even in some cases, had a stimulatory effect on ruminal fermentation at doses up to 750 µl/L. However, the cellulolytic bacteria seem to be more sensitive to LEO than amylolytic ones, because many of the ruminal fermentation parameters were affected negatively by LEO at the doses ≥ 750 µl/L when RTS was used as the fermentation substrate. Hence, these results confirm previous findings on EOs action on ruminal fermentation reporting that their effect varies according to the type of the diet (Benchaar et al., 2008; Goel and Makkar, 2012)

Effect of OEO and substrate type on ruminal fermentation

A higher value for MB with roughage-type substrate is probably related to a substantial higher PF in this group compared to CTS. Roughages with high efficiency of microbial protein synthesis, have typically a higher PF than concentrates (Blümmel et al., 1997). This means that in roughages, despite their lower content of digestible OM compared to concentrates, a greater part of degraded OM is directed to microbial protein synthesis pathway. A higher total VFA in the media containing CTS is certainly attributed to higher organic matter degradation in this group. The MB, in contrast to PF, was was decreased at 1000 µl/L of OEO, though it predictable regarding to the variation in IVTOMD under OEO action. As noticed above, a higher PF may be due to a partial inhibition of ruminal microorganisms which leads to decreased gas production from the OM initially dissolved in the matrices. otal VFA was also reduced at high doses of OEO which is a result of a lower organic matter degraded at these doses. Modifications of other parameters by OEO were different in CTS and RTS groups. The GP_{24} and the degradability of DM and OM were decreased using both roughage and concentrate type substrates as the OEO dose increased, but this decrease was more pronounced in the RTS compared to CTS group and occurred at lower doses of OEO. These findings demonstrated that OEO, similar to LEO, has a more toxic effect on ruminal cellulolytic than on amylolytic bacteria.

These results are in agreement with those reported on OEO in the literature; at lower doses (up to 300 µl/L), no inhibitory effect were reported from OEO on ruminal fermentation (Cardozo et al., 2005; Benchaar et al., 2007). However, at higher than 500 µl/L (Lin et al., 2011), or at 3000 µl/L (Busquet et al., 2006), OEO had a negative effect on ruminal fermentation by reducing gas and total VFA production. Likewise, Canbolat et al. (2010) reported that in a range of 0-800 µl/L, OEO reduced gas production, DM degradability and ammonia concentration only at doses higher than 600 µl/L.

Substantial increases in PF at high doses of OEO in RTS group is due to a sharp decrease in GP_{24}. It appears that the late phases of organic matter degradation, leading to formation of fermentation end products such as the VFA and the gas, was more inhibited by OEO in RTS than CTS group. Lower NH_3 production at the highest dose of OEO in CTS group seems to be due to a lower protein degradation caused by OEO, as it can extrapolated from a low IVTOMD at these doses. Inhibition of HAP bacteria by OEO can also contribute to lower ammonia production at high doses of OEO, as also reported by Busquet et al. (2006).

Unlike LEO, OEO exhibited a negative effect on ruminal fermentation, because regardless of the substrate type used, it reduced many of the ruminal fermentation parameters. However, similar to LEO, OEO showed a dose-response negative effect on ruminal fermentation which was more pronounced with RTS, suggesting a higher sensitivity of cellulolytic bacteria to this EO.

Effect of LEO and substrate type on ruminal methanogenesis

In spite of the increase in TG in the matrices using

CTS as the fermentation substrate, the amount of methane did not differ between CTS and RTS groups, leading to a lower value for CH_4/TG in CTS group. These results are typical regarding to the nature of forages and concentrates. Conventionally, concentrates with higher ruminal degradation compared to forages, produce a higher amount of reducing equivalents (as one of the potential precursor s of methane) during a set of time. This favors production of propionate at the expense of methane (Benchaar et al., 2001). Moreover, another precursors of methane (i.e., CO_2) is an end product of acetate producing pathway which is favored by the roughage type diets (Hungate, 1966; Russell and Wallace, 1997).

Despite the stimulatory effect of LEO on TG and CO_2 at the doses of 250, 500 and 750 µl/L, a linear decrease in CH_4 with LEO dose demonstrates that this EO had an inhibitory effect on ruminal methanogenesis. Many EOs have the potential to inhibit *in vitro* methanogenesis (Chaves et al., 2008; Patra, 2010; Jahani-Azizabadi et al., 2011) probably by directly inhibiting the methanogens (Ohene-Adjei et al., 2008) or indirectly by their negative impact on ciliate protozoa or as a consequence of reduced organic matter degradation, providing the precursors for methane production (Patra et al., 2006; Jouany and Morgavi, 2007).

The results of the present study (Table 5) demonstrated that LEO had a depressive effect on CH_4 production, accompanied by an increase in CO_2 (as precursor of methane), suggesting a direct effect of this LEO on methanogenic bacteria. Additionally, the lack of interaction effect between LEO dosage and substrate type on CH_4, as well as on CH_4/TG and CH_4/CO_2, confirmed that the depressive effect of LEO may not be related to the availability of methane precursors (CO_2 and H_2), but is a result of direct impact on methanogens. However, the CH_4/TG and of CH_4/CO_2 followed a linear and quadratic trend with LEO dosage, which their numerical increase at the highest dose of LEO was due to a greater decrease in TG and CO_2 compared to CH_4. Significant interaction effect between LEO and substrate type on TG and CO_2 (stimulatory and depressive effect on these parameters with CTS and RTS, respectively), confirmed the results obtained in the first experiment. In this regard, increased TG and CO_2 with LEO dosage in CTS group, signifies a stimulatory dose-depended effect of this EO on amylolytic bacteria which was maximum at the dose of 500 µl/L. However, in RTS group, the stimulatory effect of LEO on TG and CO_2 was recorded at a lower dose (250 µl/L of LEO); doses higher than 500 µl/L reduced TG and CO_2.

Conclusions

The LEO and OEO differently affected the ruminal fermentation, as LEO showed a moderated negative effect or in some cases a stimulatory effect on ruminal fermentation which varied depending on the type of the substrate used. However, a more general negative effect was recorded for OEO, with greater effects in RTS than CTS diets. Both LEO and OEO had a more pronounced negative effect at high doses on ruminal fermentation parameters using RTS as fermentation substrate than CTS, indicating that cellulolytic bacteria were more sensitive to these EOs. Reduced CH_4 production as well as its ratio to CO_2 by LEO, suggested that this EO had a direct inhibitory effect on ruminal methanogens. This characteristic of LEO and its depressive effect on ruminal ammonia concentration, make it a promising feed additive which can be used up to 500 µl/L with roughage and up to 750 µl/L with concentrate type diets for improving the ruminal fermentation reactions.

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