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DOSE-RESPONSE EFFECTS OF SAGE (*SALVIA OFFICINALIS*) AND YARROW (*ACHILLEA MILLEFOLIUM*) ESSENTIAL OILS ON RUMEN FERMENTATION *IN VITRO*

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Abstract

This study aimed at determining the chemical composition of sage essential oil (SEO) and yarrow essential oil (YEO), and investigate *in vitro* their impacts on gas production kinetics, ruminal digestibility and fermentation, and rumen methanogenesis at different dosages (0, 250, 500 and 750 mg L^{-1} for SEO; and 0, 250, 500, 750 and 1000 mg L^{-1} for YEO). Alpha-pinene and 1,8 cineol were two major constituents of both SEO and YEO. Both SEO and YEO had a linear and quadratic effect on asymptotic gas production (P<0.05). The gas production rate increased linearly with SEO and curve-linearly with YEO dosages (P<0.05). *In vitro* degradability of dry matter and organic matter decreased only by YEO. The partitioning factor (PF) and the microbial biomass (MB) decreased and increased linearly with YEO and SEO dosages, respectively (P<0.05). Total volatile fatty acids (VFA) were not affected by SEO, but decreased in a linear and quadratic manner with YEO dosage (P<0.05). The VFA pattern was modified in a linear and quadratic manner by both SEO and YEO (P<0.05). Ammonia concentration increased linearly only with YEO increasing doses. The methane to total gas (TG) ratio decreased quadratically only by SEO with reductions of 6.7, 13 and 4.2% at the doses of 250, 500 and 750 mg L^{-1}, respectively. These results revealed that SEO modifies the rumen fermentation positively towards producing more MB and less methane in the dose range of 0–750 mg L^{-1}, however, YEO adversely affected the rumen fermentation at all the tested doses.

Key words: essential oil, sage, yarrow, gas production, rumen fermentation

Essential oils (EOs), constituting the volatile fraction of plant secondary metabolites, have gained growing interest during the last decade as a promising natural feed additive in ruminant nutrition (Benchaar et al., 2008 a; Bodas et al., 2012). Hence, numerous studies have evaluated the potential of a large number of EOs in modulating rumen fermentation (Calsamiglia et al., 2007 b; Cobellis et al., 2016), however, the results from these studies are generally inconsistent (Benchaar et al., 2008 a; Bodas et al., 2012). This is a consequence of the existence of a variety of factors

that may affect the potential of EOs to impact the rumen fermentation and thereby animal performance. Chemical composition or the composition of active compounds present in EOs, the dose rates used and the experimental conditions (including diet type and rumen pH) are some of the variation factors that have been reported to influence the potential of EOs as feed additives (Benchaar et al., 2008 a; Hart et al., 2008; Patra and Saxena, 2009). In this connection, chemical composition is one of the principal variation factors, affecting the properties of EOs, which is itself influenced by other variation agents. These include the variations associated with the phenological stage, the environmental (geography and climate) and the agronomic (cultivation and harvesting) conditions of the plant origin, and the factors related to the processing (extraction method, storage) of the EO (Dorman and Deans, 2000; Patra and Saxena, 2010). Regarding the fact that the antimicrobial nature of EOs is an outcome of the proportions of their active compounds, as well as an eventual interaction between their components (Burt, 2004), determining the chemical composition of EOs in each experiment can provide a clear image of their overall potential as a feed additive. Identifying the active compounds in EOs can also be helpful in explaining some of the discrepancies among different studies.

Dose rate is another principal variation factor, which influences the effects of EOs as rumen modifiers. In this sense, it has well been documented that the effects of EOs on rumen fermentation are dose-dependent (Busquet et al., 2006; Calsamiglia et al., 2007 b; Benchaar et al., 2008 a), as a lack of EOs effect in most *in vivo* experiments has been attributed to a low dose of these substances (Malecky et al., 2009 a; Khiaosa-Ard and Zebeli, 2013). Hence, in addition to evaluating the trends EOs may have on rumen fermentation or animal performance, dose-response experiments provide valuable data in determining the effective doses of tested EOs, which favourably modulate the rumen fermentation.

Salvia officinalis (sage) and *Achillea millefolium* (yarrow) are two perennial flowering plants of the *Lamiaceae* and the *Asteraceae* families, respectively, naturalized in many regions of the world. Previous studies have indicated certain beneficial effects, including the propensity to partially inhibit the rumen methanogenesis (Broudiscou et al., 2000), and increasing ruminal NDF digestibility (Broudiscou et al., 2002) from sage and yarrow dried extracts, respectively. However, there is very little information available in the literature on the effects of sage or yarrow EOs on the rumen fermentation. In a recent study, Gunal et al. (2013) reported a depressive effect of sage EO on ammonia concentration *in vitro*. Hence, the objectives of the current study involved determining the active compound profiles of sage and yarrow EOs and evaluating *in vitro* their impact on rumen fermentation and rumen methanogenesis at different doses.

Material and methods

Essential oils, preparing working solutions and chemical analysis

Sage (*Salvia officinalis*) and yarrow (*Achillea millefolium*) essential oils were procured from the Barij Essence Company (Barij Essence, Isfahan, Iran). The EOs

were firstly dissolved in absolute ethanol and subsequently diluted by deionized water and ethanol (the ethanol proportion was constant in all the solutions) to obtain the working solutions with appropriate concentrations.

The quantitative and qualitative analyses of the essential oils were carried out by GC-FID and GC-MS, respectively. The GC-MS analysis was performed on an Agilent 6890N gas chromatograph coupled with an Agilent Mass Selective Detector (MSD 5973), and fitted with a HP-5 capillary column (30 m × 0.25 mm, film thickness = 0.25 μm). Helium was used as the carrier gas with a flow rate of 0.9 mL min^{-1}. The oven temperature was programmed to increase 5ºC min^{-1} at 50–180ºC and then 15ºC min^{-1} up to 250ºC. The mass scan range was 40–400 m/z, with an electron ionization energy of 70 eV. The GC-FID analysis was carried out using an Agilent 6890 gas chromatograph equipped with a nonpolar HP-5 column (30 m × 0.25 mm, film thickness = 0.25 μm). Nitrogen was used as the carrier gas with a flow rate of 0.9 mL min^{-1}. The injector and detector temperatures were held at 250ºC and 280ºC, respectively. The other operations were the same as described for GC-MS. The identification of the compounds of the essential oils was based on the comparison of their retention indices with those of reference compounds in the literature (Adams, 2007) and the mass spectra in the Wiley Library (Wiley 7.0). The relative percentage of the identified compounds was calculated using the peak area from the GC-FID analysis, without applying the FID response correction factor.

Experiments

The current study consisted of three dose-response experiments (Exp.(s)), examining SEO at 0 (control), 250 (low dose), 500 (medium dose) and 750 (high dose) mg L^{-1} and YEO at 0, 250, 500, 750 and 1000 (as high doses) mg L^{-1} of incubation culture. In a preliminary screening experiment, SEO had a strong depressive effect on gas production at 1000 mg L^{-1}, thus this dose was removed from the current study. In the first experiment (Exp. 1), the impact of SEO and YEO on gas production kinetics was tested using the incubations of 144 h. Exp. 2 was devoted to evaluate the effects of SEO and YEO on ruminal digestibility and fermentation using the incubations of 24 h. Exp. 3 was conducted to investigate the potentials of SEO and YEO to mitigate the rumen methanogenesis. Owing to the limited numbers of the syringes, SEO and YEO were tested separately in each experiment. All the experiments were repeated in two runs on two different days. The two first experiments were conducted according to the method described by Menke and Steingass (1988) and Exp. 3 was performed according to the method of Fievez et al. (2005).

Animals and rumen fluid

Rumen fluids were collected before the morning feeding from three ruminally-fistulated mature rams (50 ± 4.5 kg BW). The rams were fed a maintenance diet containing (per kg DM) 500 g alfalfa, 70 g wheat straw, 370 g barley grain, 40 g cotton seed meal, 10 g salt and 10 g mineral and vitamin supplement, providing 122 g CP and 9.6 MJ metabolizable energy per kg diet. The rumen fluids were then pooled and strained through four layers of cheese-cloth into a pre-warmed (38–39°C) insulated flask and immediately transported to the laboratory.

***In vitro* gas production**

A fattening diet for lambs, composed (per kg DM) of 400 g alfalfa, 450 g barley, 100 g wheat straw and 50 g soybean meal, providing 11.1 MJ ME and 138 g CP per kg DM, was used as fermentation substrate. In Exp. 1, a representative air-dried sample of the substrate was ground to pass through a 1 mm sieve and sub-samples of 200 mg (DM basis) were weighed into 100 mL glass syringes. The incubation of the samples was conducted in triplicate with 30 mL of buffered rumen fluid (2:1 v/v of rumen fluid to buffer) and the aforementioned doses of SEO and YEO under a continuous flow of CO_2. The buffer was composed of 474 mL distilled water, 0.12 mL microminerals solution (containing 13.2 g $CaCl_2$. $2H_2O$, 10 g $MnCl_2$. $4H_2O$, 1 g $COCl_2$ $6H_2O$ and 0.8 g $FeCl_2$, made up to 100 mL with distilled water), 237 mL buffer solution (containing 35 g $NaHCO_3$ and 4 g $(NH_4)_4$ HCO_3 made up to 1 L with distilled water), 237 mL macrominerals solution (containing 5.7 g Na_2 HPO_4, 6.2 g KH_2PO_4, 0.6 g Mg SO_4. $7H_2O$, made up to 1 L with distilled water), 1.22 mL Resazurin solution (containing 100 mg Resazurin made up to 100 mL with distilled water) and 50 mL reducing solution. Three syringes containing 30 mL of buffered rumen fluid without substrate and the EOs were considered as blanks. The syringes were then placed in a water bath at 39°C and gas volume was recorded at 2, 4, 6, 8, 12, 24, 48, 72, 96, 120 and 144 h of incubation.

In Exp. 2, a higher amount (500 mg) of the substrate was used to reduce the gravimetrical error associated with the determination of ruminal digestibility (Makkar et al., 1995; Makkar, 2005). Incubations were conducted during 24 h in triplicate with 40 mL of buffered ruminal fluid and the same doses of SEO and YEO as used in the Exp. 1. All the other handlings and conditions were the same as those described for Exp. 1. At the end of incubation, the content of syringes was transferred into centrifuge tubes and immediately placed in cold water at 4°C to stop the 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 kept frozen at -20°C until analysis for VFA and ammonia contents. The remaining residues in the tubes were oven-dried at 60°C for 48 h and then refluxed with neutral detergent solution at 100°C for 1 h to determine *in vitro* true dry matter degradability (IVTDMD). The recovered substrate was incinerated subsequently in sintered glass crucibles at 600°C to estimate *in vitro* true organic matter degradability (IVTOMD).

In Exp. 3, methane production was measured according to the method described by Fievez et al. (2005). For this purpose, 100 mg of the substrate was incubated in triplicate in 100 mL syringes containing 15 mL of buffered rumen fluid and different doses of SEO and YEO (the same doses as used in Exp. 1, and Exp. 2) for 24 h. At the end of incubation, all the syringes were immediately cooled at 4°C to stop the fermentation. After measuring the gas produced (considered as total gas, TG), 4 mL NaOH (10 M) was injected into each syringe and the remaining gas volume after the absorption of CO_2 into NaOH, was measured and considered as methane.

Chemical analysis

The crude protein content of the substrate was measured by the standard method (ID no. 984.13) of AOAC (2000). The ammonia concentration in the supernatants

was determined as illustrated by Broderick and Kang (1980). The VFA concentrations of the samples were quantified according to the method proposed by Ottenstein and Bartley (1971), using a gas chromatograph (GC-FID, PU4410-PHILIPS, England) equipped with a flame ionization detector and a 10PEG column (1.8 m × 4.6 mm i.d., glass column packed with 10% SP 1,200, 1% H_3PO_4 on 80/100 chromosorb WAW). Nitrogen was used as the carrier gas at a constant flow rate of 35 mL min^{-1}. The oven temperature was programmed as follows: initial temperature of 100ºC, held for 4 min; increased at 5ºC min^{-1} to 135ºC, and then at 10ºC min^{-1} to 200ºC and held at 200ºC for 20 min. The temperature of the injector and detector was set at 210ºC.

Calculation and statistical analysis

Data on the cumulative gas produced during the 144 h of incubation in Exp. 1 were fitted to the model proposed by France et al. (1993), as shown in the below, by the NLIN procedure of SAS (SAS, 2002).

$$GP = A\{1 - e^{-[b(t-L) + c(\sqrt{t}-\sqrt{L})]}\}$$

Where GP (mL) is the gas produced at time t, "A" (mL) is the asymptotic gas production, b and c are constants and "L" (h) is the lag time. $T_{1/2}$ (h, time of half asymptotic gas production) and μ (h^{-1}, fractional rate of gas production at $T_{1/2}$) were calculated as described by France et al. (1993).

In Exp. 2, the ratio of truly degraded organic matter (mg) to the gas produced (mL) during 24 h of incubation was used as PF (Blümmel et al., 1997 a). The mass difference between the oven-dried fermentation substrate remaining at the end of incubation and that recovered after neutral detergent extraction was considered as MB.

Data were analysed using the MIXED procedure of SAS (SAS, 2002). The model included treatment (the doses of the essential oils), day and treatment × day as fixed effects and replication in day as random effect. Orthogonal polynomial contrasts were used to evaluate the linear and quadratic responses of the variables to the increasing doses of the EOs. The statistical significance was declared at $P \leq 0.05$ and the tendency at $0.05 < P \leq 0.10$. In the case of the significant effect, the lsmeans were compared using the Tukey-Kramer test.

Results

Chemical compositions of SEO and YEO

The chemical compositions of the EOs are shown in Table 1. Based on these results, α-pinene; 1, 8-cineol; β-myrcene; trans-caryophyllene and Sabinene were the major compounds present in both SEO and YEO. However, 1-pentadecene was one of the YEO major compounds, which was absent in SEO. There were differences between SEO and YEO in terms of the composition of their minor compounds. In addition to the different proportion of some minor constituents present in both the EOs, certain minor constituents were present in only one of the EOs, as α-thujene,

nitro-benzene, tricyclene, fenchyl acetate, thymol, calarene and β-cubebene were the minor compounds present only in SEO and 1-phellandrene, gamma-cadinene, β-bisabolene, α-amorphene, cis-α-bisabolene and Bis (2-ethylhexyl) phthalate were specific minor compounds of YEO.

Table 1. Chemical composition of sage essential oil (SEO) and yarrow essential oil (YEO)

Compound	Composition (g kg^{-1})	
	SEO	YEO
α-thujene	8	-
α-pinene	213	176
sabinene	50	49
β-myrcene	102	90
1-phellandrene	-	8
tricyclene	8	-
1,8-cineol	177	197
nitro-benzene	18	-
alloocimene	7	6
fenchyl acetate	19	-
1-pentadecene	-	67
thymol	26	-
bicycloelemene	10	15
α-copaene	19	27
calarene	13	-
trans-caryophyllene	79	100
gamma-cadinene	-	7
α-bergamotene	9	12
trans-beta-farnesene	27	40
β-cubebene	21	-
germacrene D	10	39
bicyclogermacrene	14	22
β-bisabolene	-	7
α-amorphene	-	8
delta-cadinene	16	31
cis-α-bisabolene	-	7
Bis (2-ethylhexyl) phthalate	-	30
Other compounds	154	62

Ruminal gas production kinetics

The kinetic parameters of the gas produced during 144 h of incubation are shown in Table 2 for SEO and Table 3 for YEO. A combined linear and quadratic effect was

observed for "A", "L" and $T_{1/2}$ in response to the increasing dose of SEO (P<0.01). Asymptotic gas production first increased at the low dose, but decreased thereafter at the medium and high doses of SEO. However, the modifications of "L" and $T_{1/2}$ with SEO dosage were inverse to that of "A", as their lowest values were obtained at the low dose of SEO. The fractional gas production rate increased linearly with SEO increasing dose (P=0.02), with the highest values observed at medium and high doses of SEO.

Table 2. Dose-response effect of sage essential oil (SEO) on ruminal gas production kinetics

Parameters*	SEO dose (mg L^{-1})				SEM	P-values	
	0	250	500	750		linear	quadratic
A	329.3 b	369.9 a	302.7 c	269.5 d	3.78	< 0.001	< 0.001
L	0.08 c	0.07 c	0.37 b	0.48 a	0.024	<0.001	0.024
$T_{1/2}$	6.29 b	5.15 c	6.79 a	6.41 ab	0.16	0.002	0.006
μ	0.101	0.115	0.121	0.121	0.0057	0.014	0.188

*A: asymptotic gas production (mL per 200 mg DM), L: lag time (h), $T_{1/2}$: the time half of asymptotic gas production (h), μ: fractional rate of gas production (h^{-1}).

a, b, c, d – values in rows with different letters differ significantly (P≤0.05).

Table 3. Dose-response effect of yarrow essential oil (YEO) on ruminal gas production kinetics

Parameters*	YEO dose (mg L^{-1})				SEM	P-values	
	0	250	500	750		linear	quadratic
A	345.7 c	370.3 b	396.3 a	375.9 b	286.8 d	3.57	<0.001
L	0.00 b	0.00 b	0.00 b	0.00 b	0.10 a	0.012	<0.001
$T_{1/2}$	10.3 b	10.4 b	10.6 b	9.3 c	11.4 a	0.14	0.028
μ	0.052 b	0.052 b	0.052 b	0.054 a	0.053 a	0.0004	<0.001

*A: asymptotic gas production (mL per 200 mg DM), L: lag time (h), $T_{1/2}$: the time of half asymptotic gas production (h), μ: fractional rate of gas production (h^{-1}).

a, b, c, d – values in rows with different letters differ significantly (P≤0.05).

Gas production kinetic was also affected by YEO, as all the estimated parameters changed linearly or non-linearly with YEO dosage (P<0.01). The asymptotic gas production was enhanced by YEO at the doses up to 750 mg L^{-1}, with the highest amount observed at 500 mg L^{-1}, but it decreased at the highest dose of YEO. A short lag time was observed only at the highest dose of YEO. The half time of asymptotic gas production remained unaffected at low and medium doses, but decreased and subsequently increased at 750 and 1000 mg L^{-1} of YEO, respectively. Similarly, the fractional gas production rate was not changed at 0 to 500 mg L^{-1} of YEO, however, it increased at high doses of YEO with the highest rate observed at 750 mg L^{-1}.

***In vitro* ruminal digestibility and fermentation indices**

Data on SEO effects on ruminal digestibility and fermentation are presented in Table 4. Despite a treatment effect on IVTDMD (data not shown), as this variable increased at low dose of SEO, no specific trend was observed either in IVTDMD,

or in IVTOMD under the effect of SEO (P>0.05), IVTOMD, however, tended to increase (P=0.072) quadratically with SEO dosage. A linear and quadratic effect was observed on the gas produced after 24 h of incubation (GP_{24}) with increasing doses of SEO, this variable was lowered by medium and high doses of SEO (P<0.01). The partitioning factor and MB increased linearly (P<0.01) with increasing doses of SEO in the cultures. Total VFA concentration was not affected by SEO dosage, however, the VFA pattern was modified (iso-butyrate was not separated from propionate, therefore it was included in the propionate peak), as the molar proportion of propionate decreased (P=0.003) and that of valerate increased (P=0.014) linearly with incremental doses of SEO. Moreover, SEO had no significant effect on the acetate to propionate ratio, but this ratio tended to increase linearly with the SEO dosage (P=0.063). Ammonia concentration had a tendency to increase quadratically (P=0.056) with increasing doses of SEO.

Table 4. Dose-response effect of sage essential oil (SEO) on ruminal digestibility and fermentation indices

Variables*	SEO dose (mg L^{-1})				SEM	P-values	
	0	250	500	750		linear	quadratic
IVTDMD	0.612 b	0.649 a	0.625 ab	0.639 ab	0.0085	0.159	0.209
IVTOMD	0.672	0.697	0.673	0.667	0.0080	0.327	0.072
GP_{24}	122.3 a	124.8 a	116.7 b	111.8 c	1.20	<0.001	0.008
PF	2.80 b	2.93 ab	2.98 ab	3.14 a	0.049	<0.001	0.856
MB	162.6 b	180.6 ab	187.0 ab	202.9 a	8.97	0.007	0.906
Total VFA (mM)	80.7	81.7	79.1	72.4	8.97	0.665	0.399
VFA (mol per 100 mol)							
acetate	55.7	55.2	52.4	56.3	1.40	0.887	0.160
propionate + isobutyrate	24.2 a	24 a	23.2 ab	22.7 b	0.32	0.007	0.675
butyrate	15.3	14.7	16.3	14.5	0.75	0.805	0.481
isovalerate	1.92	2.29	3.50	1.82	0.597	0.721	0.138
valerate	2.84 b	3.88 ab	4.61 a	4.70 a	0.449	0.014	0.335
acetate: propionate	2.30	2.30	2.26	2.49	0.063	0.100	0.114
Ammonia (mM)	9.2 b	9.4 ab	10.9 a	9.4 ab	0.39	0.250	0.056

*IVTDMD: *in vitro* true dry matter degradability; IVTOMD: *in vitro* true organic matter degradability; GP_{24} (mL per 500 mg DM): gas produced after 24 h of incubation; PF: partitioning factor; MB (mg): microbial biomass; VFA: volatile fatty acids.

a, b, c, d – values in rows with different letters differ significantly (P≤0.05).

In contrast to SEO, YEO had a depressive effect on ruminal digestibility (Table 5) as IVTDMD decreased linearly (P<0.001) and IVTOMD was depressed in a linear and quadratic manner (P<0.05) by increasing doses of YEO. There was also a linear and quadratic effect (P<0.001) from YEO on GP_{24}, as it decreased at the highest dose of YEO. The partitioning factor and MB decreased linearly (P<0.01) with increasing YEO doses. yarrow essential oil had also a linear and quadratic ef-

fect on both total VFA concentration and the VFA pattern. Total VFA concentration decreased only at the highest dose of YEO. The acetate molar proportion increased at the doses up to 750 mg L^{-1}, but decreased subsequently at the highest dose of YEO. In contrast, other volatile fatty acids were changed in a pattern opposite to that of acetate, as their proportions decreased at 0–750 mg L^{-1}, and increased subsequently at 1000 mg L^{-1} of YEO. Increasing inclusion doses of YEO in the cultures, resulted in a linear increase (P<0.001) in ammonia concentration.

Table 5. Dose-response effect of yarrow essential oil (YEO) on ruminal digestibility and fermentation indices

Variables*	YEO dose (mg L^{-1})					SEM	P-values	
	0	250	500	750	1000		linear	quadratic
IVTDMD	0.662 a	0.636 a	0.579 b	0.563 b	0.484 c	0.0084	<0.001	0.056
IVTOMD	0.750 a	0.729 a	0.673 b	0.654 b	0.574 c	0.0080	<0.001	0.012
GP_{24}	131.7 b	135.6 a	132.4 ab	130.8 b	124.3 c	0.83	<0.001	<0.001
PF	2.73 a	2.57 b	2.43 c	2.40 c	2.21 d	0.030	<0.001	0.683
MB	160.6 a	158.1 a	154.4 a	149.7 ab	127.1 b	6.03	0.001	0.085
Total VFA (mM)	93.0 a	92.8 a	93.6 a	95.9 a	80.6 b	1.66	0.003	0.002
VFA (mol per 100 mol)								
acetate	55.1 c	56.0 bc	57.5 ab	58.9 a	43.6 d	0.52	<0.001	<0.001
propionate + isobutyrate	22.2 b	21.8 b	21.3 b	21.3 b	23.9 a	0.34	0.029	0.002
butyrate	17.8 b	18.0 b	17.7 b	16.0 c	21.1 a	0.37	0.004	<0.001
isovalerate	2.94 b	2.08 bc	1.82 c	1.68 c	7.93 a	0.282	<0.001	<0.001
valerate	2.07 b	2.04 b	1.71 b	1.56 b	3.54 a	0.153	0.001	<0.001
acetate: propionate	2.49 b	2.57 b	2.70 a	2.76 a	1.83 c	0.037	<0.001	<0.001
Ammonia (mM)	12.7 d	13.1 d	14.5 c	17.8 a	16.5 b	0.31	<0.001	0.219

*IVTDMD: *in vitro* true dry matter degradability; IVTOMD: *in vitro* true organic matter degradability; GP_{24} (mL per 500 mg DM): gas produced after 24 h of incubation; PF: partitioning factor; MB (mg): microbial biomass; VFA: volatile fatty acids.

a, b, c, d – values in rows with different letters differ significantly (P≤0.05).

In vitro methane production

Methane production was not changed with the inclusion of SEO in the cultures (Table 6), however, the methane to TG ratio was modified quadratically (P=0.045) with SEO increasing doses, as it decreased by 6.7, 13 and 4.2% at 250, 500 and 750 mg L^{-1}, of SEO, respectively.

Yarrow essential oil, however, had a linear and quadratic effect on both TG (P<0.01) and methane (P<0.05) (Table 7). The highest dose of YEO lowered methane and TG, however, CH_4 to TG ratio did not change with increasing doses of YEO (P>0.05).

Table 6. Dose-response effect of sage essential oil (SEO) on rumen methane production

Variables*	SEO dose (mg L^{-1})				SEM	P-values	
	0	250	500	750		linear	quadratic
TG (mL)	23	22.5	23.2	22.5	0.89	0.846	0.928
CH_4 (mL)	5.5	5.0	4.8	5.2	0.27	0.381	0.163
CH_4:TG	0.239 a	0.223 ab	0.208 b	0.229 ab	0.0076	0.251	0.045

*TG (mL per 100 mg substrate, DM basis): total gas; CH_4 (mL per100 mg substrate, DM basis).
a, b – values in rows with different letters differ significantly (P≤0.05).

Table 7. Dose-response effect of yarrow essential oil (YEO) on rumen methane production

Variables*	YEO dose (mg L^{-1})					SEM	P-values	
	0	250	500	750	1000		linear	quadratic
TG (mL)	20.8 a	20.8 a	21.0 a	21.0 a	14.7 b	0.99	0.005	0.008
CH_4 (mL)	5.0 a	5.0 a	5.0 a	5.3 a	3.0 b	0.36	0.013	0.012
CH_4:TG	0.241	0.240	0.238	0.254	0.205	0.0142	0.244	0.182

*TG (mL per 100 mg substrate, DM basis): total gas; CH_4 (mL per 100 mg substrate).
a, b – values in rows with different letters differ significantly (P≤0.05).

Discussion

Chemical composition of SEO and YEO

Chemical composition analysis revealed that monoterpenes hydrocarbons were the major constituents of both the essential oils tested. However, trans-caryophyllene as a sesquiterpene and 1,8 cineol as a monoterpene alcohol were also among the major components of SEO and YEO. Among terpenoids, monoterpene hydrocarbons have the lowest antimicrobial activity, and in some cases, they stimulate the rumen microbial activity. In contrast, oxygenated terpenoids have been found to possess stronger inhibition to the growth and activity of rumen microbes (Benchaar et al., 2008 a). These results also indicated that the chemical compositions of SEO and YEO were different from those reported in the literature. For instance, Russo et al. (2013) and Nadim et al. (2011) reported completely different chemical compositions of SEO and YEO, respectively, from those in our study. These expected contrasts are due mainly to the differences associated with the geography, the phenological stage and the agronomic condition of the plant origin and also different extraction and storage procedures of the EOs (Dorman and Deans, 2000; Patra and Saxena, 2010). The results from the current study showed also a high similarity between SEO and YEO in terms of the composition of their major compounds; however, a different pattern was observed between their minor compounds profiles. Among the minor compounds, thymol (as a phenolic compound) was present only in SEO. This substance has extensively been studied as a potential additive in modulating the rumen fermentation (Calsamiglia et al., 2007 a; Benchaar et al., 2008 a), however,

its proportion in SEO was too low to have a significant contribution in the overall antimicrobial property of SEO.

Gas production kinetics

Experiments on gas production kinetics, deliver not only certain parameters, which may be useful in determining the modifications occurred in rumen active microbial communities, but also provide credible data on the impacts of EOs on the rumen fermentation, especially regarding a long exposure time of rumen microbes to EOs and the fact that some rumen microbial populations may adapt to EOs (Cardozo et al., 2004; Hart et al., 2008). The treatment-caused variation of asymptotic gas production indicated that SEO has a dose-response effect on the rumen microbial activity, as this parameter was enhanced by low dose, but lowered subsequently by medium and high dose of SEO. The rumen fermentation modulatory characteristic of EOs is generally attributed to their dose dependent antimicrobial activity (Calsamiglia et al., 2007 a; Macheboeuf et al., 2008). At high doses, depending on their chemical composition, EOs have been shown to have a general inhibitory effect on most rumen microbes, some of them, however, have exhibited a selective effect on the rumen microbial ecosystem at low and moderate doses (Benchaar et al., 2008 b; Khiaosa-Ard and Zebeli, 2013). In most studies, some beneficial effects of EOs, including the reduction of ammonia and methane production, as well as the modification in the VFA pattern, have been linked to their inhibitory impact on some rumen microbial populations (Wallace et al., 2002; Calsamiglia et al., 2007 b; Benchaar and Greathead, 2011). However, there are certain studies that report a stimulatory effect of EOs on rumen fermentation (Newbold et al., 2004; Benchaar et al., 2006; Sallam et al., 2011). Stimulating rumen fermentation by some EOs or certain plant secondary metabolites has partly been linked to the development of some bacterial communities favoured by the inhibition of rumen protozoa and fungi (Newbold et al., 1997; Goel et al., 2008). In total, any change in the rumen gas production may be a result of different causes. In the current study, an increased asymptotic gas production at low dose of SEO might be related to an improved substrate digestibility, this was consistent with a numerical increase in IVTOMD at the same dose of SEO (Table 4). Moreover, there are indications, suggesting that some active compounds of EOs, especially those with lower antimicrobial activity such as monoterpenoids with hydrocarbon and alcohol structure, may be degraded and used as a carbon source by some rumen microorganisms (Benchaar et al., 2008 a; Malecky and Broudiscou, 2009; Malecky et al., 2009 b, 2012). This may be a probable explanation for the stimulatory effect of SEO on asymptotic gas production in the present study. This is supported regarding the chemical composition of SEO mainly composed of monoterpene hydrocarbons and 1,8 cineol. However, a decreased asymptotic gas production at medium and high doses of SEO is likely a result of the shift in the rumen fermentation from the gas and VFA production pathway to that of MB production (Blümmel et al., 1997 b). This was confirmed by an increase in PF and MB with increasing doses of SEO (Table 4). A moderately longer lag time observed at medium and high doses of SEO compared to the control indicates that SEO has retarded substrate colonization by rumen microorganisms at these doses

(Wallace et al., 2002). The variation in asymptotic gas production with increasing doses of SEO was accompanied with a modification in $T_{1/2}$ and the fractional gas production rate. This is likely a reflection of the modification in the rumen predominant microbial populations involved in gas production, which needs to be clarified through further research.

Alike SEO, YEO exhibited a dose-response effect on gas production kinetics, which was stimulatory at doses up to 750 mg L^{-1}. In contrast to SEO, a higher asymptotic gas production at the doses of 250–750 mg L^{-1} is due probably to a shift in rumen fermentation from MB to the gas-producing pathway; this was in agreement with the results of PF and MB in Exp. 2 (Table 5). However, a decreased asymptotic gas production, accompanied with a longer lag time and $T_{1/2}$ at the highest dose of YEO indicated that this essential oil has a general negative impact on rumen gas production at the doses higher than 750 mg L^{-1}.

Ruminal digestibility and fermentation indices

In Exp. 2, the modification observed in GP_{24} with the SEO dosage was consistent with that of asymptotic gas production observed in Exp. 1. Despite the lack of SEO dose effect on ruminal degradability of dry matter and organic matter, IVTOMD tended to increase at low dose of SEO, this may explain, in part, the increase in GP_{24} at this dose. A linear increase in PF with increasing doses of SEO could be indicative of the redirection of digested organic matter from fermentation pathways (producing gas and VFA) to that of microbial biomass production (Blümmel et al., 1997 b). This is supported by a linear increase in MB with the incremental inclusion of SEO in the cultures, thereby providing further evidence indicating a higher partitioning of digested organic matter into microbial cells than towards gas production. Despite the lack of treatment effect on IVTOMD, an increased PF with SEO dosage is also likely the principal cause of a low GP_{24} at medium and high doses of SEO. In an earlier study, Broudiscou et al. (2002) reported no effect of sage dry extract at 15 g kg^{-1} DM on ruminal organic matter degradability and VFA production examined in the continuous culture system. It should be noted that the bioactive compounds in sage dry extract may be completely different from those in its essential oil. Consistent with results of Gunal et al. (2013), total VFA concentration in the present study was not significantly affected by SEO, its numerical variation with SEO dosage was, however, coherent with that of GP_{24}. In contrast, the VFA pattern was altered by SEO. A linear decrease in the propionate proportion was mainly due to an increase in the concentrations of other volatile fatty acids, mainly valerate and to a lesser extent isovalerate, rather than to a decrease in propionate concentration (data not shown). These results were inconsistent with those reported by Gunal et al. (2013), who reported no significant effect from sage essential oil on propionate and valerate proportions in a dose range of 0-500 mg L^{-1}. Similarly, in another experiment, Castillejos et al. (2008) examined the impact of sage essential oil on rumen fermentation at 0–500 mg L^{-1}. They reported increased proportions of propionate and valerate at the expense of those of acetate and butyrate only at 500 mg L^{-1} of sage essential oil, but similar to our results, total VFA was not affected. These contrasts may be associated to the difference in the fermentation substrate,

dose range and eventually the difference in the bioactive substances composition of the essential oil.

Rumen fermentation and digestibility were also modified by YEO in a dose-response manner. The IVTDMD and IVTOMD decreased with increasing inclusion doses of YEO in the cultures, this decrease was more pronounced at the highest dose of YEO. This is more likely a result of general inhibition of rumen microbial activity, as reported previously with high doses of most EOs (Benchaar et al., 2008 b; Khiaosa-Ard and Zebeli, 2013).

Total VFA and GP_{24} were also modified non-linearly by YEO dosage, as they remained unaffected at the doses up to 750 mg L^{-1}, but lowered by the highest dose of YEO. The lack of treatment effect on GP_{24} and total VFA in the dose range of 0–750 mg L^{-1}, despite decreasing organic matter degradability, could be a consequence of a linear decrease in PF. Indeed, a higher partitioning of the digested organic matter towards fermentation pathway than into microbial cells appears to have counteracted the negative impact of a depressed IVTOMD on gas and VFA production at 0–750 mg L^{-1} of YEO. The results of VFAs indicated a modification of the VFA pattern by YEO, which varied depending on the included dose. At doses 0–750 mg L^{-1}, the acetate molar proportion increased linearly at the expense of the other VFAs. In contrast, at the highest dose, YEO had an inverse effect on the proportion of all the VFAs, as the molar proportion of acetate decreased, while those of other VFAs increased. These results suggest that YEO has favoured the growth of acetate-producing bacteria at the doses lower than 1000 mg L^{-1}. However, at the highest dose, the acetate producing bacteria seem to be more inhibited in comparison with the bacteria producing the other VFAs. Consistent with our results, Broudiscou et al. (2002) reported that despite moderately depressing organic matter digestibility, yarrow dry extract enhanced NDF digestibility *in vitro*, supporting the hypothesis that the yarrow secondary metabolites may have a positive impact on the growth of cellulolytics as the major acetate producing bacteria. A linear increase in ammonia concentration may be a result of an increased digestion of CP with YEO dosage. This hypothesis is supported by the findings of Broudiscou et al. (2002), who reported an increase in CP digestion with the inclusion of yarrow dry extract in continuous culture. Moreover, regarding the fact that ammonia concentration in culture (*in vitro*) is a balance between its release from protein degradation and its uptake by the rumen bacteria (Nolan and Dobos, 2005), it is possible that a linear increase in ammonia is a consequence of a linear decrease in MB.

Based on these results, especially regarding a reduction in ruminal degradability of dry matter and organic matter, YEO seems to have had a moderate inhibitory effect on the rumen microbiome at 0–75 mg L^{-1}. However, the change in PF, as well as the alteration of VFA pattern at the same dose of YEO, indicate also some sort of modification in the metabolic pathways of rumen fermentation as a result of the shift in the composition of the rumen active microbial populations. However, a decrease in total VFA and GP_{24} accompanied with a more pronounced reduction in IVTDMD, IVTOMD and MB at 1000 mg L^{-1} of YEO, all are indications of a general and marked negative impact of YEO on the rumen microbial ecosystem.

Regarding the relatively comparable profile of SEO and YEO in terms of their major components, a different impact observed from these EOs on rumen fermentation could be indicative of the important role that their minor compounds play in determining their overall bioactivity. Previous studies have revealed that there are different interactions (being synergistic or antagonistic) among the active compounds of EOs and the outcome of these interactions determines their overall bioactivity, in which the role of minor compound could be critical (Burt, 2004; Calsamiglia, 2007 a).

***In vitro* methane production**

Methane production was not significantly modified with SEO dosage, though it decreased numerically at low and medium doses of SEO. However, the methane to TG ratio decreased quadratically with increasing doses of SEO. Likewise, Broudiscou et al. (2000) found a depressive effect from sage extract on the rumen methane production. Moreover, these results were numerically consistent with those of Gunal et al. (2013), who reported a linear reduction in methane production by the sage EO tested in dose ranges of 0–500 mg L^{-1}. Previous studies have suggested different modes of action for the anti-methanogenic property of some EOs, mainly through: limiting the availability of the methane production precursors (i.e. CO_2 and H_2) by decreasing acetate and butyrate to propionate ratios, inhibiting protozoa that harbour archaea, and directly inhibiting methanogenic bacteria (Russell and Wallace, 1997; Calsamiglia et al., 2007 b; Jouany and Morgavi, 2007; Kamra et al., 2008). Regarding the results of the VFA pattern in the present study, it is less probable that the reduction in methane proportion to be due to a limited presence of its precursors in the cultures, but rather, it is likely a result of the direct inhibition of methanogens. The absence of the significant effect of SEO on methane production in the current study might also be related to the method used for quantifying methane production. In this method, the gas remaining after the injection of NaOH, being considered as methane, is not purely methane, but also contains some other gases such as H_2. This is of more importance in the case of the direct inhibition of methanogens, resulting in the accumulation of H_2 (Bodas et al., 2012), which naturally enters into methane portion. Therefore, this might have resulted in an underestimate of SEO potential in mitigating the rumen methanogenesis. Among the large number of EOs tested during last decade, a few have exhibited the potential to mitigate rumen methanogenesis (Bodas et al., 2008, 2012). Moreover, in most cases, a significant reduction in methane production by some EOs has been accompanied with a general inhibition of rumen fermentation (Benchaar et al., 2008 a; Chaves et al., 2008; Patra and Saxena, 2010). This has been attributed to the high resistance of methanogenic archaea to the EOs tested (McIntosh et al., 2003). Thus, identifying the products of plant origin or the EOs with the potential to abate rumen methanogenesis without adversely affecting the rumen functions would be of great value. Given the chemical composition of SEO, composed mainly of monoterpenoids, which possess a low antimicrobial activity (Oh et al., 1967; Cox et al., 2001; Benchaar et al., 2008 a), a marked impact is not expected from SEO on rumen methanogenesis. However, regarding the fact SEO had no adverse, but even exhibited a positive impact on rumen fermentation at the doses

up to 750 mg L^{-1}, this demonstrates its potential to mitigate rumen methanogenesis, which merits further investigations using accurate methane-measuring methods such as gas chromatography.

The YEO lowered methane production, but at the highest dose, this was accompanied with a concomitant decrease in TG. In Exp. 2, it was observed that all the variables indicating the rumen microbial activity, including the IVTDMD and IVTOMD, GP_{24}, MB and total VFA were reduced by the highest dose of YEO, signifying a general inhibition of rumen fermentation. In addition, the ratio of methane to TG remained the same at all included doses, suggesting that YEO has no selective effect on methanogens or other microbial populations, which may have an indirect contribution in methane production. These results, therefore, indicated no beneficial effect from YEO on mitigating the rumen methane production.

Conclusions

Collectively, these results indicated a dose-response effect from both SEO and YEO on gas production kinetics and rumen digestibility and fermentation. However, the nature of this impact was different for each one of the EOs tested. The SEO had no adverse effects, but a positive impact on the rumen fermentation by increasing gas production, likely as a result of an improved organic matter degradability at the low dose. Moreover, SEO modified rumen fermentation towards producing more microbial protein than gas and VFA, which is considered beneficial for the host animal by supplying more microbial protein and increasing the efficiency of feed utilization. These effects, accompanied with a reduction in the methane to total gas production ratio, make SEO as a potential promising candidate to positively modify the rumen fermentation, which merits to be investigated further *in vitro* and *in vivo*. The YEO, however, had no beneficial effect on the rumen fermentation. A linear decrease in ruminal digestibility, as well as a modification of rumen fermentation towards producing more gas and VFA at the expense of microbial protein, an increased acetate to propionate ratio and ammonia concentration, suggest that YEO had an overall negative impact on the rumen fermentation.

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