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Effect of essential oils on ruminal fermentation, microbial population and methane emission *in vitro*

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Abstract. The effect of adding increasing levels (0, 25, 50 and 75 µl/75 ml buffered rumen fluid) of essential oils (EOs) to a basal substrate (50% roughage: 50% concentrate) on ruminal fermentation characteristics was evaluated *in vitro* by a semi automatic gas production (GP) technique. The EOs used were from A*chillea santolina* (AS_{25}, AS_{50}, AS_{75}), A*rtemisia judaica* (AJ_{25}, AJ_{50}, AJ_{75}), S*chinus terebenthifolius* (ST_{25}, ST_{50}, ST_{75}) and *Mentha microphylla* (MM_{25}, MM_{50}, MM_{75}). The main components of EO were piperitone (49.09%) and camphor (34.49%) in AJ, 1,6-dimethyle 1,5-cyclooactaiene (60.5%) in AS, piperitone oxide (46.7%), *cis*-piperitone oxide (28%) in MM and γ-murolene (45.25%), α-thujene in ST. The administration of EOs affected (P<0.05) the cumulative gas production (GP). The AS_{50} increased (P<0.05) the GP but ST_{50}, ST_{75} and all levels of MM decreased (P<0.05) the GP. The EOs from AS, AJ and ST increased (P<0.05) methane production with all investigated doses except AS_{75} which decreased it (P>0.05). The MM oils inhibited methane production along with a significant reduction of protozoa count. Partitioning factor (PF) was used as an index of the efficiency of microbial protein synthesis *in vitro*. There was no significant effect of EOs on PF except MM_{50} and MM_{75} which improved (P<0.05) the PF. The inclusion of AS, AJ and ST 25 did not affect (P>0.05) true digestibility of dry matter and organic matter, while ST_{50}, ST_{75}, MM_{50} and MM_{75} decreased (P<0.05) the true digestibility of dry matter and organic matter. The supplementation of $AS7_5$, ST_{50}, ST_{75}, MM_{50} and MM_{75} decreased (P<0.05)) protozoa population. The NH_3-N concentration was dramatically declined with MM inclusion. This study suggested that EOs have the potential to affect ruminal fermentation efficiency. The EOs from MM and the third dose of AS_{75} could be a promising methane mitigating agent.

Keywords. Essential oils – Gas production – Methane – Protozoa – Degradation.

***Effet des huiles essentielles sur la fermentation ruminale, la population microbienne et l'émission de méthane* in vitro**

Résumé. *L'effet de l'apport de niveaux croissants (0, 25, 50 et 75 µl/75ml du liquide ruminale tamponné) des huiles essentielles à un substrat de base (50% fibres : 50% de concentré) sur les caractéristiques de la fermentation ruminale a été évalué in vitro par la technique semi-automatique de production de gaz (GP). Les huiles essentielles ont été obtenues d'*Achillea santoline *(AS25, AS50, AS75),* Artemisia judaica *(AJ25, AJ50, AJ75),* Schinus terebenthifolius *(ST25, ST50, ST75) et* Mentha microphylla *(MM25, MM50, MM75). Les principaux composants des huiles ont été piperitone (49,09%) et du camphre (34,49%) dans AJ, 1,6-dimethyle 1,5-cyclooactaiene (60,5%) dans AS, piperitone (oxyde 46,7%), cis-piperitone oxyde (28%) dans le MM et murolene (45,25%), α-thujene dans ST. Il y avait des différences (P<0,05) dans la production de gaz (PG) pour les différents niveaux d'huiles essentielles. AS50 augmentait (P<0,05)) la GP, mais ST50, ST75 et tous les niveaux de la MM ont abaissé (P<0,05) la PG. Les huiles essentielles de l'AS, AJ et ST augmentaient (P<0,05) la production de méthane avec toutes les doses, sauf AS75 qui a légèrement diminué (P>0,05) la production de méthane. Les huiles MM inhibaient la production de méthane avec une réduction (P<0,05) des*

protozoaires. Le facteur de partition (FP) a été utilisé comme un indice de l'efficacité de la synthèse des protéines microbiennes in vitro. Il n'y a pas eu d'effet (P<0,05)) sur les huiles essentielles, sauf PF MM50 et MM75 qui a amélioré (P<0,05) le FP. L'apport de l'AS, AJ et ST 25 n'a pas affecté (P>0,05) la digestibilité de la matière sèche et de la matière organique, tandis que ST50, ST75, MM50 et MM75 diminuaient (P<0,05) la digestibilité de la matière sèche et de la matière organique. L'adjonction de AS75, ST50, ST75, MM50 et MM75 diminuait la population de protozoaires. La concentration de N-NH_3 a nettement chuté (P<0,05) avec l'incorporation de MM. Il ressort de cette étude que les huiles essentielles ont le potentiel d'affecter la fermentation ruminale, et que les huiles essentielles de MM et de AS (75 µl/75 ml) ont un bon pouvoir d'inhibition de la production de méthane.

Mots-clés. *Huiles essentielles – Production de gaz – Méthane – Protozoaires – Dégradation.*

I – Introduction

The use of antibiotic growth promoters proved to be an interesting tool to improve feed efficiency and to prevent rumen acidosis in cattle (Page, 2006). However, public concerns regarding the use of antibiotics in livestock production have increased because of the development of multi drug-resistant bacteria. This has prompted interest in seeking more natural approaches to feed antibiotics, such as plant-derived essential oils (EO), as an option for improving rumen fermentation, feed efficiency and animal performance. Essential oils are complex mixtures of secondary metabolites and volatile compounds extracted from plants by distillation, in particular steam distillation (Greathead, 2003). Essential oils have antimicrobial activities against both gram-negative and gram-positive bacteria, a property that has been attributed to the presence of terpenoid and phenolic compounds (Conner, 1993; Dorman and Deans, 2000; Calsamiglia *et al.*, 2007).

Recently, many *in vitro* studies demonstrated that EO or their components have the potential to favorably alter rumen metabolism (McIntosh *et al.*, 2003; Busquet *et al.*, 2006). For example, McIntosh *et al.* (2003) showed that a commercial blend of EO inhibited the rate of deamination of amino acids and the number of hyper-ammonia-producing bacteria in 48-h *in vitro* batch cultures. Essential oils from different sources altered the bacterial growth and metabolism of several types of bacteria, including rumen bacteria (Wallace, 2004). Busquet *et al.* (2005) reported that garlic oil altered fermentation by reducing the proportion of acetate and increasing that of propionate in a manner similar to monensin (MO) in a continuous culture. Chiquette and Benchaar (2005) showed inhibitory effects of garlic oil and juniper berry EO on the production of methane in vitro. Therefore, the objective of this study was to evaluate the antiprotozoal and antimethanogenic activities of several natural plant extracts with the *in vitro* gas production test.

II – Materials and methods

1. Plant materials

Various parts of 4 plant species: *Achillea santolina* L. (aerial parts), *Artemisia judaica* L. (aerial parts), *Schinus terebenthifolius* Raddi (fruits) and *Mentha microphylla* C. Koch (leaves) were harvested during flowering stage from different locations of Alexandria State and Sinai Peninsula, Egypt in August 2006 and April 2007. The plant materials were identified and classified using the Flora of Egypt guide (Tackholm, 1974) and confirmed by Prof. Dr. Fath Allah Zieton, of Alexandria University. Voucher specimens have been deposited at the Department of Pesticide Chemistry, Faculty of Agriculture, Alexandria University, Egypt.

2. Extraction of essential oils

The plant material was dried at room temperature for 5 days. Essential oils were extracted by hydrodistillation in a Clevenger-type apparatus for 2 h. The oils were dried over anhydrous sodium sulfate and stored at 4ºC for biological analyses and GC-MS analyses.

3. Analyses of essential oils by GC-MS

Essential oils were diluted in diethyl ether and 1µl was injected into a gas chromatography (TRACE GC 2000, THERMO)/ mass spectrometry (SSQ 7000, FINNIGAN) (GC/MS) set up. The GC column was a 60 m (0.25 mm i.d.) DB-5 (5% phenyl) Methylpolsiloxane capillary column.

4. Treatments and experimental design

The different levels of EO were added to the diet samples. Four levels (0, 25, 50 and 75 µl/75ml buffered rumen fluid) of each EO were investigated as follow: No additive, *Achillea santolina* (AS_{25}, AS_{50}, AS_{75}), *Artemisia judaica* (AJ_{25}, AJ_{50}, AJ_{75}), *Schinus terebenthifolius* (ST_{25}, ST_{50}, ST_{75}) and *Mentha microphylla* (MM_{25}, MM_{50}, MM_{75}). The total mixed ration (50% roughage: 50% concentrate) was used as substrate incubated with buffered rumen fluid (2:1, v/v) in 160 ml serum bottles for 24 h. The chemical composition of the total mixed ration used was 922.4, 131.0, 718.0, 343.0 and 20.0 g kg^{-1} for dry matter, crude protein, neutral-detergent fibre, acid-detergent fibre and ether extract, respectively. Five adult rumen cannulated sheep grazing tropical grass pasture and a supplement based on maize and soybean meal (0.7 kg/100 kg of live weight, 20% crude protein) plus a mineral mixture were used as inoculum donor. Both solid and liquid rumen fractions (50% solid: 50% liquid) were collected before the morning feeding through the cannula using a stainless steel probe (2.5 mm screen) attached to a large capacity syringe.

The *in vitro* gas production (GP) assay was carried out using a pressure transducer and data logger (LANA/CENA-USP, Piracicaba/SP, Brazil) for measuring the gas produced in 160 ml serum bottles incubated at 39ºC (Mauricio *et al.*, 1998). Ground samples (0.5 g) were incubated in 75 ml of diluted rumen fluid (25 ml mixed rumen fluid + 50 ml of Menke's buffered medium) (Longo *et al.*, 2006). Once filled, all the bottles were closed with rubber stoppers shaken and placed in the incubator at 39ºC. The bottles were hand shaken after recording the gas headspace pressure at 12 and 24 h incubation using a pressure transducer (Theodorou *et al.*, 1994). Three GP runs were performed. Bottles of each run included, four bottles containing only buffered rumen fluid without substrate (blank), four bottles for substrate without additive (control), four bottles for each concentration of EO and four bottles with internal standard were used for adjustments and variation between the runs. Gas samples were collected at 12 h and 24 h incubation (5.0 ml each time and accumulated in vacutainer tubes). Methane concentration was analysed with a Shimadzu 2014 gas chromatography equipped with a thermal conductivity detector and shincarbon ST micro packed column. Methane production at the end of incubation period was estimated from the volume of gas and the gas composition data as CH_4 = [GP + HS] x Conc; where CH_4 is the volume (ml) of methane, GP is the volume (ml) of gas produced at the end of the incubation, HS is the volume (ml) of the headspace in the serum bottle and Conc is the percentage of methane in the gas sample analyzed (Tavendale *et al.*, 2005). After termination of the incubation, two bottles content were used for determination of true digestibility of dry and organic matter (TDDM, TDOM) and PF according to Blummel and Becker (1997) and Blummel *et al.* (1997). The contents of two other bottles were used for determining the NH_3-N concentration and protozoa counting. The NH_3-N concentration was measured according to Preston (1995). Protozoa were counted microscopically following the procedure described by Kamra *et al.* (1991).

5. Statistical analysis

Data were subjected to analysis of variance (ANOVA), using the General Linear Model procedure of the SAS software package (2000). The model was: Y= μ+ Fi + e, where μ is overall mean, Fi the treatment effect. Experimental units were runs. Replicates in the same run were considered as repetitions. Significant differences between individual means were identified using Tukey test (SAS, 2002).

III – Results

The main components in different EOs (Table 1) were different. The main constituents were Piperitone (49.09%) and Camphor (34.49%) in Artemisia EO, 1,6-dimethyle 1,5-cyclooactaiene (60.5%) in Achillea EO, Piperitone oxide (46.7%), cis-piperitone oxide (28%) in Menthe EO and γ-Muurolene (45.25%), α-Thujene in Schinus EO.

Table 1. Main constituents (%) of the essential oils isolated from four Egyptian plants

Component	RT (min)	AJ	AS	MM	ST
α-Pinene	10.72	–	–	1.87	–
α-Thujene	10.75	–	–	–	15.95
Sabinene	12.25	–	0.52	3.50	0.72
Myrcene	12.84	–	–	0.37	–
1,8-Cineole	14.09	–	8.64	13.34	–
δ-3-Carene	14.38	–	–	–	7.83
β-Thujone	16.74	–	8.96	–	–
Camphor	17.99	34.49	5.41	–	–
Borneol	19.25	3.90	–	–	–
4-Terpineol	19.49	–	2.33	–	–
Linalyl propionate	20.49	–	–	1.36	–
Fragranol	20.65	–	10.52	–	–
cis-Piperitone oxide	21.42	–	–	28.00	–
Piperitone	21.67	49.09	–	–	–
1,6-dimethyl,1,5-cyclooactadiene	23.58	–	60.52	–	–
Torreyol	24.48	–	–	–	0.79
Piperitone oxide	24.61	–	–	46.70	–
γ-Cubebene	25.61	–	–	–	1.60
(-)-Isoledene	25.95	–	–	–	3.63
Germacrene	27.30	–	–	0.15	–
γ-Muurolene	27.36	–	–	–	45.25
α-Ylangene	27.68	–	–	–	1.45
Cadinene	28.27	–	–	–	6.76
(-)-Spathulenol	30.24	1.70	–	–	3.46

AJ = *Artemisia judaica*; AS = *Achillea santolina*; MM = *Mentha microphylla*; ST = *Schinus terebenthifolius.*

There were differences ($P<0.05$) in cumulative gas production (GP) after subtracting the blank gas volume for different levels of the EOs (Table 2). The first (AS_{25}) and second dose (AS_{50}) of Artemisia increased the GP by 16 and 22%, respectively, while the third dose (AS_{75}) depressed the GP by 12%. All levels of Eos from AJ increased ($P<0.05$) GP up to 13% while the second and third dose of ST depressed ($P<0.05$) the GP by about 13% and 17%, respectively. The inclusion of MM oil produced half of GP with the third dose and decreased the GP by 35% with the second dose. The EOs from AS, AJ and ST increased methane production with all investigated

doses except the third dose from AS which decreased (P>0.05) methane production. The MM oils inhibited completely methane production.

Table 2. Effect of different levels of essential oils on gas (GP, ml/g DM) and methane production *in vitro* for 24 h incubation

Treatments	Levels	GP	% change	CH_4 (ml/g DM)	CH_4 (ml/g TDOM)
No additive	–	137.2^{bcd}	–	9.9^{ab}	17.8^{abc}
A. santolina	AS_{25}	158.9^{ab}	16+	13.6^{ab}	25.4^{ab}
	AS_{50}	167.1^{a}	22+	15.5^{a}	14.8^{bc}
	AS_{75}	125.1^{de}	12-	6.9^{bc}	13.9^{bc}
A. judaica	AJ_{25}	151.8^{abc}	11+	14.5^{ab}	27.1^{a}
	AJ_{50}	153.5^{abc}	12+	14.2^{ab}	27.6^{a}
	AJ_{75}	154.3^{ab}	13+	9.5^{ab}	19.5^{ab}
S. terebenthifolius	ST_{25}	132.0^{cde}	4-	10.7^{ab}	20.0^{ab}
	ST_{50}	119.1d^{ef}	13-	9.9^{ab}	21.4^{ab}
	ST_{75}	114.6^{ef}	17-	10.2^{ab}	26.9^{a}
M. microphylla	MM_{25}	131.3^{fg}	4-	0.8^{d}	2.8^{d}
	MM_{50}	88.8^{gh}	35-	0^{d}	0^{d}
	MM_{75}	72.0^{h}	48-	0^{d}	0^{d}
SEM†		7.9	–	2.9	2.5

Values within the same column sharing the same letter are not significantly different (P>0.05).
† SEM: standard error of difference between means.

The second and third dose of ST and MM decreased (P<0.05) the TDDM and TDOM (Table 3). Irrespective their sources, the EOs did not affect (P>0.05) the PF which is considered as an index of microbial protein synthesis except the second (MM_{50}) and third dose (MM_{75}) of Mentha that improved (P<0.05) the PF. The protozoa count decreased (P<0.05) with the supplementation of the second dose of ST_{50}, the third dose of AS_{75} and $ST_{75.}$ All doses of MM decreased (P<0.05) the protozoa count.

Table 3. Effect of different levels of essential oils on partition factor (PF, mg truly digested organic matter/ml gas at 24 h), true digestibility of dry and organic matter (TDDM, TDOM), NH_3-N concentration (mg/l) and protozoa count (x10^5 ml^{-1})

Treatments	Levels	TDMM	TDOM	PF	Protozoa	NH_3-N
No additive	–	591^{a}	555^{a}	3.53^{b}	5.25^{a}	119^{ab}
A. santolina	AS_{25}	559^{a}	533^{a}	2.95^{b}	4.73^{ab}	117^{ab}
	AS_{50}	560^{a}	522^{a}	2.69^{b}	4.95^{ab}	138^{a}
	AS_{75}	535^{ab}	496^{ab}	3.37^{b}	2.85^{c}	95.7^{bc}
A. judaica	AJ_{25}	578^{a}	540^{a}	3.11^{b}	5.25^{a}	118^{ab}
	AJ_{50}	570^{a}	526^{a}	3.01^{b}	5.40^{a}	114^{ab}
	AJ_{75}	568^{a}	530^{a}	3.08^{b}	5.18^{a}	105^{b}
S. terebenthifolius	ST_{25}	560^{a}	527^{a}	3.41^{b}	5.03^{ab}	128^{a}
	ST_{50}	497^{b}	461^{b}	3.35^{b}	4.13^{b}	130^{a}
	ST_{75}	418^{bc}	388^{c}	2.97^{b}	3.08^{c}	133^{a}
M. microphylla	MM_{25}	556^{a}	524^{a}	3.43^{b}	4.58^{ab}	84.5^{c}
	MM_{50}	495^{b}	459^{bc}	4.51^{a}	4.13^{b}	98.0^{bc}
	MM_{75}	426^{bc}	402^{c}	4.72^{a}	2.63^{c}	77.0^{c}
SEM†	–	10.5	11.1	0.78	0.35	9.8

Values within the same column sharing the same letter are not significantly different (P>0.05).
† SEM: standard error of difference between means.Values within the same column sharing the same letter are not significantly different (P>0.05).

IV – Discussion

The antimicrobial activity of the EOs is attributed to a number of secondary plant metabolites, which include saponins, terpenoids and phenylpropanoids present in the essential oil fraction of many plants. The main compound of AS are 1,6-dimethyle 1,5-cyclooactaiene (60.5%) which may negatively affect rumen microbes activity. The reduction of GP and methane production with the third dose of AS_{75} may be due to this compound which also decreased the protozoa count. In agreement with our results, wormwood (*Artemisia vularis*) has a high concentration of EOs and bioactive compounds such as camphor, cineole, catechol and vanillin (Lee *et al.*, 1999). Kim *et al.*, (2006) reported that substituting the concentrate mixture with 30-50 g/kg DM of wormwood (*Artemisia montana*) increased N retention, ruminal VFA and NH_3–N concentrations, microbial yield and ether extract digestibility in sheep. Riddle *et al.* (1996) reported positive correlations of specific monoterpenes (camphor) with juniper intake by goats. Because of piperitone (46.7%) and *cis*-piperitone oxide (28%) and 1,8-cineole (13.3%) were the main constituents in menthe oil, the methane emission, GP and protozoa count were decreased significantly compared to the control substrate without additive. Cook *et al.* (2007) analyzed *Mentha spicata* plants from 3 locations in Zakynthos Island in Greece. The main constituents were trans-piperitone oxide, piperitenone oxide and 1,8-cineole. They observed variation in *Mentha spicata* EO composition among locations and plant organs in July would not appear to be directly related to the climatic conditions but related to local differences in soil water availability and retention and plant shading, or genetic differences between the plants, which together affect the physiology of EO biosynthesis. In addition, 1,8-cineole was reported to be the major constituent (29 %) of one oil from *Mentha longifolia* plants in Israel, together with piperitone (14 %) and cis-piperitone oxide (15 %) (Fleisher and Fleisher, 1991). There may be potential to select EO compounds that reduce methane by selectively inhibiting protozoa, which would be expected to decrease methane production because ruminal protozoa provide a habitat for methanogens that live on and within them. A decrease of feed degradability by EOs of *Mentha* and *Schinus* could be due to phenolic compounds such as tannins, piperitone oxide, *cis*-piperitone oxide, γ-Muurolene and α-Thujene. Digestibility depression is a function of the competition between rates of digestion and passage (Van Soest, 1994).The degree of inhibition depended, however, on the chemical structure of the EO compound added. Of the compounds evaluated, oxygenated monoterpenes, particularly monoterpene alcohols and aldehydes, strongly inhibited growth and metabolism of rumen microbes, whereas monoterpene hydrocarbons slightly inhibited and, sometimes, stimulated activity of rumen microbes. In fact, monensin affects only some gram-positive bacteria, while EOs inhibit gram positive and gram-negative bacteria (Helander *et al.*, 1998).

Ruminal gram-positive bacteria are involved in fermentation processes that produce acetate, butyrate, formate, lactate, hydrogen, and ammonia. Ruminal gram negative bacteria are involved in fermentation processes associated with the production of propionate and succinate (Russell and Strobel, 1989). It could be hypothesized that the fermentation pattern observed in EO is mediated through a stronger inhibition of the gram negative rumen bacteria, in contrast to monensin that inhibits mainly gram-positive rumen bacteria. Several studies reported that the addition of BEO decreased the effective degradability and the rate of ruminal degradation of some protein supplements (Molero *et al.*, 2004; Newbold *et al.*, 2004).The reduction in ammonia N in the current trial suggested that the EOs of MM reduced amino acid deamination when thymol was supplemented (Broderick and Balthrop, 1979). Inhibition of amino acid deamination has practical implications because it may increase ruminal escape of dietary protein and improve the efficiency of N use in the rumen (Van Nevel and Demeyer, 1988). A consistent finding when saponins are supplied to ruminants is a reduction in ruminal ammonia N concentration (Wallace *et al.*, 1994; Hristov *et al.*, 1999). These effects have been generally attributed to the pronounced antiprotozoal activity of saponins (Francis *et al.*, 2002), protozoa being the primary rumen ammonia producers. However, ruminal ammonia N concentration may increase (Hristov *et al.*, 1999) or

decrease (Devant *et al.*, 2000) depending on the amount of degradable protein and on the amount and type of dietary carbohydrates available for microbial use (Russell *et al.*, 1983). This study suggested that the EOs have the potential to affect ruminal fermentation efficiency, and MM and AS essential oils could be a promising methane mitigating agent. Few in vivo studies evaluated effects of EO and their main components on methane emissions, and no studies have assessed long-term effects of EO and their constituents on methane production.

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