Effect of Different Levels of Citrus Essential Oil and its Active Component on Rumen Microbial Fermentation and Methane Emission *in Vitro*

Sobhy M. Sallam
Department of Animal Production, Faculty of Agriculture, Alexandria University, 21545-El-Shatby, Alexandria, Egypt.

Received on: 12 /1/2011 *Accepted: 20/2/2011*

ABSTRACT

The fermentation characteristics of adding different levels (0, 25, 50 and 75 µl/75ml buffered rumen fluid) of citrus essential oil or its bioactive component (limonene, 0, 30, 45 and 60 µl/75ml buffered rumen fluid) to a basal substrate (50% roughage: 50% concentrate) were evaluated *in vitro* by semi automatic gas production (GP) technique. The investigated essential oil was *Citrus reticulata* (CR_{25}, CR_{50}, CR_{75}), and Limonene (L_{30}, L_{45}, L_{60}). The analyses of the citrus essential oils by GC/MS showed that the main components were *dl*-Limonene (83.9%) and γ- Terpinene (10.75%). There were significant differences ($P < 0.05$) in cumulative GP after subtracting the blank gas volume for different levels of citrus essential oil or limonene. All levels of citrus essential oil and limonene significantly ($P<0.05$) decreased the GP compared with the substrate with no additive. The second and third dose of citrus essential oil or limonene decreased ($P<0.05$) methane emission when expressed on dry matter basis, but when expressed on the basis of digested organic matter the third dose from citrus essential oil decreased ($P<0.05$) methane emission only *in vitro*. The inhibition of methane production was accompanied with a significant reduction in protozoal count. Partitioning factor (PF) was used as an index of the efficiency of microbial protein synthesis *in vitro*. There was no significant effect of citrus essential oils on PF, while limonene supplementation decreased ($P<0.05$) the PF values. The inclusion of citrus essential oil or limonene affected negatively the true digestibility of dry and organic matter. The NH_3-N concentration dramatically declined with the inclusion of high level of citrus or limonene. This study suggested that the citrus essential oil has the potential to affect ruminal fermentation efficiency, and could be a promising methane mitigating agent due to its rich content of limonene.

Key words: citrus, limonene, essential oil, gas production, methane, protozoa, degradation.

INTRODUCTION

Because of the residues appearance and bacterial resistant strains of antibiotics used as growth promoters in animal feeds, the antibiotic use has been prohibited in the European Union since January 2006 (Regulation 1831/2003/EC). This has prompted interest in seeking more natural approaches such as plant-derived essential oils (EO), as natural alternative feed additives 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 methods, 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). Chemically, essential oils are variable mixtures of principally terpenoids, especially monoterpenes (C10) and sesquiterpenes (C15), although diterpenes (C20) may be also present. Most essential oils are classified as generally recognized as safe, and have been approved for food and beverage consumption by the US Food and Drug Administration (www.cfsan.fda.gov). However, few plant extracts have been tested for their effects on ruminal microbial fermentation (Cardozo *et al.*, 2004; Castillejos *et al.*, 2005; Busquet *et al.*, 2006; Calsamiglia *et al.*, 2007) especially methane emission. Therefore, the objective of this study was to evaluate the effects of different levels of citrus essential oil and its active component (limonene) on rumen microbial fermentation activities and methane emission *in vitro*.

MATERIALS AND METHODS

Plant materials

Various parts of *Citrus reticulata* Balanco (fruit peel), were collected during flowering stage from different location of Alexandria Province and Sinai Peninsula, Egypt in August 2006 and April 2007. The plant materials were identified and classified with the guidance of the student's Flora of Egypt book (Tackholm, 1974) and confirmed by Dr. Fath Allah Zieton, Professor of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt. Voucher specimens have been deposited at the Department of Pesticide Chemistry, Faculty of Agriculture, Alexandria University, Egypt.

Isolation of essential oils

The plant materials were dried at room temperature for five days. Essential oils were extracted by hydro distillation in a Clevenger-type apparatus for 2hr. The oils were dried over anhydrous sodium sulfate and stored at 4°C for biological analyses and GC-MS analyses.

Analyses of essential oils by GC-MS

Essential oils were diluted in diethyl ether and 1 µl was injected into a gas chromatography (TRACE GC 2000, THERMO)/ mass spectrometry (SSQ 7000, FINNIGAN) (GC/MS) set up. The GC column was a 60m (0.25mm i.d.) DB-5 (5% phenyle) Methylpolsiloxane capillary column. The GC conditions were as follows: injector temperature, 220°C, column temperature, isothermal at 40°C for 2 min, then programmed to 250°C/2min and held at this temperature for 2 min; ion source temperature, 200°C. The Helium was used as the carrier gas at a rate of 1 ml/min. The effluent of the GC column was introduced directly into the ion source of the MS. Spectra were obtained in the EI mode with 70 ev ionization energy. The sector mass analyzer was set to scan from 40 to 400 amu for 5 s.

Treatments and experimental design

The different levels of citrus essential oil (0, 25, 50 and 75 µl/75ml buffered rumen fluid) or limonene (0, 30, 45 and 60 µl/75ml buffered rumen fluid) were added to the diet sample. The total mixed ration (50% roughage: 50% concentrate) was used as substrate incubated with buffered rumen fluid (2:1, v/v) into 160 ml serum bottles for 24 h. The proximate analyses of the used total mixed ration 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 39°C (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) into 160 ml serum bottles. Once filled, all the bottles were closed with rubber stoppers shaked and placed in the incubator at 39 °C. The bottles were shaked manually after recording of the gas headspace pressure at 12 and 24 h incubation using a pressure transducer (Theodorou *et al.*, 1994). Methane determination was done in a Shimadzu 2014 gas chromatography equipped with a thermal conductivity detector. Separation was achieved using shincarbon ST micro packed column, helium was the carrier gas with a flow rate of 10 ml/min. The detector and column temperature were 250 and 60°C, respectively. The test of linearity and calibration were accomplished using the standard gas curve in the range of probable concentration of the samples. Methane production at the end of incubation period was estimated from the volume of gas and the gas composition data as "CH4 = [GP + HS] x Conc"; where CH4 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 bottle contents were used for determination of true digestibility of dry and organic matter (TDDM, TDOM) *in vitro*. Another two bottle contents were used for determining the PF according to Blummel and Becker (1997) and Blummel *et al.* (1997). The NH_3-N concentration was measured according to Preston (1995). Protozoa were counted microscopically following the procedure described by Kamra *et al.* (1991). Short chain fatty acids (SCFA) were calculated according to the Getachew *et al.* (2002).

Statistical analysis

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

RESULTS AND DISCUSSION

The chemical profiles by GC-MS of the citrus essential oils extracted from Egyptian plants are given in Table 1. The analyses of the citrus essential oils showed that the main components were *dl*-limonene (83.9%) and γ- terpinene (10.75%). Also, the citrus essential oil has traces from α-pinene (1.44%), sabinene (0.97%), myrcene (0.71%) and α-terpinolene (0.30%).

Effect of different levels (0, 25, 50 and 75 µl/75ml buffered rumen fluid plus 500 mg total mixed ration) of citrus essential oils or limonene (0, 30, 45 and 60 µl/75ml buffered rumen fluid plus 500 mg total mixed ration) on gas and methane production *in vitro* for 24h incubation are presented in Table.2. There were significant differences ($P<0.05$) in cumulative gas production (GP) after

subtracting the blank gas volume for different levels of the essential oil. All levels of citrus essential oil and limonene significantly (P<0.05) decreased the GP compared with the substrate with no additive. The citrus essential oil supplementation decreased linearly GP with the increasing level of supplementation from 10.5 to 34.7%. Inclusion of L_{30}, L_{45} or L_{60} decreased (P<0.05) GP by 11.4, 23.6 and 22.3%, respectively. The second and third dose of citrus essential oil or limonene decreased (P<0.05) methane emission when expressed on dry matter basis, but when expressed on the base of digested organic matter only the third dose from citrus essential oil decreased (P<0.05) methane emission *in vitro*. The inhibition of methane production was accompanied with a significant reduction in protozoal count.

The effect of different levels from either citrus essential oils or limonene on true digestibility of dry and organic matter (TDDM, TDOM, g/kg DM), partitioning factor (PF), protozoa count, NH_3-N concentration and short chain fatty acids (SCFA) are shown in Table 3. The inclusion of either citrus essential oil or limonene affected negatively the true digestibility of dry and organic matter. The inclusion of citrus essential oil decreased the TDDM, TDOM compared to the control but this reduction was not significant among levels. Partitioning factor (PF) was used as an index of the efficiency of microbial protein synthesis *in vitro*. There was no significant effect of citrus essential oils on PF, while limonene supplementation decreased (P<0.05) the PF values. The protozoal count declined significantly (P<0.05) with the supplementation of the second and third doses of citrus essential oil, while all doses of limonene decreased the protozoal count significantly (P<0.05). The NH_3-N concentration declined dramatically with inclusion of high levels of citrus or limonene. The SCFA decreased (P<0.05) linearly with increasing level of citrus essential oil supplementation.

Plant essential oils and extracts have been used for long time (Jone, 1996) in food preservation, pharmaceuticals, alternative medicine and natural therapeutics (Reynolds, 1996 and Lis-Balchin and Deans, 1997). The antimicrobial activity of the essential oils 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 bioactive components of citrus essential oil are *dl*-Limonene (83.9%) and γ- Terpinene (10.75%) which may affect negatively the rumen microbes activity. The reduction of GP and methane production could be due to these compounds which also decreased the protozoal count. There is a possibility to select essential oil (EO) compounds that reduce methane by selectively inhibiting protozoal numbers, which may lead a decrease in methane production because ruminal protozoa provide a habitat for methanogens that live on and within them.

Table 1: Main constituents (%) of the Egyptian citrus essential oil.

Component	RT (min)	*Citrus reticulata*
'inene	10.72	1.44
)inene	12.25	0.97
Myrcene	12.84	0.71
α-Terpinolene	13.69	0.30
imonene	14.13	83.93
:rpinene	15.07	10.75

RT: Retension time

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^{a}	-	10.3^{ab}	17.8^{abc}
Citrus	CR_{25}	122.8^{b}	10.5	10.1^{ab}	18.1^{c}
	CR_{50}	105.7^{c}	23.0	8.6^{c}	19.0^{bc}
	CR_{75}	89.6^{d}	34.7	6.8^{d}	13.3^{d}
Limonene	L_{30}	121.5^{b}	11.4	11.8^{a}	22.0^{a}
	L_{45}	104.8^{c}	23.6	8.2^{c}	20.2^{ab}
	L_{60}	106.6^{c}	22.3	9.2^{bc}	21.3^{a}
SEM‡		7.5	-	0.89	1.53

a,b,c;d within column, means with different superscripts are differ significantly (Tukey test; P<0.05).
‡ SEM: standard error of difference between means.

Table.3 Effect of different levels of essential oils on true degradation of dry and organic matter (TDDM, TDOM, g/kg DM), partition factor (PF, mg truly digested organic matter /ml gas at 24 h), protozoa count (x10^5 ml^{-1}), NH_3-N concentration (mg/l) and predicted short chain fatty acids (SCFA, mM).

Treatments		TDDM	TDOM	PF	protozoa	NH_3-N	SCFA
No additive	-	591^{a}	555^{a}	3.5^{ab}	5.25^{a}	119.2^{ab}	63.4^{a}
Citrus	CR_{25}	515^{b}	480^{b}	3.8^{ab}	5.10^{ab}	112.5^{b}	54.4^{b}
	CR_{50}	462^{b}	451^{c}	3.3^{b}	3.38^{c}	109.9^{bc}	46.8^{c}
	CR_{75}	473^{b}	457^{c}	4.0^{a}	3.38^{c}	89.1^{d}	39.7^{d}
Limonene	L_{30}	483^{b}	474^{b}	3.1^{b}	2.78^{d}	115.9^{b}	53.9^{b}
	L_{45}	318^{c}	310^{d}	2.3^{c}	2.70^{d}	126.0^{a}	46.4^{c}
	L_{60}	292^{c}	287^{d}	1.9^{d}	3.15^{c}	104.0^{c}	47.2^{c}
SEM‡	-	12.4	11.6	0.11	0.21	4.6	2.3

a,b,c;d within columns, means with different superscripts differ significantly (Tukey test; $P<0.05$).
‡ SEM: standard error of difference between means.

Limonene is the most abundant monocyclic monoterpene in lemons (*Citrus limonum*), oranges (*Citrus aurantium*), grapefruit (*Citrus paradisi*), peppermint (*Mentha piperita*), spearmint (*Mentha spicata*), and other oils (Turner *et al.*, 1999). Dorman and Deans (2000) demonstrated the antimicrobial activity of limonene, mainly against gram-negative bacteria. Castillejos *et al.* (2006) reported that limonene at 50 and 500 mg/L reduced total VFA concentration (−4.5 and −5.6%, respectively), suggesting that these doses were toxic to rumen bacteria. In addition, limonene at a dose of 500 mg/L reduced ammonia N (−14.6%) and branched chain VFA (−6.6%) concentrations, suggesting that deamination of AA was inhibited (Allison et al., 1962).

A depression in feed degradability by either citrus essential oil or limonene 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, however, depended 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 essential oils inhibit gram positive and gram-negative bacteria (Helander *et al.*, 1998). In agreement with our results, several studies showed that the addition of blended essential oil decreased the effective degradability and the rate of ruminal degradation of some protein supplements (Molero *et al.*, 2004; Newbold *et al.*, 2004).

Ruminal gram-positive bacteria are involved in the fermentation processes that produce, among other end products, acetate, butyrate, formate, lactate, hydrogen, and ammonia. On the other hand, ruminal gram-negative bacteria are involved in fermentation processes associated with the production of propionate and succinate (Russell and Strobel, 1989). We may hypothesize 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.

The reduction in ammonia N in the present trial suggested that citrus essential oil reduced amino acid deamination, as was indicated by Broderick and Balthrop (1979) with thymol. 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).

In agreement with our results, Nagy *et al.* (1964), Nagy and Tengerdy (1968) and Dziba *et al.*, (2006) revealed that the antibacterial effects of terpenes adversely affect rumen fermentation, dry matter digestibility, nutritive value, retard *in vitro* fermentation of cellulose and reduce production of volatile fatty acids (VFA). In contrast to previous

findings that suggest sagebrush (rich in terpenes) inhibits *in vivo* dry matter digestibility (Ngugi *et al.*, 1995), the addition of terpenes to either barely-based diet or beet pulp-based diet increased the digestibility of dry matter, neutral detergent fiber, and acid detergent fiber but decreased concentrations of total VFA and acetate (Villalba *et al.* ,2006). Terpenes also depressed butyrate concentration in the barley-based diet. Propionate concentrations were not affected by terpenes in either feed.

CONCLUSION

It is concluded that the citrus essential oil has the potential to positively affect ruminal fermentation efficiency, and could be a promising methane mitigating agent due to its rich content of limonene. This study indicates the need of conducting long term *in vivo* experiment to investigate the potential effect of citrus essential oil on rumen microbial fermentation and methane emission.

Acknowledgements

This work was supported by the Third World of Academic Science (TWAS) in Italy, the National Council for Scientific and Technological Development (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) in Brazil.

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الملخص العربي

تأثير مستويات مختلفة من زيت اليوسفي المتطاير والمركب الفعال به علي تخمرات الكرش الميكروبية وإنتاج الميثان معمليا

صبحي محمد عبدالله سلام

قسم الإنتاج الحيواني - كلية الزراعة بالشاطبي - جامعة الإسكندرية - جمهورية مصر العربية

أجريت الدراسة بهدف تقييم إضافة مستويات مختلفة (صفر، ٢٥، ٥٠ ، ٧٥ ميكروليتر لكل ٧٥ مل من سائل الكرش والمحلول المنظم للحموضة) من مستخلص زيت اليوسفي المتطاير المستخلص بالتقطير المائي والليمونين (المركب الفعال به بمعدلات صفر، ٣٠، ٤٥، ٦٠ ميكروليتر لكل ٧٥ مل من سائل الكرش والمحلول المنظم للحموضة) إلي عليقة أساسية مكونة من ٥٠% مخلوط مركز و ٥٠% علف مالئ علي خصائص التخمرات الميكروبية في الكرش معملياً باستخدام تقنية إنتاج الغاز شبه الألي. أوضحت نتائج تحليل مستخلص زيت اليوسفي المتطاير بجهاز كروماتوجرافي الغاز/مطياف الكتلة أن المركبات السائدة في مستخلص زيت اليوسفي هي الليمونين (٨٣،٩%) والتربين (١٠،٧٥%). كما أظهرت النتائج حدوث إنخفاض معنوي في إنتاج الغاز عند إضافة المستويات المختلفة من زيت اليوسفي والليمونين مقارنة بالكنترول. إضافة المستوي الثاني أو الثالث من زيت اليوسفي أو الليمونين أدي لحدوث إنخفاض معنوي في إنتاج الميثان (مل لكل جم مادة جافة محضنة) بينما إضافة المستوي الثالث من زيت اليوسفي أحدث إنخفاضاً في إنتاج الميثان عند التعبير عنه بالمل لكل جم مادة عضوية مهضومة حقيقيا مقارنة بالكنترول. إنخفاض إنتاج الميثان كان متصاحبا مع إنخفاض في عدد البروتوزوا. تم تقدير الـ partitioning factor (PF) وهو العلاقة مابين المادة العضوية المهضومة حقيقيا وحجم إنتاج الغاز كمقياس لكفاءة البروتين الميكروبي معمليا. وأوضحت النتائج عدم وجود إختلافات معنوية بين إضافة المستويات المختلفة من زيت اليوسفي علي قيمة PF مقارنة بالكنترول، بينما أحدثت إضافة الليمونين إنخفاضاً معنوياً في قيمة الـ PF. كما كان هناك إنخفاضاً معنوياً في الهضم الحقيقي للمادة الجافة والعضوية وتركيز الأمونيا نتيجة إضافة زيت اليوسفي المتطاير أو الليمونين مقارنة بالكنترول. وتشيرهذه الدراسة إلي أن إضافة زيت اليوسفي المتطاير لعلائق المجترات يؤثر علي كفاءة تخمرات الكرش الميكروبية ويعتبر من الإضافات الواعدة لخفض إنتاج الميثان في المجترات وذلك لمحتواه العالي من مركب الليمونين.