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ANTIMETHANOGENIC AND ANTIPROTOZOAL EFFECT OF SOME ESSENTIAL OILS *IN VITRO*

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SUMMARY

Ruminal inoculum enriched with particle-associated microorganisms was collected from rumen cannulated Santa Inês wethers grazing tropical grass pasture and a supplement based on ground corn and soybean before feeding and used to evaluate effects of some commercial essential oils (EO) on ruminal fermentation and methane emission in vitro. The investigated EOs were orange EO (Citrus sinensis, CrS_{25}, CrS_{50}, CrS_{75}), lemon EO (Citrus limonum, CrL_{25}, CrL_{50}, CrL_{75}), mint EO (Mentha arvensis L., MeA_{25}, MeA_{50}, MeA_{75}) and garlic EO (Allium sativum L., AS_{25}, AS_{50}, AS_{75}), which supplemented to basal diet by four levels (0, 25, 50 and 75 µl/75ml buffered rumen fluid). The results showed that the Citrus limonum, Mentha arvensis and Allium sativum essential oils were decreased significantly cumulative gas production (GP), while Citrus sinensis essential oil had no significant effect on cumulative GP. The Citrus limonum and Mentha arvensis essential oil decreased ($P<0.01$) methane emission by 40 and 38%, respectively, while the third dose of Mentha arvensis and all doses of Allium sativum essential oil supplementation completely inhibited methane emission compared to the control diet. The reduction in methane emission was accompanied with decreases in true degradation of dry and organic matter, total protozoa count, short chain fatty acids but improved the partitioning factor (index of the microbial protein synthesis efficiency) and no significant differences in NH_3-N concentrations compared to the control diet. It is concluded that Citrus limonum essential oil had potential effects as antimethanogenic and antiprotozoal without detrimental effects on rumen degradation and fermentation in vitro.

Keywords: Gas production, methane, protozoa, degradation, ammonia

INTRODUCTION

Globally, ruminant livestock produce approximately 80 million t of CH_4 annually, accounting for approximately 28% of anthropogenic emissions (Beauchemin *et al*., 2008). Methane is an important greenhouse gas, and its release into the atmosphere is directly linked with animal agriculture, particularly ruminant production. Production of CH_4 has long been regarded as an inefficiency in the ruminant digestive process, representing a loss of ingested gross energy of between 2 and 15% (Johnson *et al*., 2007). There are many attempts to reduce methanogenesis by addition of chemicals such as halogen compounds and inonophores. Animal nutritionists are actively seeking alternatives to antibiotic additives and growth promotants because the use of these compounds has become increasingly controversial (Parveen *et al*., 2006).

Essential oils (EO) are being promoted as natural feed additives, which modulate rumen fermentation to improve nutrient utilization, animal performance and depress methane emission. Studies have shown that some EO 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 (Dorman and Deans 2000; Benchaar *et al*., 2008). There are some recent reviews (e.g., Calsamiglia *et al*., 2007; Benchaar *et al*. 2008) where different EO compounds, their possible modes of action and their effects on rumen fermentation and methane emission have been described. In addition, essential oils from a variety of sources have been shown to inhibit methane production, alter the bacterial growth and modify the profiles of rumen fermentation (Evans and Martin 2000, Wallace 2004, Busquet *et al*. 2005a,b, Garcia-Gonzalez *et al*., 2005, Fraser *et al*., 2007). The objective of this study was to evaluate the inclusion effects of four commercial Brazilian essential oils by four concentrations on the fermentation patterns and methane production *in vitro*.

MATERIALS AND METHODS

This study was conducted at the Centre for Nuclear Energy in Agriculture (CENA), University of São Paulo (USP), Sao Paulo, Brazil. It was utilized the *in vitro* gas production technique described by Theodorou *et al*. (1994) and adapted to the semi automatic system of Mauricio *et al*. (1999), using a pressure transducer and a data logger (Pressure Press Data 800, LANA, CENA–USP, Piracicaba, Brazil).

Essential oils:

Four commercial essential oils were investigated *in vitro*. The used essential oils were orange essential oil (*Citrus sinensis*), lemon essential oil (*Citrus limonum*), mint essential oil (*Mentha arvensis*) and garlic essential oil (*Allium sativum L.*). The essential oils were obtained from the Química Bpar Ltda., Sao Paulo, Brazil. For biological assays, four concentrations (0, 25, 50 and 75 µl per 75ml buffered rumen fluid plus 500 mg of substrate) of each EO were investigated as follow: No additive (control diet), orange essential oil (*Citrus sinensis*, CrS_{25}, CrS_{50}, CrS_{75}), lemon essential oil (*Citrus limonum*, CrL_{25}, CrL_{50}, CrL_{75}), *Mentha arvensis* (MeA_{25}, MeA_{50}, MeA_{75}) and *Allium sativum L.* (AS_{25}, AS_{50}, AS_{75}). Each concentration was added to the bottles which contain 75ml buffered rumen fluid and 500mg of the substrate.

Description of substrate:

The substrate used was 50:50 concentrate:forage diet (50.0% Tifton-85 (*Cynodon sp*) hay, 32.7% ground corn, 15.0% soybean meal, 1.0% limestone, and 1.3% mineral premix) on dry matter (DM) basis; 92.4% DM, 13.1% crude protein (CP), 2.0% ether extract (EE), 4.3% ash, 71.8% neutral detergent fiber (NDF), and 34.3% acid detergent fiber (ADF). The substrate was ground by using a Wiley mill (Marconi, Piracicaba, SP, Brazil) to pass a 1-mm screen. The DM was determined by oven drying at 105°C for 24 h, and organic matter (OM) after ashing at 550°C for 4 h (AOAC, 1990). Ether extract was also determined according to AOAC (1990). The CP (N × 6.25) was determined by using a Leco FP528 (Leco Corporation, St. Joseph, MI, USA) combustion nitrogen analyzer (AOAC, 1997). Concentrations of dietary NDF and ADF were ash-corrected and determined by the non-sequential method

using beakers according to Van Soest *et al*. (1991) and Goering and Van Soest (1970), respectively. The NDF analysis was performed with the addition of heat stable α-amylase (Ankom Technology, Tecnoglobo Equipamentos, Curitiba, Brazil) and sodium sulfite.

Inoculum donors and preparation:

Five adult rumen cannulated Santa Inês wethers (50 kg of BW) grazing tropical grass pasture and a supplement based on ground corn 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 contents were collected separately before morning feeding through the cannula using a stainless steel probe (2.5 mm screen) attached to a large capacity syringe. Liquids and solids were placed in pre-warmed (39ºC) insulated flasks and transported under anaerobic conditions to the laboratory. Pooled rumen contents (50:50 v/v) were squeezed through four layers of cheese-cloth and kept in a water bath at 39ºC with CO_2 saturation until inoculation took place.

In vitro gas production:

The *in vitro* gas production (GP) assay was carried out as described by Theodorou *et al*. (1994) and adapted to the semi automatic system of Mauricio *et al*. (1999), using a pressure transducer and a data logger (Pressure Press Data 800, LANA, CENA–USP, Piracicaba, Brazil) in 160 ml serum bottles incubated at 39 ºC for 24 h.

Ground substrate (0.5 g as-fed) were incubated in 75 ml of diluted rumen fluid (25 ml mixed rumen fluid + 50 ml of Menke's buffered medium) in serum bottles (Longo *et al*., 2006). Once filled all the bottles were closed with rubber stoppers shaken and placed in the incubator at 39 °C. The bottles were shaken manually after the recording of the gas headspace pressure at 12 and 24 h incubation using a pressure transducer (Theodorou *et al*., 1994). The amount of GP at each measuring time was calculated according to the regression equation obtained in our system and conditions from unpublished data on 500 samples between gas volume versus pressure: GP (ml) = 0.0112 psi^2 + 7.3358 psi (r^2=0.98).

Three GP runs were performed. Bottles of each run included, four bottles contain only buffered rumen fluid without substrate (blank), four bottles for substrate without additive (control), four bottles for each concentration of EO and four bottles for Tifton hay as an internal standard to correct the variation between runs. The gas values were expressed as ml per g of incubated DM.

Methane emission and analyses:

The gas samples were collected by syringe from the bottles (5.0 ml each time and accumulated in vacutainer tubes) twice at 12 and 24 h incubation for methane analyses. 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 10.0 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 "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 headspace

volume (ml) of the serum bottle and Conc is the percentage of methane in the gas sample analyzed (Tavendale *et al*., 2005).

Degradation partitioning factor ammonia-N and protozoa count:

After termination of the incubation at 24h two bottles content were used for determination of true digestibility of dry and organic matter (TDDM, TDOM) and partitioning factor (PF) as an index of microbial protein efficiency. The bottles content were quantitatively transferred into a 600 ml spout-less beaker with a total of 70 ml of ND solution (double strength Blummel & Becker 1997) and refluxed for 3 h at 105^{o}C. Residual DM and ash were determined. The partitioning factor (PF) is the ratio between mg of organic matter truly degraded and gas volume (ml) at 24 h incubation (Blummel and Becker, 1997; and Blummel *et al*., 1997). Another two bottles content 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). Short chain fatty acids (SCFA) were calculated according to the Getachew *et al*. (2002).

Statistical analysis:

The data were analyzed using generalized linear model ANOVA procedures. Doses of essential oils were the source of variation, and linear, quadratic and cubic effects of increasing dosage of essential oils were determined using polynomial contrasts. The statistical significance was expressed in the table as linear (L), cubic (C) and quadratic (Q) effects, respectively (SAS, 2002).

RESULTS

The effects of inclusion different levels (0, 25, 50 and 75 µl/75ml buffered rumen fluid plus 500 mg total mixed ration) of Brazilian essential oils on gas and methane production *in vitro* for 24h incubation are given in Table 1. The results showed that the incremental addition of *Citrus sinensis* EO had no significant effect on cumulative gas production (GP) and CH_4 emission when expressed on the basis of milliliter per gram of truly digested organic matter, while the reduction in CH_4 emission as a milliliter per gram of dry matter was ($P<0.05$) quadratic effect only. Cumulative GP and methane emission declined significantly (linear, cubic or quadratic) with the increasing dose of *Citrus limonum*, *Allium sativum* and *Mentha arvensis* EO. All the investigated doses from *Allium sativum* EO and third dose of *Mentha arvensis* EO were completely inhibited CH_4 emission either expressed as milliliter per g incubated dry matter or milliliter per g truly digested organic matter compared to the control diet without EO.

The effect of EO supplementation on pH, true degradation 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) concentration are presented in Table 2. The results indicated that the inclusion of *Mentha arvensis* EO increased significantly the mean values of the pH, while *Citrus sinensis*, *Citrus limonum* or *Allium sativum* EO did not affect significantly on the mean value of pH. The results of true degradation showed variable responses to dose supplementation of EO. True degradation of dry and organic matter (g/kg DM) was depressed significantly by supplementation of the *Mentha arvensis* EO. The reduction significance in TDDM was not linear or quadratic effect but it was cubic effect when *Citrus sinensis* EO was

supplemented. While, *Citrus limonum* EO reduced (P<0.05) TDDM by quadratic and cubic effect not linear response. On the other hand, the results showed a linear significant decline in TDDM when *Allium sativum* EO was supplemented to the control diet. Although, inclusion of *Citrus limonum* and *Citrus sinensis* EO decreased true degradation of organic matter (TDOM) but the responses were not significant, while the *Allium sativum* EO supplementation was linearly depressed (P<0.05) the TDOM. All investigated EO's by various doses had no significant effect on mean values of partitioning factor (PF) or ammonia-N concentration. The significance of effect on protozoa count was declined linearly when *Citrus sinensis* or *Mentha arvensis* EO was supplemented, while *Allium sativum* EO supplementation had not significant effect on protozoa number. There was a dose dependent inhibition of protozoa number by inclusion of *Citrus limonum* EO. The results of the short chain fatty acids (SCFA) showed that *Citrus limonum*, *Mentha arvensis* or *Allium sativum* EO supplementation were lowered signicantly SCFA concentrations, while inclusion of *Citrus sinensis* EO had no (*P*> 0.05) effect on the SCFA concentrations.

Table 1. Effect of different levels of essential oils on gas and methane production *in vitro* for 24 h incubation

Treatments	Levels	GP (ml/g DM)	CH_4 (ml/g DM)	CH_4 (ml/g TDOM)
Control	**No additive**	127.7	11.2	21.2
	CrS_{25}	115.5	11.6	24.8
Citrus sinensis	**CrS_{50}**	102.8	8.2	19.9
	CrS_{75}	88.9	6.9	13.2
	L	**NS**	**NS**	**NS**
Significance of effect	**Q**	**NS**	*****	**NS**
	C	**NS**	**NS**	**NS**
	CrL_{25}	119.8	12.6	30.3
Citrus limonum	**CrL_{50}**	93.3	7.7	18.8
	CrL_{75}	79.4	6.7	15.5
	L	*******	******	********
Significance of effect	**Q**	*****	*******	********
	C	*****	******	********
	MeA_{25}	131.0	12.6	30.6
Mentha arvensis	**MeA_{50}**	80.1	4.3	10.0
	MeA_{75}	51.0	0	0.4
	L	****	***	**
Significance of effect	**Q**	****	****	**
	C	***	***	**
	AS_{25}	81.7	0	0
Allium sativum	**AS_{50}**	59.6	0	0
	AS_{75}	57.7	0	0
	L	******	********	********
Significance of effect	**Q**	*****	********	********
	C	*****	********	********
SEM[1]		7.8	1.1	3.8

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
[1] SEM: standard error of means; NS: not significant

Table 2. Effect of different levels of the investigated essential oils on degradation and rumen fermentation *in vitro*

Treatments	Levels	pH	TDDM	TDOM	PF	protozoa	NH_3_N	SCFA
Control diet	**0**	6.68	544	558	4.00	4.39	**22.9**	56.7
Citrus sinensis	**CrS_{25}**	6.71	500	515	3.90	2.93	24.3	51.6
	CrS_{50}	6.73	440	454	4.11	1.99	24.2	45.7
	CrS_{75}	6.76	435	483	4.85	1.60	26.3	39.5
Significance of effect	**L**	**NS**	**NS**	**NS**	**NS**	*	**NS**	**NS**
	Q	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
	C	**NS**	*	**NS**	**NS**	**NS**	**NS**	**NS**
Citrus limonum	**CrL_{25}**	6.70	510	521	3.94	2.25	24.3	53.2
	CrL_{50}	6.77	409	450	4.29	2.05	23.7	41.4
	CrL_{75}	6.79	393	405	4.64	1.28	25.9	35.3
Significance of effect	**L**	**NS**	**NS**	**NS**	**NS**	****	**NS**	***
	Q	**NS**	*	**NS**	**NS**	***	**NS**	*
	C	**NS**	*	**NS**	**NS**	***	**NS**	*
Mentha arvensis	**MeA_{25}**	6.70	512	525	3.63	2.78	23.7	58.2
	MeA_{50}	6.82	357	374	4.28	1.46	27.1	35.6
	MeA_{75}	6.86	339	354	6.35	1.39	26.4	22.6
Significance of effect	**L**	*	***	***	**NS**	*	**NS**	****
	Q	**	****	****	**NS**	**NS**	**NS**	****
	C	**	****	****	**NS**	**NS**	**NS**	***
Allium sativum	**AS_{25}**	6.69	458	470	5.34	3.90	26.1	36.3
	AS_{50}	6.73	412	435	5.74	3.15	23.4	28.9
	AS_{75}	6.75	357	374	5.84	2.65	262	25.6
Significance of effect	**L**	**NS**	*	**	**NS**	**NS**	**NS**	**
	Q	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	*
	C	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	*
SEM[1]	-	0.018	22	24	0.41	0.28	2.7	1.9

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
TDDM: True digestibility of dry matter (g/kg DM), TDOM: True digestibility of organic matter (g/kg DM), PF: partitioning factor (mg of truly digested organic matter /ml gas at 24 h), NH_3-N: Ammonia nitrogen concentration (mg/l), Protozoa count ($x10^5$ ml^{-1}), SCFA: Short chain fatty acids (mM), [1]SEM: standard error of means; NS: not significant

DISCUSSION

Essential oils are complex mixtures of secondary metabolites and volatile compounds extracted from plants through distillation methods. 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). The main components of the investigated essential oils were limonene, menthol and diallyl disulfide and allicin in citrus sp, mint and garlic essential oils, respectively. These components were responsible on the antimicrobial

activity of these essential oils (Cowan, 1999; Benchar *et al*., 2008; and Patra and Saxena, 2010). The active components of the mint are differ according to the species e.g. piperitone oxide (46.7%) and cis-piperitone oxide (28%) in *Mentha microphylla*, 1,8-cineole (29%) in *Mentha longifolia*, piperitenone oxide and 1,8-cineole in *Mentha spicata*, menthone (23%) and menthol (40%) in *Mentha piperita*, menthone (20%) and menthol (41%) in *Mentha canadensis* (Cooke *et al*. 2007; Jirovetz *et al*. 2009). Effects of EO on microbial fermentation were considered positive when total VFA concentration and propionate proportion increased, when the acetate proportion or acetate to propionate ratio decreased, and/or when ammonia N concentration decreased (Castillejos *et al*. 2008). The limited number of EO and EO compounds evaluated to date show some promise in this regard. The varied response among EO products evidently reflects differences in chemical structure, which influences their effects on microbial activity.

The antimicrobial activity of EO has prompted interest in whether these compounds could be used to inhibit methanogenesis in the rumen. The challenge is to identify EO that reduces methane production without a concomitant reduction in feed digestion. Chiquette and Benchaar (2005) showed inhibitory effects of garlic EO on the production of methane *in vitro*. Kamra *et al*. (2005 and 2008) investigated methanol and ethanol extracts of various spices, including fennel, clove, garlic, onion, and ginger for effects on methane production *in vitro*. Among the extracts tested, methanol extract of garlic was the most effective suppressant of methane, with 64% reduction *in vitro* and no adverse effects on feed digestibility. Similarly, Busquet *et al*. (2005a) reported that garlic oil (312 mg/l) decreased methane production *in vitro*. Effects of garlic oil and its main components, Busquet *et al*. (2005b) observed, in batch culture, that garlic oil and diallyl disulfide (300 mg/l of ruminal fluid) reduced methane production by 74 and 69% respectively, without altering digestibility. This depression in methane emission could be due to the direct inhibition of rumen methanogenic archaea. In agreement with our data, Patra *et al*. (2005) reported that ethanol and methanol extracts of cloves and the methanol extract of fennel also inhibited methane production *in vitro*, but digestibility of the feed was also reduced. Ando *et al*. (2003) reported that feeding 200 g/day (*i.e.*, 30 g/kg of total dietary DM) of peppermint (*Mentha piperita* L.) to Holstein steers decreased the total number of protozoa. Agarwal *et al*. (2009) reported that methane emission was reduced ($P<0.001$) by 19.9%, 46.0% and 75.6% at 0.33, 1.0 and 2.0 µl/ml of incubation medium of *Mentha piperita* essential oil, respectively. The mitigation of methane emission was accompanied with inhibiting protozoal numbers, which would be expected to decrease methane production because ruminal protozoa provide a habitat for methanogens that live on and within them. However, the antiprotozoal effects of EO have been inconsistent and variable among EO and EO active components.

A depression in feed degradability by higher doses of tested essential oils could be due to their active compounds. 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. We may hypothesize that the fermentation pattern observed in EO is mediated through a stronger inhibition of the gram negative rumen bacteria. Several studies observed that the addition of blended EO decreased the effective degradability and

the rate of ruminal degradation of some protein supplements (Molero *et al*. 2004; Newbold *et al*. 2004). Partitioning factor is defined as the ratio of organic matter truly degraded (mg) to the volume of gas (ml) produced by it. Blummel *et al*. (1997a&b) and Al-Masri *et al*. (2003) reported that there was a negative correlation between GP and microbial protein synthesis. A higher PF as noticed with the supplementation of higher doses of *Mentha arvensis, Citrus sinensis, Citrus limonum* or all doses of *Allium sativum* EO means that proportionally more of the degraded organic matter was incorporated into microbial mass, i.e. the efficiency of microbial protein synthesis as higher. The results showed that higher PF was accompanied with lower of CH_4 output and protozoa count as reported by Blummel *et al*. 2003. Ushida *et al*. (1991) reported that there was a reduction in microbial yield when a large number of protozoa were present in the fermentation media, owning to the turnover of the bacterial cells utilized as sources of protein for the protozoa.

Recently, a number of *in vitro* studies have 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 AA and the number of hyper-ammonia-producing bacteria in 48-h *in vitro* batch cultures. In agreement with our finding, using dual-flow continuous culture fermenters for 8 days of incubation, and maintained at constant pH, Castillejos *et al*. (2005) observed that addition of mixture of EO (MEO) at 1.5 mg/l had no effect on NH_3–N concentration. The lack of effect of MEO on N metabolism was attributed to the dose of 1.5 mg/l, which may have been too low to alter activity of ruminal bacteria. However, when Castillejos *et al*. (2007) used the same MEO at higher concentrations (*i.e.*, 5, 50, and 500 mg/l) there was still no effect of MEO on ruminal concentrations of NH_3–N in continuous fermenters for 9 days of incubation maintained at constant pH. McIntosh *et al*. (2003) and Newbold *et al*. (2006) suggested that concentrations of MEO above 35 mg/l would be required to substantively alter N metabolism in the rumen, a level that may be difficult to achieve *in vivo*. Indeed, Benchaar *et al*. (2007) observed no change in ruminal NH_3–N concentration and N digestibility when lactating dairy cows were supplemented with MEO at doses of 0.75 or 2 g/day. However, McIntosh *et al*. (2003) speculated that local concentrations of EO, some of which are often sparingly soluble, may be higher on the surface of ingested plant materials, which may increase the bactericidal effects of EO *in vivo*.

The reduction in SCFA concentrations at the current study were result of the antimicrobial effects of EO and may be dose dependent. Busquet *et al*. (2006) studied effects of various plant extracts (*i.e.*, anise oil, cade oil, capsicum oil, cinnamon oil, clove, bud oil, dill oil, fenugreek, garlic oil, ginger oil, oregano, oil, tea tree oil, and yucca), and secondary plant metabolites (*i.e.*, anethol, benzyl salicylate, carvacrol, carvone, cinnamaldehyde, and eugenol) on ruminal fermentation in a 24 h batch culture. Each treatment was supplied at varying doses up to 3 g/l of culture fluid. None of the EO or EO compounds increased total VFA concentration but, at the highest concentration, most treatments decreased total VFA concentration, a possible reflection of decreased feed digestion. Similar effects were reported by Castillejos *et al*. (2006) for eugenol, guaiacol, limonene, thymol, and vanillin using doses up to 5 g/l. These EO compounds generally had no effect on total VFA concentration, with

the exception of the highest dose, which decreased total VFA concentration in cultures for all compounds.

CONCLUSION

It is concluded that the inhibition of methanogenesis and protozoa number by *Citrus limonum* EO had no adverse effects on *in vitro* feed degradability of organic matter, pH, PF and NH_3-N concentration. On the other hand, the methane mitigation by *Mentha arvensis* or *Allium sativum* EO was accompanied with a depression in *in vitro* feed degradability.

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التأثير المضاد لبعض الزيوت المتطايرة علي البكتريا المنتجة للميثان والبروتوزوا معمليا

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تم تجميع سائل الكرش الغني بالأحياء الدقيقة المرتبطة بجزيئات العلف قبل التغذية من كباش السانتانيز البرازيلية المفستلة في الكرش والتي كانت ترعي علي نباتات المناطق الحارة بالإضافة إلي إضافة الذرة المطحون وفول الصويا وذلك لتقييم تأثير إضافة بعض الزيوت المتطايرة التجارية بمستويات مختلفة علي تخمرات الكرش وإنتاج الميثان معملياً باستخدام تقنية إنتاج الغاز شبه الألي. الزيوت المتطايرةالمختبرة هي زيت البرتقال والليمون والنعناع والثوم وأضيفت إلى عليقة مكونة من ٥٠% مخلوط مركز و٥٠% علف مالئ بأربعة مستويات صفر, ٢٥, ٥٠ , ٧٥ ميكروليتر لكل ٧٥ مل من سائل الكرش والمحلول المنظم للحموضة.

أوضحت النتائج إن إنتاج الغاز المتراكم إنخفض معنوياُ مع إستخدام الزيوت المتطايرة لكل من الليمون والنعناع والثوم بينما لم يتأثر إنتاج الغاز المتراكم معنوياً مع إستخدام زيت البرتقال. إستخدام زيت الليمون والنعناع أدي الي إنخفاض غاز الميثان (٤٠، ٣٨% علي الترتيب)، بينما الجرعة الثالثة من زيت النعناع وكل الجرعات من زيت الثوم ثبطت إنتاج الميثان كلياً بالمقارنه بالعليقة الكنترول. إنخفاض غاز الميثان كان مصحوب بإنخفاض في التحلل الحقيقي للمادة الجافة والعضوية والعدد الكلي للبروتوزوا والاحماض الدهنية قصيرة السلسلة لكنها حسنت من Partitioning factor (وهو دليل علي كفاءة تخليق البروتين الميكروبي) ولم توجد إختلافات معنوية في تركيزات الأمونيا بالمقارنه بالعليقة الكنترول. وتشيرهذه الدراسة إلي أن زيت الليمون لة تأثيرات قوية كمادة مضادة لنمو البكتريا المنتجة للميثان والبروتوزوا وبدون تأثيرات ضارة علي درجة هضم المادة الجافة والعضوية وتخمرات الكرش معملياً.