

## Dose Response to Carvone Rich Essential Oils of Spearmint (*Mentha spicata* L.): *in Vitro* Ruminal Fermentation Kinetics and Digestibility

M. Taghavi Nezhad<sup>1</sup>, D. Alipour<sup>1\*</sup>, M. Torabi Goudarzi<sup>2</sup>, P. Zamani<sup>1</sup>, and G. Khodakaramian<sup>3</sup>

### ABSTRACT

The aim of this study was to assess the effect of several doses of spearmint essential oil (SEO; 0, 250, 500, 750 or 1,000  $\mu\text{g ml}^{-1}$  buffered rumen fluid) on the fermentation kinetic and digestibility using *in vitro* gas production technique. A total mixed ration (30% roughage: 70% concentrate) was incubated with buffered rumen fluid. *In vitro* gas production, asymptotic gas production (A), rate of gas production ( $\mu$ ), partitioning factor (PF), microbial biomass (MB), ammonia concentration and digestibility were determined. Increasing the dose of SEO decreased the parameters A and  $\mu$ . Adding SEO, however, increased PF, ammonia concentration, apparent *in vitro* dry matter digestibility and true *in vitro* organic matter digestibility at the lower levels of SEO (250 and 500  $\mu\text{g ml}^{-1}$ ). But, at the level of 1,000  $\mu\text{g ml}^{-1}$ , a decrease was observed for these parameters. The increment in PF and digestibility illustrate that SEO has a potential to modulate the rumen fermentation, which may be beneficial (at low doses) for improving nutrient utilization.

**Keywords:** Carvone, Essential oil, *In vitro* gas production, Partitioning factor, Spearmint.

### INTRODUCTION

Access to cheap antimicrobial compounds has been associated with overuse of antibiotic, especially in the developing countries. Some of these antibiotics such as ionophore monensin are used extensively for their ability to improve efficiency of feed utilization. However, there is global concern to reduce feed antibiotics because of their relation to the increase in the number of bacteria that are resistant to antibiotics used by humans [26]. Thus, new commercial additives are required that offer more safety and also have the property of manipulating rumen fermentation. Essential oils (EO) have

antimicrobial properties that make them suitable alternatives to antibiotics. Recently, the *in vitro* experiments have demonstrated that EOs or their components could be used favorably to modulate rumen microbial activities [5, 22]. Some effects could be due to a decrease in the number of proteolytic bacteria or lower methanogenesis [12, 20]. Currently, it is necessary to evaluate the effects of various EOs from different plants on rumen parameters.

In ethnomedicine, spearmint (*Mentha spicata*) is traditionally used as medicinal plant in digestive disorders. Spearmint essential oils (SEO) have been proved to possess an inhibitory effect against some gram-positive and gram-negative pathogenic

<sup>1</sup> Department of Animal Science, Faculty of Agriculture, Bu-Ali Sina University, Hamadan, Islamic Republic of Iran.

\* Corresponding author, e-mail: alipourd@basu.ac.ir

<sup>2</sup> Jundishapur Research Center of Medicinal Plants, Kashan, Islamic Republic of Iran.

<sup>3</sup> Department of Plant protection, Faculty of Agriculture, Bu-Ali Sina University, Hamadan, Islamic Republic of Iran.



bacteria [24]. There are few experimental data on the effects of SEO on rumen fermentation kinetics and feed digestibility. Therefore, the objective of this study was to assess the effects of SEO on the fermentation kinetics, efficiency of microbial biomass synthesis, and feed digestibility using the *in vitro* gas production technique.

## MATERIALS AND METHODS

### Rumen Inoculum

Rumen liquor was taken from four ruminally fistulated mature Mehraban sheep (50±4.5 kg body weight) fed diet containing 70% alfalfa hay and 30% concentrate plus mineral and vitamin supplements. Rumen contents were collected from all parts of rumen before morning feeding. Pooled rumen contents were strained through four layers of cheese clothes into an insulated thermos and transferred immediately to laboratory. The strained rumen fluid was continuously purged with oxygen free CO<sub>2</sub> and kept at 39°C in a water bath before use in the *in vitro* incubations.

### Essential Oil and Fermentation Substrates

The essence of spearmint (*Mentha spicata*) standardized at 55% carvone was obtained from Barij Essence Pharmaceutical Company (Kashan, Iran). The essence tightly bottled was stored in refrigerator at 4°C until the beginning of the experiment.

The diet used as a fermentation substrate was the one typically fed to fattening lambs containing alfalfa hay (17%), wheat straw (12%), barley grain (61%), soybean meal (9%), and mineral and vitamin premix (1%). The chemical composition of substrate was OM, 93.48 g; CP, 12.89 g; NDF, 33.98 g; ether extract, 1.73 g; NFC, 44.88 g; metabolizable energy 15.07 MJ on kg DM basis. A representative sample of this diet

was oven-dried and ground through a 1 mm screen mill for use in the incubations.

### *In vitro* gas Production and Fermentation Parameters

The gas production was measured using 100 ml glass syringes according to Menke and Steingass [23]. In the first trial 200 mg substrate were weighed into the syringes that were subsequently filled with 7.5 ml strained rumen fluid and 22.5 ml phosphate-bicarbonate buffer kept at 39°C [23]. The gas volume was recorded at 2, 4, 6, 8, 12, 48, 72, 96 and 120 hours after incubation.

In the second trial, fermentation parameters and substrate digestibility were measured in syringes containing 500 mg substrates and inoculated 40 ml buffered rumen fluid. After 24 hours of incubation the volume of gas production was recorded and the syringes contents were filtered through pre-weighed polyester bags (40 µm pore size) to measure the apparent *in vitro* dry matter digestibility (AIVDMD). The pH was measured and immediately the filtrates were stored at -20°C until analyzed for ammonia. Bags containing digestion residues were oven-dried at 60 °C for 48 hours. Then, the dried bags were weighed and boiled in the neutral detergent solution for one hour [28]. For determining the true *in vitro* organic matter digestibility (TIVOMD), thoroughly washed bags were oven-dried again, as mentioned above, and weighed. The residuals in bags were precisely withdrawn and ashed at 600°C to calculate the disappeared organic matter. The ratio of fermented organic matter to feed organic matter was considered as TIVOMD. At the beginning of two trials, different levels of essential oils (0, 250, 500, 750 and 1,000 µg ml<sup>-1</sup>) were added to the syringes.

### Measurements and Calculations

In order to estimate the kinetic parameters of gas production, gas production data were

fitted using the exponential model proposed by France *et al.* [14] as follows:

$$G=A[1-e^{-\mu t}]$$

Where  $G$  (ml) denotes the cumulative gas production at time  $t$ ;  $A$  (ml) is the asymptotic gas production, and  $\mu$  ( $\text{h}^{-1}$ ) is the fractional rate of gas production.

The concentration of ammonia in filtrates was determined using phenol-hypochlorite [4]. The ratio of organic matter truly degraded (mg) to gas volume (ml) at 24 hours incubation was used as the partitioning factor [2]. The mass difference of original residue and NDS-boiled residue were taken as a rough estimate of microbial mass (MB).

### Statistical Analyses

The data were analyzed using generalized linear model ANOVA procedures (SAS, 8.1) and polynomial linear (L), quadratic (Q) and cubic (C) contrasts were used to test the effect of levels of SEO on parameters. All experiments were carried out with three replicates.

## RESULTS

### Kinetics of Gas Production

The effect of SEO on the gas production at different incubation times is presented in Figure 1. As the amount of SEO in the syringes was increased, the volume of gas decreased at all times of incubation. The addition of SEO led to lower asymptotic gas

productions (L and Q effects,  $P < 0.001$ ; C,  $P < 0.01$ , Table 1). A drastic drop in parameter  $A$  occurred between concentrations  $750 \mu\text{g ml}^{-1}$  and  $1000 \mu\text{g ml}^{-1}$ . The parameter  $\mu$  was influenced in a quadratic manner by SEO (Q,  $P < 0.001$ ). The highest decrease in  $\mu$  was observed in the ranges between  $500 \mu\text{g ml}^{-1}$  and  $750 \mu\text{g ml}^{-1}$ .

### Fermentation Parameters and digestibility

The effect of different levels of SEO on  $\text{GP}_{24}$ , pH, ammonia concentration, MB, PF, AIVDMD and TIVOMD is given in Table 2. The  $\text{GP}_{24}$  was decreased by different levels of SEO (L and Q,  $P < 0.001$ ). The minimum level for  $\text{GP}_{24}$  decrease was  $250 \mu\text{g ml}^{-1}$ . However, an increase in pH was observed with the increment levels of SEO (L,  $P < 0.001$ ; Q,  $P < 0.05$ ). The concentration of ammonia sharply increased in the ranges between  $250 \mu\text{g ml}^{-1}$  to  $750 \mu\text{g ml}^{-1}$  (L and Q,  $P < 0.001$ ) A significant quadratic effect of doses was observed for PF (Q,  $P < 0.001$ ). The MB was not affected by SEO levels. However, at the levels of  $500 \mu\text{g ml}^{-1}$  and  $250 \mu\text{g ml}^{-1}$  the highest values were seen for AIVDMD (L,  $P < 0.01$ ; Q,  $P < 0.001$ ) and TIVOMD (L,  $P < 0.05$ ; Q,  $P < 0.01$ ) respectively.

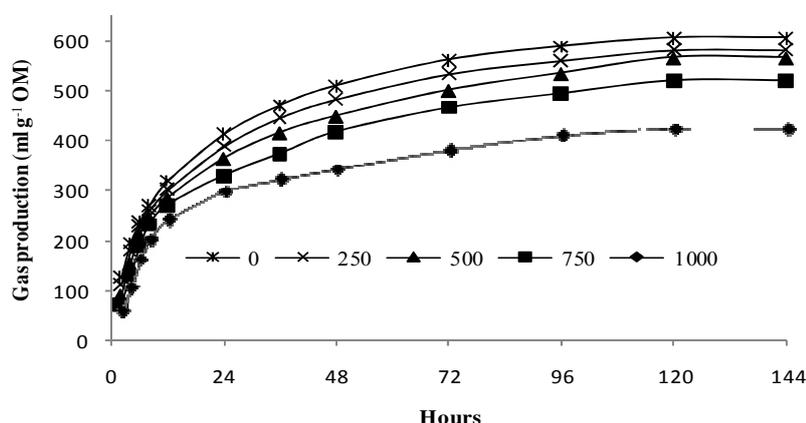
## DISCUSSION

In agreement with the results of this study, Macheboeuf *et al.* [21] found that using the

**Table 1.** Effects of different doses of spearmint essential oil (SEO) on the estimated parameters of *in vitro* gas production.

Parameters	Doses of SEO ( $\mu\text{g ml}^{-1}$ )					SEM <sup>c</sup>	Contrasts		
	0	250	500	750	1000		L <sup>d</sup>	Q <sup>e</sup>	C <sup>f</sup>
A <sup>a</sup>	570.4	546.1	524.7	484.0	396.8	11.41	***	***	*
$\mu$ <sup>b</sup>	0.071	0.070	0.063	0.062	0.074	0.003	NS <sup>g</sup>	***	*

<sup>a</sup> Asymptotic gas production(ml); <sup>b</sup> Fractional rate of gas production ( $\text{h}^{-1}$ ); <sup>c</sup> Standard error of the means; <sup>d</sup> Linear; <sup>e</sup> Quadratic; <sup>f</sup> Cubic; <sup>g</sup> Non-significant; \*  $P < 0.05$ ; \*\*\*  $P < 0.001$ .



**Figure 1.** Cumulative gas production curves for diets receiving different doses of SEO ( $\mu\text{g ml}^{-1}$ )

essence of *Anethum graveolens*, which contained a high percentage of carvone, reduced 24-hours gas production more than 50 percent. Gas production reflects fermentation of feed organic matter [16]. It is postulated that the decreasing feed fermentation results in lower gas production. In the case of this trial, the reduction of gas production was not consistent with the observed increment in digestibility. Reduction of  $\mu$  shows the lower rate of substrate degradation *in vitro*.

The  $\text{GP}_{24}$  also decreased with the increasing level of SEO. However, the pattern of reduction was not similar to the

pattern of Figure 1. This could be due to the basis at which doses were added to the fermentation medium. In both trials, doses were added based on the amount per ml of buffered rumen fluid. The feed to fluid ratio was 200/30 and 500/40 mg feed per ml of incubation medium in the first and second trials, respectively. This may be the reason for the different pattern obtained for gas volume reduction. Amount of substrate and volume of buffered rumen fluid in the syringes may be an important factor affecting gas production [25].

Consistently with this study, Macheboeuf *et al.* [21] found that adding EO of *Anethum*

**Table 2.** Effects of different doses of spearmint essential oil (SEO) on the fermentation parameters and digestibility.

Parameters	Doses of SEO ( $\mu\text{g ml}^{-1}$ )					SEM <sup>f</sup>	Contrasts		
	0	250	500	750	1000		L <sup>g</sup>	Q <sup>h</sup>	C <sup>i</sup>
GP <sub>24</sub> <sup>a</sup>	297.9	279.0	272.3	273.3	269.8	4.48	***	**	NS <sup>j</sup>
pH	6.58	6.76	6.78	6.80	6.82	0.125	***	*	NS
Ammonia (mmol)	5.88	6.08	6.81	7.38	6.36	0.193	***	***	***
PF <sup>b</sup>	2.37	2.70	2.67	2.65	2.44	0.165	NS	***	NS
MB <sup>c</sup>	73	82	78	74	68	6.1	NS	NS	NS
AIVMD <sup>d</sup>	521	563	590	535	487	9.1	**	***	NS
TIVMD <sup>e</sup>	676	721	694	693	630	9.7	*	**	NS

<sup>a</sup> Volume of gas after 24 hours of incubation ( $\text{ml g}^{-1}$  OM); <sup>b</sup> Partitioning factor; <sup>c</sup> Microbial biomass (mg) ; <sup>d</sup> apparent *in vitro* dry matter digestibility ( $\text{g kg}^{-1}$  DM); <sup>e</sup> True *in vitro* organic matter digestibility ( $\text{g kg}^{-1}$  OM); <sup>f</sup> Standard error of the means; <sup>g</sup> Linear; <sup>h</sup> Quadratic; <sup>i</sup> Cubic; <sup>j</sup> Non-significant; \* P< 0.05; \*\* P< 0.01, \*\*\* P< 0.001.

*graveolens* increased final pH in 16-hours *in vitro* batch culture incubations. A similar effect on pH was also observed in the current study and by Busquet *et al.* [5] who reported that 3  $\mu\text{g ml}^{-1}$  carvone increased pH in 24-hours batch culture fermentation. In this study, the amount of the total volatile fatty acids (TVFAs) was not measured. However, increased pH was likely due to the reduction of TVFAs [29].

In some previous studies [1, 15], the increased pH was associated with a reduction in TVFAs, reflecting a decrease in diet fermentability. However, in this study addition of SEO at moderate levels had led to an increased feed utilization as evidenced by increased PF, MB (slightly) and *in vitro* digestibility. The PF is considered an index of efficiency of microbial biomass synthesis [2]. Proportional to the amount of substrate degraded, lower gas production (high PF) is an indicator of greater microbial biomass synthesis [3]. At the dose of 250  $\mu\text{g ml}^{-1}$ , higher PF was observed, probably a greater amount of digested organic matter were directed towards the growth of microbial cell rather than towards VFA production. This is partly indicated by a slight increase of MB. An increase in MB contradicts antimicrobial activity of the EOs. A number of EOs has antimicrobial effects in a dose dependant manner [21]. At different doses, they may possess specific inhibitory or stimulatory effects on microorganisms [17]. Some of EOs have an inhibitory effect on the growth of rumen protozoa [8], amino acid fermenting bacteria [13] and methanogenic *Archaea* [6]. These could be an explanation for increased MB, which is the main determinant in PF [11, 18].

Despite many studies cited previously [1, 7, 21], inclusion of SEO led to an increase in ammonia concentration. In an *in vitro* batch fermentation, EO of *Anethum graveolens* decreased ammonia concentration [20]. Similar to our finding, Busquet *et al.* [5] reported that adding pure carvone increased ammonia concentration in fermentation media. Using Anethol (20  $\mu\text{g ml}^{-1}$ ), garlic oil (100  $\mu\text{g ml}^{-1}$ ), juniper berry oil (20  $\mu\text{g ml}^{-1}$ ) and *p*-

cymene (20  $\mu\text{g ml}^{-1}$ ) increased ammonia concentration in 6-h *in vitro* incubation of trypticase as substrate [10]. Increasing of ammonia concentration i.e., at levels of 250, 500 and 750  $\mu\text{g ml}^{-1}$ , could be due to (I) at the end of 24 hours fermentation, a substantial numbers of bacteria were in stationary phase, and it can be hypothesized that the increase in ammonia production was a result of increased bacterial lysis [21]. It has been reported that a blend of EO did not influence growth of pure culture of *Ruminobacter amylophilus*, but greatly affected its lysis in stationary phase [22, 30]. (II) SEO might have increased proteolytic activity of rumen microorganisms as previously described by Cardozo *et al.* [7]. (III) Combination of I and II. The protein metabolism by rumen microorganisms is a complex process and also the effect of EO on protein metabolism depends on the dose, the chemical composition of Eos, and the chemical structure of EO [9]. These factors vary considerably from study to study and make interpretation of the results difficult.

Decreased ammonia concentration at the level of 1,000  $\mu\text{g ml}^{-1}$  may be due to the inhibitory effect on proteolytic activity of microorganisms at this dose [10].

The influence of EO on digestibility varies among literature. In agreement with this study, Yang *et al.* [31] found that garlic oil and Juniper berry oil increased digestibility of OM and CP in dairy cows. They concluded that increased digestibility of CP led to higher OM digestibility. Kongum *et al.* [19] observed garlic oil and coconut oil improved true *in vitro* OM digestibility. They reported that the population of a group of cellulolytic bacteria i.e. *Ruminococcus albus*, increased. It could be speculated that SEO has an inhibitory effects, which are specific, and not general on all microbes at the moderate doses i.e., 250 and 500  $\mu\text{g ml}^{-1}$ . However, it seems that at the highest dose (1,000  $\mu\text{g ml}^{-1}$ ), SEO has a general and non-specific inhibitory effect on rumen microorganisms, which has diminished the measured parameters.

One issue sometimes linked to using the EOs in ruminant nutrition is a decrease in diet digestibility that could lower animal



performance. However, in this experiment, SEO had no negative effect on, and even increased, *in vitro* digestibility at levels of 250 and 500  $\mu\text{g ml}^{-1}$ . The main constituent of SEO in this study was carvone, which has lower activity than carvacrol and thymol due to lack of phenolic ring [27]. These two latter compounds have a strong antimicrobial activity against broad spectrum of microorganisms that may lower the overall diet fermentability. It would also be noteworthy to point out that spearmint contains the other main components such as limonene, linalool and 1,8- cineole [24]. These constituents have their own antimicrobial characteristics that are also important to modulate rumen fermentation and must be further investigated in the future.

### CONCLUSIONS

Based on the results of PF and *in vitro* digestibility, it seems that SEO at the doses of 250  $\mu\text{g ml}^{-1}$  and 500  $\mu\text{g ml}^{-1}$  improved dietary utilization. It must be noted that *in vitro* methods have some limitations for evaluating the biological effects of EOs. More investigation on the effects of SEO on the growth and enzymatic activity of pure cultures for specific groups of rumen bacteria i.e. fibrolytics, amylolytics, proteolytic and amino acid fermenting bacteria, is suggested. Finally, carrying out an *in vivo* experiment with emphasis on animal performance gives a better judgment about these essential oils.

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## اثر اسانس روغنی سرشار از کاروون بر کینتیک برون تنی تخمیر شکمبه‌ای و قابلیت هضم

م. تقوی نژاد، د. علیپور، م. ترابی گودرزی، پ. زمانی، و غ. خداکرمیان

### چکیده

هدف از انجام این پژوهش بررسی اثرات سطوح مختلف (صفر، ۲۵۰، ۵۰۰، ۷۵۰ و ۱۰۰۰ میکرو گرم در میلی‌لیتر مایع بافری شکمبه) اسانس روغنی نعنای اخضر (SEO) بر کینتیک تخمیر و قابلیت هضم با استفاده از آزمون تولید گاز بود. یک نمونه از جیره کامل مخلوط (۷۰ درصد کنسانتره و ۳۰ درصد علوفه) در مایع شکمبه و بافر انکوباسیون شد. مقدار تولید گاز، تولید گاز در نقطه مجانب (A)، سرعت تولید گاز ( $\mu$ )، عامل بخش پذیری (PF)، توده میکروبی (MB)، غلظت آمونیاک، قابلیت هضم ظاهری ماده خشک و قابلیت هضم حقیقی ماده آلی اندازه گیری شد. افزایش مقدار SEO فراسنجه‌های A و  $\mu$  را کاهش داد. در سطوح پایین تر (۲۵۰ و ۵۰۰ میکرو گرم در میلی‌لیتر) افزودن SEO مقدار PF، غلظت آمونیاک، قابلیت هضم ظاهری و قابلیت هضم حقیقی ماده آلی افزایش یافت. در سطح ۱۰۰۰ میکروگرم در میلی‌لیتر فراسنجه‌های فوق‌الذکر با کاهش مواجه شدند. افزایش مقدار PF و قابلیت هضم ظاهری و حقیقی نشان دهنده توانایی این اسانس برای تغییر در تخمیر شکمبه است که می‌تواند در سطح پایین استفاده از مواد مغذی را بهبود ببخشد.