The Effect of Essential Oils of Spearmint on the *in Vitro* Rumen Fermentation, Growth, and Deaminative Activity of Amino Acid Fermenting Bacteria

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Abstract

The aim of this study was to assess the effect of essential oils of spearmint (SEO) on the in vitro gas production (GP) kinetics, fiber digestibility and proteolysis by rumen microbial community using Menke syringes. Asymptotic GP, half time of GP and the sigmoidisity degree of the GP curve were determined using a typical growing lamb diet. Ammonia production and in vitro fiber digestibility in response to different doses SEO was measured. The effect of this oil on the deaminative activity of mixed rumen microbes and a recently isolated amino acid-fermenting bacterium were studied. SEO decreased asymptotic GP and increased degree of sigmoidicity. While In vitro fiber digestibility was negatively affected by SEO, the concentration of ammonia in fermentation syringes increased. The tested oil inhibited ammonia production and the specific rate of ammonia production by the isolate (Clostridium SPP. MT8). Similarly, such results were observed for ammonia production by mixed rumen microbes. Overall, SEO had an antimicrobial activity and anti-proteolysis effect, and therefore modulating effect on rumen fermentation.

Keywords: bacteria, deaminatvie activity, growth, in vitro, spearmint

Introduction

In ruminants, nitrogen retention is inefficient because of deamination of amino acids in the rumen, which leads to the excess of NH_3 in the blood stream that is converted to urea and lost. Antibiotic growth promoters (i.e., ionophores) have been successfully used to enhance N utilization efficiency in ruminants. These antibiotics inhibit the growth of a group of rumen bacteria called the ammonia hyper-producing bacteria (HAP), which have the capacity to produce ammonia despite their relatively low number compared to other predominant rumen bacteria (Russell *et al*, 1988). Recently, antibiotic use in animal ration has faced many criticisms due to the emergence of antibiotic resistance. An alternative to antibiotics is plant

natural compounds that can be used as feed additives. Essential oils (EO) are one of category of compounds, which have been considered for their potential to modulate the rumen fermentation. Spearmint (*Mentha spicata*) is a medicinal plant which has many uses in Iranian ethnomedicine. The antimicrobial effect of its essence against a broad spectrum of microorganisms has been documented (Mkaddem *et al*., 2009). However, little information is available about the effect of this EO on fermentation parameters of mixed rumen microorganisms and activity of ruminal amino acid-fermenting bacteria. Therefore, the aim of this study was to assess the effect of different doses of spearmint EO (SEO) on the fermentation parameters of mixed rumen microbes and the activity of an amino acid-fermenting bacterium isolated from the rumen of Mehraban sheep.

Materials and Methods

Rumen inoculum was taken from four ruminally fistulated mature Mehraban sheep fed a diet containing 70% alfalfa hay and 30% concentrate plus mineral and vitamin supplements. For kinetics measurement 200 mg substrates (i.e., atypical diet for growing lambs) were weighed into 100 ml glass syringes and subsequently filled with 30 ml buffered rumen fluid. Gas volume was recorded at 1 to 144 hours after incubation. The kinetic parameters were estimated using mono-phasic model (Groot *et al*., 1996) of $GP=A/(1+(B/t)^s)$ where GP is the cumulative gas production (ml/g incubated OM), A is the estimated asymptotic gas production (ml/g incubated OM), B is the time (h) after incubation at which half of the asymptotic gas production has been reached and S represents a constant that determines the sharpness of the switching characteristic of the profile. In a separate run of incubation fiber digestibility (IVFD) and ammonia were measured in syringes containing 500 mg of the substrates and inoculated with 40 ml of buffered rumen fluid according to Makkar (2010).

In another trial an amino acid fermenting bacteria was isolated from the rumen of Mehraban sheep and identified as described by Flythe and Andris (2009). The accession number of isolated bacteria (Clostridium SPP. MT8) was JN804563. Also, a suspension of mixed ruminal bacteria was provided (Flythe, 2009). The effect of essences on the growth of isolated bacteria was evaluated by measuring optical density (absorbance at 600 nm) of stationary phase cultures after 24 hours of incubation (39ºC). Ammonia production and protein of pure culture of bacteria were determined at 0 and 6 hours of incubation. Specific rate of ammonia production was calculated from these two values using the averages of 0 and 6 hours protein samples. The ammonia production by mixed rumen microbes were measured by differences between the values of 0 and 24 hours incubations. At the beginning of all trials, different dosed of SEO (0, 250, 500, 750 and 1000 mg/L) were added to the fermenting media. The data were analyzed using generalized linear model ANOVA

procedures (SAS, 8.1). For all analyses, specific orthogonal contrasts were used to test 1) control vs. the average of SEO doses and 2) linear (L), quadratic (Q) and cubic (C) effects of EO doses on parameters.

Results and Discussion

The effect of SEO on the fermentation parameters are shown in Table 1. Inclusion of SEO decreased asymptotic GP and controls were significantly different from treatments. Half-time was decreased with the increased concentration of SEO, while addition of SEO increased the switching factor. SEO had led to the increase in the ammonia concentration. However, IVFD were markedly decreased by inclusion of SEO especially at the highest doses. The decrease in GP in response to inclusion of SEO is indicative of their antimicrobial effects, which have been demonstrated previously (Taghavi-nezhad *et al*., 2011). The degree of sigmoidicity represents the possible lag process occurring at the early stages of incubation (Groot *et al*. 1996). Also, a more sigmoidal GP curve indicates that incubated substrates have lower nutritive value than the exponential mode (Groot *et al*., 1996) or an altered fermentation (France *et al*., 2000).

In general, lowered GP as a result of SEO shows that this EO have a general and dose dependant antimicrobial activity. Trends to sigmoidicity with addition of SEO point out a specific inhibitory effect against some microorganisms in addition to its general effect. The decrease in IVFD shows the negative effect of these essences on the degradation of organic matter and structural carbohydrates at high doses.

The effects of different doses of SEO are shown in Table 2. The growth of isolated bacterium was decreased by SEO. Also, ammonia production, microbial protein and specific rate of ammonia production were lowered by inclusion of SEO.

Table 1. The effect of different doses of SEO on the kinetics of *in vitro* ruminal fermentation

Parameters	SEO doses					Contrasts			
	0	250	500	750	1000	Control vs. SEO	L	Q	C
A	507.4	473.1	443.6	370.8	264.4	***	***	***	NS
B	12.3	10.1	11.0	9.2	5.4	***	***	***	*
S	1.16	1.18	1.2	1.3	1.9	***	***	***	*
Ammonia	5.88	6.08	6.81	7.38	6.36	***	***	***	***
IVFD	0.33	0.34	0.27	0.22	0.16	***	***	**	NS

A: asymptotic GP (ml/g OM); B: half time of asymptotic gas production (h); S: regulating the switching characteristics of the GP profiles; L: linear; Q: quadratic; C: cubic; * P<0.05; **: P<0.01 *** P<0.001; NS: non-significant; SEO: spearmint essential oils. IVFD: in vitro fiber digestibility.

Table 2. The effect of different doses of ZEO and SEO on the ammonia production (mmol), microbial protein (µg) and specific rate of ammonia production (mmol/mg of protein) by isolated bacterium

Parameters	SEO doses					Contrasts			
	0	250	500	750	1000	Control vs. SEO	L	Q	C
Growth of bacteria (O.D)	0.416	0.262	0.181	0.095	0.051	***	***	***	NS
Ammonia	6.7	5.1	2.7	1.98	2.84	***	***	**	NS
Microbial protein	504.4	281.1	334.6	317.7	303.1	***	**	*	*
Specific rate of NH_3 production	14.02	19.48	7.83	6.21	9.6	NS	*	NS	**

O.D: optical density after 24 hours of incubation; SEO: spearmint essential oils. L: linear; Q: quadratic; C: cubic; * P<0.05; **: P<0.01; *** P<0.001; NS: non-significant

Addition of SEO had a negative effect on the ammonia production by the mixed rumen bacteria suspension and at the highest doses of SEO (750 and 1000 µg/ml) small amounts of ammonia were produced (Figure).

The inhibitory effects of EO on amino acid fermenting bacteria have been cited previously (McIntoch *et al*., 2003). Carvone (the main constituent of spearmint) is suggested to exhibit its antimicrobial activity via disrupting metabolic energy status of cells (Burt, 2004). However, the small amount of ammonia production at the highest doses of SEM instead of complete inhibition of bacterial growth could be due to the facilitated diffusion and other transport mechanisms in absence of ion-motive forces (Flythe and Russel, 2005).

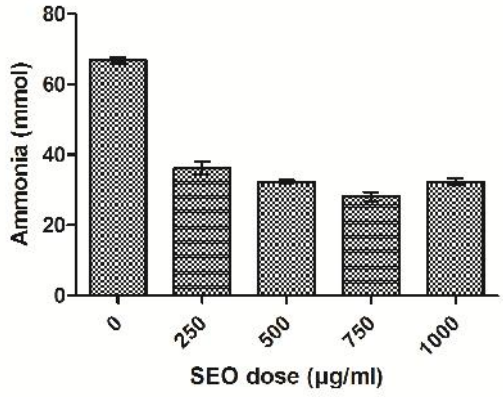

Figure 5. The effect of SEO on ammonia production by mixed rumen microbes

Conclusions

One important aspect of using phytochemicals in ruminants nutrition is the improvement of nitrogen utilization efficiency which can be achieved by lowering proteolysis and ammonia production in the rumen. Decreased ammonia production by mixed rumen microorganisms and a pure culture of a novel amino acid-fermenting bacterium showed that SEO has the potential to manipulate protein metabolism in the rumen. At the lower doses,SEO can modulate rumen fermentation without negatively affecting organic matter and fiber degradation. Further research is needed to elucidate *in vivo* effects of this EO, such as lactation and fattening performance of ruminants.

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