Effect of Essential Plant Oil Used as an Additive to Alter Silage Fermentation in Ruminant by *In Vitro*

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http://dx.doi.org/10.13005/bbra/2429

(Received: 15 February 2017; accepted: 01 March 2017)

The subject of this research was to evaluate the effect of adding different levels (0, 2ml) essential oils of thyme (Thymus) and Mentha piperita (Carvacrol) on alfalfa silage degradability by *in vitro* gas production and *in vitro* gas production kinetics. *In vitro* gas production values were determined by using rumen liquor from three lactation Holstein cows in times of 2, 4, 6, 8, 12, 16, 24, 48, 72 and 96 hours. The results of this paper report that, the effects of essential oils of thyme (Thymus) and Mentha piperita (Carvacrol) were significant. *In vitro* gas production was decreased by essential oils of thyme (Thymus) and Mentha piperita (Carvacrol). These essential oils and their different doses or combinations in alfalfa silage could be used to improve the performance of ruminants. Moreover, essential oils may act at different levels in energy and protein metabolic pathways, therefore their careful selection and combination may be a useful tool to effectively manipulate rumen fermentation.

Keywords: Essential Oils, Thyme, Mentha Piperita, *In Vitro*.

Whereas, Rumen is a diverse and unique microbial ecosystem composed of bacteria, protozoa and fungi. In rumen, hydrogen is produced during the anaerobic fermentation of nutrients. This hydrogen can be used during the synthesis of volatile fatty acids (VFAs) and microbial protein synthesis. The excess hydrogen from NADH is eliminated primarily by the formation of methane produced by methanogens. Also, Methane production in the ruminants is an energetically (2-15% of ingested GE) wasteful process, since the portion of animal's feed.

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Thus, for decades one of the goals of ruminant microbiologists and nutritionists has been to manipulate the ruminal microbial ecosystem to improve the efficiency of converting feeds to animal products edible by humans. The use of composition as feed additives as antibiotics; ionophore has proved to be a useful tool in reducing energy (methane) and nitrogen (ammonia) losses (McGuffey *et al*., 2001).

The use of antibiotics as feed additives and growth promoters in animal nutrition has been banned after January 2006 in the European Union due to the risk of antibiotics residues in animal products (milk and meat) and its subsequent effects on human health. For this reason, in the last few years nutritionists have become increasingly more interested in bioactive plant factors as a safe means

of ruminal fermentation modulators. Many plant extracts have antimicrobial activities against a wide range of rumen microorganisms (Chao *et al*., 2000; Voda *et al*., 2003; Burt, 2004).

Also, the possibility of using biologically active plant compounds for modulating changes in the rumen was first reported in 1911. In response to the requirements of animal production, the animal feed industry has marketed animal feed additives containing secondary plant metabolites. Many researchers have demonstrated potentially favorable effects of essential oils (Szumacher-Strabel and Cieœlak, 2010).

Essential oils are aromatic oily liquids extracted from plant material via expression, fermentation or commonly a distillation method. Contrary to their name, those aren't true oils (lipids) and are most commonly associated with the fragrance, the *Quinta essentia* of plants. Chemically, Essential oils are secondary metabolites composed primarily of isoprenes or terpenes ($C_{10}H_{16}$) and may contain mixtures of diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}), hemiterpenes (C_5), and sesquiterpenes (C_{15}). When isoprenes are associated with additional elements, usually oxygen, they are termed terpenoids (Cowan, 1999).

This composition has been shown to accumulate in cell membranes and disrupt their integrity, leading to leakage of enzymes and metabolites (Smid and Gorris, 1999).

Structure-function relationships associated with polar groups, number of double bonds, molecular size, and molecular solubility may have marked effects on their activities (Kamel, 2001).

Recent studies have shown that, plant secondary metabolites have antimicrobial properties against different types of microorganisms including bacteria, protozoa, and fungi (Greathead, 2003).

These compounds have been shown to modulate ruminal fermentation to improve nutrient utilization in ruminants (Wang *et al*., 1996; Hristov *et al*., 1999).

Plant Secondary Metabolites have been shown to suppress protozoa population, increase bacteria and fungi population, propionate production, partitioning factor, yield and efficiency of microbial protein synthesis and decrease methanogens hence improve performance in ruminants.

Plant secondary metabolites may have applications in ruminant nutrition because fermentations in the silo and rumen are dependent on microbial activities that may be affected by their use (Kung *et al*., 2008).

Similarly, the well documented antimicrobial activity of essential oils and their active components has prompted a number of scientists to examine the potential of these secondary metabolites to manipulate rumen microbial fermentation to improve production efficiency in ruminants. A number of *in vitro* studies have been recently published on the effects of Essential oils and their components on ruminal fermentation and metabolism (McIntosh *et al*., 2003; Busquet *et al*., 2006).

Results from those studies revealed variable effects of Essential oils and their derivatives on rumen bacteria and ruminal fermentation (Busquet *et al*., 2006; Castillejos *et al*., 2006). Discrepancies between studies were attributed to different types and doses of Essential oils (Busquet *et al*., 2006), but also to the *in vitro* technique used (Fraser *et al*., 2007).

The objective of this study was to examine *in vitro* the effects of two Essential oils (thyme and Mentha piperita oils) and Essential oils components (thymol and carvacrol) on rumen microbial fermentation by gas production technique.

MATERIALS AND METHODS

Animals and Mini Silo Experiments

The ruminal fluid was obtained from three ruminally fistulated, lactating Holstein cows (620 ± 8.9kg body weight; 45 ± 13d in milk) fed a total mixed ration (CP=18%, NDF= 33%) consisting of whole crop barley silage (46.6%), alfalfa hay silage (4.5%), dry ground corn (6.8%), steam rolled barley (17.6%), pelleted dairy supplement (24.5%).

The pellets contained [dry matter (DM) basis] ground barley grain (14.1%), ground corn grain (0.05%), heat-processed canola meal (20.8%), beet pulp (11.9%), heat-treated soybean meal (20.6%), corn gluten meal (17.0%), dry molasses (6.5%), limestone (1.7%), di calcium phosphate (2.7%), sodium bicarbonate (1.6%), and salt,

minerals, and vitamins mixture (2.7%). The diet was formulated to meet nutrient requirements of the cows (NRC, 2001) and was provided twice daily (9:00 and 16:00) ad libitum. At the laboratory, the ruminal fluid was filtered through four layers of cheesecloth into pre-warmed thermos flasks.

In 2014, Alfalfa forage was harvested at 28-30% of DM with a New Holland FP230 pull-type harvester (New Holland North America, New Holland, PA) with an on-board kernel processor.

Chop length was set to achieve a theoretical cut of 0.95cm. Three piles of chopped forage were treated with the following: 1) nothing, 2) 10mL of Thyme essential oil, 3) 10mL of Mentha piperita essential oil (carvacrol). Alfalfa was ensiled in 3 trials (500g of DM/kg) of October 1 to November 12, respectively. Silos were stored in the dark at ambient temperatures (20°C) and opened after 42 days of ensiling.

Aerobic Stability

After opening silos, the contents of each silo were thoroughly mixed and 1kg of silage samples were transferred into separate 1-L containers (3 containers per treatment). Each container was embedded with two Thermochron buttons (Embedded Data Systems, Lawrenceburg, KY) in the lower and mid layers of the silage mass to record the temperature every 15min. The containers were each covered with a double layer of cheesecloth and stored at ambient temperature (20 to 22°C) for 7d. ambient temperature was also simultaneously measured at 15min intervals during this period. Silage was sampled from each container after 1, 3 and 7d of aerobic exposure for chemical and microbial analysis, and for measurement of pH.

Chemical analysis

Dry matter (DM) was determined by drying samples at 105ºC for 24h, ash content, by ashing in a muffle furnace at 550ºC for 8h.

Nitrogen (N) content was estimated using the Kjeldahl method (AOAC, 1990).

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) determinations were based on the method of Van Soest *et al*. (1991) using an ANKOM fiber analyzer.

Non-Fibrous Carbohydrate (NFC) is calculated using the equation of (NRC, 2001), NFC =100 – (NDF + CP + EE + Ash).

Essential oils were bought from a commercial factory in Kashan Province. All chemical analyses were carried out in triplicate.

***In vitro* gas production technique**

The method of Menke *et al*. (1979) was used for the gas production procedure.

Approximately 200mg dry weights of samples (Alfalfa silage non thyme essential oil and Alfalfa silage with 2% of thyme essential oil) were weighed in triplicate into 100ml calibrated glass syringes following the procedures of Menke and Steingass (1988). The syringes were pre-warmed at 39ºC before the injection of 30 ml rumen fluid-buffer mixture (1:2) into each syringe and incubated in a water bath at 39ºC.

Preparation of artificial saliva was done according to the method of Menke and Steingass (1988). The artificial saliva containing buffer solution ($NaHCO_3$, 39g/l), macrominerals solution (Na_2HPO_4, 5.7g; KH_2PO_4, 6.2g and $MgSO_4.7H_2O$, 0.6g per 1L of distilled water), microminerals solution ($CaC_{l2}.2H_2O$, 13.2g; $MnC_{l2}.4H2O$, 10g; $CoC_{l2}.6H_2O$, 1g; $FeC_{l3}.6H_2O$, 8g per 100ml of distilled water) and potential redox indicator (resazurine, 0.1g/100ml) was prepared the day before incubation and stored at 39°C.

The reducing agent ($Na_2S.9H_2O$, 0.625g; NaOH 1N, 4ml; distilled water, 95ml) was then prepared the incubation of the samples. Artificial saliva and ruminal fluid were mixed in a 2:1 ratio. 200mg of vetch-oat hay (ground to pass a 1-mm screen) were weighed into 60ml syringes and incubated at 39°C with 30ml of inoculum. In each trial and for each level, three syringes were used. The syringes were gently shaken 30min after the start of incubation and every hour for the first 10h of incubation. Gas test was evaluated as the volume of gas in the calibrated syringes and was recorded before incubation 2, 4, 6, 8, 12, 16, 24, 48, 72 and 96 hours.

McIntosh *et al*., (2003) reported that essential oil levels lower than 35, 80 and 100ppm for various bacteria could not affect rumen fermentation.

Cardozo *et al*., (2005) also reported that, they studied five different doses (0, 0.3, 3, 30 and 300ppm) of essential oils in their experiment. To determine the effects of essential oils on *in vitro* gas production and gas production kinetics, doses of 0 (control), 50, 100 and 150ppm were used. Gas volumes were recorded at 3, 6, 9, 12, 24, 48, 72 and

96h of incubation. Five repetitions of each sample were used in the *in vitro* gas production experiment. Cumulative gas production data were fitted to the model of Orskov and McDonald (1979) as follows:

$Y = a + b (1-e^{-ct})$

Where;

a= the gas production from the immediately soluble fraction (ml/200mg DM),

b= the gas production from the insoluble fraction (ml/200mg DM),

c= the gas production rate constant for the insoluble fraction ((ml/h),

t= incubation time (hours),

(a+ b) = the potential extent of gas production (ml/ 200mg DM),

Y= gas produced at time "t".

Statistical analysis

Data on apparent gas production parameters were subjected to one-way analysis of variance using the analysis of variation model ANOVA of SAS (2006). Multiple comparison tests used Duncan's multiple- t-test (1980). Significance between individual means was identified using the Duncan's multiple range tests. Mean differences were considered significant at ($p<0.05$). Standard errors of means were calculated from the residual mean square in the analysis of variance. All data obtained from three replicates n = 3.

RESULTS

Chemical composition of used alfalfa silage is shown in Table1. There was considerable variation between forages in terms of chemical composition. The crude protein content of forages ranged from 20.05 to 20.21%. Silage A was very rich in crude protein and higher than that of the other silages. As can be seen from Table 1, there were significant differences between silages in terms of NDF and ADF.

The chemical composition of forage (alfalfa silage), concentrate and wheat straw of feedstuffs used for cows, from which rumen fluid was obtained, is presented in Table2.

The effect of incubating the materials *In Vitro* during 2, 4, 6, 8, 12, 24, 48, 72 and 96 hours by different dose of essential oils of thyme and Mentha piperita on gas production and the their parameters evaluated from gas test is shown in Table3.

The effects of dose of essential oils of thyme and Mentha piperita on *in vitro* gas production of alfalfa silage are presented in Table3. The cumulative volume of gas production increased with increasing time of incubation.

Gas produced after 96 h incubation ranged between 68.82 and 60.89 ml per 200 g of substrate. There were significant ($p<0.001$) differences between silages in terms of gas production at all incubation times except 2 h. Gas production at 4 h incubation of silages was significantly ($p<0.001$) higher than the others, possibly due to a higher water soluble carbohydrate content, but at 6 h incubation gas production of control silage was significantly ($p<0.001$) higher than other silages. At 24 h incubation, gas production of silage content thyme was significantly ($p<0.001$) higher than the other. At 72 h incubation, gas production values of silage no added essential oils were significantly ($p<0.001$) higher than the others.

All incubation times, showed that generally experimental silages produced less gas than control silage, since content silage had higher protein content than all other silages.

Protein in alfalfa silage is poorly utilized by ruminants, especially when high-forage diets are fed with relatively low available energy (Buxton, 1996).

Extensive degradation occurs during harvest and storage of silage (Albrecht and Muck, 1991) followed by further microbial degradation in the rumen (Buxton, 1996). Under *in vitro* conditions, gas is produced both directly as a result of fermentation (CO_2 and CH_4) and indirectly, from the acidifying effect of VFAs on CO_2 released from the bicarbonate buffer solution (Getachew *et al*., 1998).

The degradation of protein yields ammonia, which combines with H+ from the buffer to form NH_4^+, which remains in solution so inhibiting the indirect gas production. This may be one of the reasons why control silage had generally a lower gas production value than the other silages.

The main compounds of the essential oils were determined to be,%: carvacrol (0.03),

octadecadienoic acid methyl ester (51.04), hexadecanoic acid methyl ester (16.17), oleic acid methyl ester (23.13) in Nigella sativa; ã-terpinene (21.20), thymol (0.53) and carvacrol (57.01) in oregano; á-piene (10.03), â-myrcene (14.71), eugenol (0.02) and 1.8 cineol (41.52) in laurel; (69.57), acetaugenol (6.58) and cinnamyl acetate (3.83) in cinnamon; linoleic acid (34.75), diallyldisulphide (25.00), dially tetrasulphide (1.69), diallytrisulphide (19.48) and allylmethyltrisulphide (5.24) in garlic; elaidic acid methyl ester (18.08), cuminyl aldehyde (13.13), safrana (3.36) and hexadecanoic acid methyl ester (15.49) in cumin; and isoanethol (2.49), limoene (1.14), cuparene (3.37) and anethol (79.56) in anise.

The findings of the present study indicate that essential oils, and doses had significant effects ($P<0.01$) on gas production. Each essential oil, each incubation time and each feedstuff gave different results depending on the dose.

DISCUSSION

Cardozo *et al.*, (2004) reported that essential oils changed rumen VFA proportions and that the results obtained by adding garlic suggested that deamination was inhibited. In this study, essential oil decreased *in vitro* gas production of all feeds. Garlic-150 decreased *in vitro* gas production except for soyabean, in which

Table 1. Chemical composition of alfalfa and their silage (%)

Item	DM %	CP	NDF	ADF	pH
$Alfalfa_{(C)}$	29.11	20.05	34.2	26.31	5.72
$alfalfa_{(Thyme)}$	32.61	20.21	33.55	24.79	5.59
$alfalfa_{(M.piperita)}$	29.36	20.16	32.5	28.33	5.72

DM: dry matter, CP: crude protein, EE: ether extract, NDF: neutral detergent fiber, ADF: acid detergent fiber.

Table 2. Chemical compositions of the feedstuffs and forage and concentrate fed to animals, g/kg DM

Feeds	DM g/kg	CP	EE	NDF	ADF	Ash g/kgDM
Forage (alfalfa silage)	320.1	110.5	30.3	465.6	290.8	75.5
Concentrate	910.5	275.5	42.2	275.2	95.25	65.8
wheat straw	89.9	45.1	15.99	820.5	575.25	86.9

DM: dry matter, CP: crude protein, EE: ether extract, NDF: neutral detergent fiber, ADF: acid detergent fiber.

Table 3. Incubation different time of different treatments.

Traetment	Alfalfa Silage								
	2	4	6	8	12	24	48	72	96
Control	22.76^{b} ±1.392	31.87^{c} ±0.91	37.38^{c} ±1.069	43.03^{c} ±1.064	48.22^{c} ±0.645	56.4^{b} ±0.655	61.16^{b} ±0.576	64.02^{d} ±0.779	68.82^{c} ±0.947
Thyme10mL	24.51^{c} ±0.698	29.37^{c} ±0.693	34.92^{c} ±0.809	40.54^{b} ±0.5	44.27^{b} ±1.049	48.49^{a} ±1.264	52.5^{a} ±1.168	56.45^{b} ±1.355	65.93^{c} ±1.38
Mentha. p-10mL	20.00^{ab} ±0.694	26.71^{b} ±1.108	31.96^{b} ±0.739	37.78^{b} ±0.858	42.77^{b} ±0.881	46.66^{c} ±0.854	50.88^{c} ±0.559	55.48^{c} ±0.322	60.89^{c} ±0.531
Pr<F	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

it had no effect. These results are in agreement with the findings of Cardozo *et al*., (2004). Decreased *in vitro* gas production by essential oils may indicate more efficient utilization of energy due to the inhibited loss of energy as methane. An increasing trend for live weight gain in lamb fattening by using oregano, oregano oil, and a commercial essential oil mix can be attributed to this effect (Akkan *et al*., 2006).

In vitro gas production decreased for all feeds by increasing doses of essential oils in this study. Morover, essential oils showed the lowest *in vitro* gas production, which was similar to literature findings.

Benchaar *et al*., (2007) reported that *in vitro* gas production of carvacrol, thymol and eugenol decreased compared with controls. Those results are in agreement with the findings for oregano in this study. Oregano possesses more carvacrol and eugenol compounds respectively than other essential oils.

Therefore, garlic and oregano decreased *in vitro* gas production in barley. It is well known that there is a high correlation between *in vitro* gas production and both *in vivo* digestibility and/ or microbial growth (Menke *et al*., 1979). These results showed that essential oils could be used to improve digestion of slowly degradable starch, and to control degradation of highly degradable starch sources in the rumen to maintain ruminal pH within the physiological range.

Cardozo *et al*., (2004) reported that essential oils could inhibit deamination in the rumen. Similarly, lower gas production for soyabean meal with essential oils and higher gas production with essential oils may also suggest that protein degradation could be controlled by essential oils. Furthermore, essential oils might improve nitrogen utilization in the rumen if the diet was based on slowly degradable protein sources. *In vitro* gas production was decreased significantly by essential oils. Some of essential oils may be used to improve cellulose digestion and could be considered a feed additive.

Busquet *et al*., (2005) reported that *in vitro* gas production decreased as doses were increased. Patra *et al*., (2006) investigated the effects of water, methanol and ethanol extracts of garlic on rumen fermentation and methanogenesis. An aqueous garlic extract caused higher gas production and the ethanol and methanol extracts of garlic secondary metabolites appeared to have a potential to reduce rumen methanogenesis without adversely affecting rumen fermentation.

The effects of *Cordia verbenacea D.C.* essential oil on ruminal fermentation were determined by using the *in vitro* gas production technique (Araujo *et al*., 2010). Inclusion of essential oils inhibited methanogenesis when hay was used as the substrate, but this effect was not seen with concentrate. These results showed that essential oils from *Cordia verbenacea D.C.*was able to modify *in vitro* ruminal fermentation when hay was the substrate. In this study, GAR-150 decreased *in vitro* gas production compared with controls.

The effects of essential oils on rumen fermentation differ significantly. Carvacrol and thymol have strong antimicrobial activity against a wide range of gram-positive and -negative bacteria. Both are found in ORE (Sivropoulou *et al*., 1996).

Castillejos *et al*., (2006) reported that low doses of thymol (50 mg/l) had no effects on *in vitro* rumen microbial fermentation. But at higher doses of thymol or oregano total VFA decreased (Castillejos *et al*., 2006) and decreased total gas production (Akkan *et al*., 2006; Benchaar *et al*., 2007; Kamalak *et al*., 2011). Furthermore, several *in vitro* studies have suggested that the effects of thymol are diet and pH dependent (Cardozo *et al*., 2005; Castillejos *et al*., 2006).

Eugenol is one of the main active components of Cyanamid (accounts for up to 69.57%). The eugenol content in Cyanamid in this study is in agreement with the findings of Davidson and Naidu (2000), who suggested that, when used at optimal doses, the efficiency of energy and protein utilization in the rumen was improved. Eugenol may improve VFA production and profile, and N utilization in the rumen of lactating animals (Castillejos *et al*., 2006).

Different doses of Cyanamid essential oils led to similar *in vitro* gas production levels in barley and soyabean meal. *In vitro* gas production levels of wheat straw were found to be lower for CIN-150 dose at 12 h incubation. Cyanamid containing EUG might be used for improving rumen

fermentation in forage-based diets. Therefore, this result is in agreement with the findings of Castillejos *et al*., (2006).

A commercial blend of essential oil compounds, the major components of which are carvacrol, thymol, eugenol, vanillin and limonene, can be used to manipulate rumen fermentation (McIntosh *et al*., 2003; Benchaar *et al*., 2007). In this study, CUM showed the highest *in vitro* gas production compared with controls for all of the feeds. ORE-150 showed the lowest *in vitro* gas production for all of the feeds. Moreover, there were important and varied interactions of feeds, doses and incubation times, which are in agreement with the findings of McIntosh *et al*., (2003). Benchaar *et al*., (2007) reported that the changes in rumen fermentation caused by essential oil compounds (thymol, carvacrol and eugenol) may not beneficial for dairy cattle. They suggest that the types and concentrations of EO and EO compounds must be carefully defined.

CONCLUSIONS

The finds of this investigate suggest that the estimated essential oils and their combinations influence rumen fermentation in a manner depending upon the essential oils and feeds used. *In vitro* results should be tested in *in vivo* studies.

ACKNOWLEDGMENTS

This research was carried out as Ph.D thesis in Animal sciences at Ferdowsi University of Mashhad, Iran. The authors gratefully thank the Ferdowsi University of Mashhad for support, and they also especially like to thank prof. A. Naserian, prof. A. Tahmasbi and Dr. A. Vakili for their comments.

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