IJAS Iranian Journal of Applied Animal Science

The Effects of Various Essential Oils of Medical Plant Seeds and Spices on Digestion Characteristics and Population Changes of Ruminal Anaerobic Fungi in *in vitro* Condition

Research Article

M. Sadjadian[1], M. Danesh Mesgaran[1*] and A.R. Vakili[1]

[1] Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

Received on: 7 Jun 2016
Revised on: 18 Jan 2017
Accepted on: 31 Jan 2017
Online Published on: Jun 2017

*Correspondence E-mail: danesh@um.ac.ir

Online version is available on: www.ijas.ir

ABSTRACT

The effect of essential oils (EO) of medical plant seeds and spices on rumen microbial fermentation of alfalfa hay, sugar beet pulp and barley grain (as substrate) were evaluated under *in vitro* conditions. *In vitro* incubations were carried out using the gas production method with glass syringes. Treatments were as follows; a control (no additive), monensin, EO of cinnamon, black pepper seed, cumin seed, fennel seed and garlic oil (200 and 400 µL/g DM). Monensin was used as a positive control in the medium at 5 µmol. Data on gas production were fitted using an exponential equation. Results showed that compared to control treatments, monensin had a significant increase on gas production ($P<0.05$), and cumin seed EO decreased gas production of the feed samples (200 and 400 µL). The effects of treatments on *in vitro* ruminal fermentation characteristics were tested using an *in vitro* culture inoculated by mixed rumen microbes. The test treatments were as follows; control (no additive), EO of cinnamon, black pepper seed, cumin seed and fennel seed. Evaluations were made for medium pH, ammonia nitrogen concentration and dry matter disappearance after a 48 h incubation period. To evaluate the effect of EO on *in vitro* ruminal fungi populations, a sample was taken from the medium after a 120 h incubation period and fungal population was determination by real-time polymerase chain reaction. Compared to the control treatment, cumin and cinnamon additions resulted in a significant decrease ($P<0.05$) on disappearance of dry matter in the feed samples. In the present study, additions of all tested EO to alfalfa hay treatment showed a significant increase in the final pH of the culture ($P<0.05$). However, cinnamon addition resulted in a significant decrease in medium ammonia nitrogen concentration for each of the feed samples ($P<0.05$). Results of the present study also demonstrate that addition all of the tested EO to alfalfa hay had a significantly decrease on *in vitro* ruminal fungal population ($P<0.05$).

KEY WORDS alfalfa hay, essential oil, fungal population, real-time PCR.

INTRODUCTION

In ruminants, symbiotic relationship is established with rumen microorganisms, by which the animal provides the nutrients and optimal environmental conditions to allow fermentation of their feed by the rumen microorganisms. Instead, microorganisms degrade fiber and synthesize microbial protein to supply energy and protein needs of the host animal (Van Soest, 1982). However, this symbiotic relationship is somewhat inefficient through loss of energy (methane) and protein ($N\text{-}NH_3$) (Van Nevel and Demeyer, 1988). These losses not only reduce production performance, but they also contribute to release of environmental pollutants (Tamminga, 1996). Ruminant nutritionists have long been interested in modulating competition among different microbial populations with the objective of improv-

ing the efficiency of energy and protein utilization in the rumen. This has been achieved through optimized diet formulations and use of feed additives that modify fermentation in the rumen and enhance or inhibit specific microbial populations (Calsamiglia *et al*. 2006). Antibiotic ionophores have been very successful in reducing energy and protein losses in the rumen (Van Nevel and Demeyer, 1988). However, the use of antibiotics in animal feed is becoming less socially acceptable because of residue and production of resistant strains of bacteria. The use of antibiotics in animal feed has been banned in the European Union since January 2006 (Directive 1831/2003/CEE, European Commission, 2003). For this reason, scientists have become interested in developing alternative ways to modulate rumen fermentation such as application of yeasts, organic acids, plant extracts, probiotics and antibodies (Calsamiglia *et al*. 2006). Essential oils (EO) are complex mixtures of secondary metabolites and volatile compounds extracted from plants by methods of distillation that seem to have no direct function in their growth and development (Balandrin and Klocke, 1985; Benchaar *et al*. 2008). However, they are responsible odor and color in plants (Guenther, 1948). Application of EO can be a useful strategy to improve efficiency of nutrient utilization by ruminants (Benchaar *et al*. 2008; Tajodini *et al*. 2014). EO have an important role in plants, for example protection against invasive insects, bacteria, viruses and fungi, as well as in attracting pollinators (Bakkali *et al*. 2008). EO have demonstrated antimicrobial activity against a variety of microorganisms, including gram positive and gram-negative bacteria, protozoa, yeast and fungi (Helander *et al*. 1998; Greathead, 2003; Reichling *et al*. 2009). The mechanism by which EO are thought to exert their antimicrobial activity is by disrupting the cell wall structure, affecting electron transport, ion gradients, protein translocation, phosphorylation steps and other enzyme-dependent reactions (Ultee *et al*. 1999; Dorman and Deans, 2000). The antimicrobial activity of plant extract is attributed to a number of secondary plant metabolites, including saponins, terpenoids and phenylpropanoids present in the EO fraction of many plants (Dorman and Deans, 2000). A number of recent *in vitro* studies have evaluated the effects of various EO on ruminal microorganisms and ruminal metabolism and have reported varied results. Many studies have reported the stimulatory and inhibitory effects of EO on a large variety of microorganisms under *in vitro* and *in vivo* conditions (Newbold *et al*. 2004; Benchaar *et al*. 2007; Nanon *et al*. 2014; Khorrami *et al*. 2015).

Fernandez *et al*. (1997) showed that a commercial product of a blended of EO compounds inhibits protein degradation in the rumen, thus has the potential to increase protein supply to the post-ruminal tract.

McIntosh *et al*. (2003), reported that EO can cause a decrease in the ruminal fungal population. The objective of this study was to evaluate the effects of various medicinal plant EO on *in vitro* ruminal fermentation of alfalfa hay, sugar beet pulp and barley grain and *in vitro* ruminal fungal population.

MATERIALS AND METHODS

Substrates and EO preparation

The tested feed samples were as follows; alfalfa hay (AH), barley grain (BG) and sugar beet pulp (BP); these were ground to pass through a 1mm screen (AOAC, 1990). Treatments were feed samples without EO (as controls) or substrates plus 200 or 400 µL/g DM dose of EO of cinnamon, black pepper, cumin, fennel or garlic oil. Monensin was also used as a positive control in the medium at 5 µmol. These values were selected based on our previous experiments. The EO content was obtained for each plant with hydro-distillation of ground samples using Clevenger apparatus (Jahani-Azizabadi *et al*. 2014). The EO samples were stored in a refrigerator (4 ˚C) until they were used in the experiment.

Gas production technique

Two sheep (45±2 kg, body weight) fitted with rumen cannulae were used as donors for rumen fluid. They were fed 1.5 kg DM alfalfa hay and 0.4 kg DM concentrates (165 g CP/kg DM) per head per day. Rumen content was collected before feeding in the morning. Rumen fluid was strained through 4 layers of cheesecloth and incubated at 39 ˚C.
The gas production method of Menke and Steingass (1988) was used.

Rumen fluid was immediately strained through four layers of cheesecloth and mixed in a 2:1 with buffer, then, 40 mL of diluted fluid was added to the syringes. Each syringe was gassed with CO_2 then incubated at 38.6 ˚C. The volume of gas produced was determined at 2, 4, 8, 12, 24, 36, 72 and 96 h after incubation. Gas production data were fitted using an exponential equation of

$$P= b(1-e^{-ct})$$

Where:
b: volume of gas produced.
c: fractional rate constant of gas production (/h).
t: incubation time (h).
P: volume of gas produced at time t.

Ruminal quantification of fungi using batch culture

Feed samples were alfalfa hay (AH), barley grain (BG) and sugar beet pulp (BP). Treatments were substrates without

EO (as control) and substrates plus 200 µL/g DM of EO of cinnamon, black pepper, cumin and fennel.

Samples were incubated in a medium prepared as described in Arroquy *et al*. (2005). The fermentation medium was pre-reduced anaerobically sterilized ruminal fluid medium consisting 150 mL/L of mineral mixtures I (3 g/L K_2HPO_4), 150 mL/L mineral mixture II (3 g/L KH_2PO_4, 6 g/L $(NH_4)_2SO_4$, 6 g/L NaCl, 0.6 g/L $MgSO_4\_7H_2O$, 0.6 g/L $CaCl_2$), 0.05 g/L of cellobiose, 400 mL/L of cell-free ruminal fluid, 1 mL/L resazurin, 300 mL/L of distilled water, 4 g/L $NaHCO_3$ and 0.5 g/L of cysteine-HCL. The fermentation medium was autoclaved for about 20 minutes, cooled and then 4 g/L sodium bicarbonate was added. An amount of 45 ml of medium was supplied to a 100 mL bottle that contained 0.45 g of a feed sample (4 replicates per each sample).

Then, each bottle was inoculated under carbon dioxide with 5 mL of mixed rumen microbes. Rumen fluid was obtained from three sheep (49.5±2.5 kg) each fitted with a rumen fistulae before the morning feed, and immediately strained through four layers of cheesecloth.

The animals were fed with 1.5 kg of DM alfalfa hay and 0.4 kg DM concentrates (165 g CP/kg of DM) per head per day. The bottles were incubated for 120 h at 39 ˚C. Then, each bottle content was filtered through a 42 µm filter, and unfiltered residue was dried using a forced-air oven at 60 ˚C for 48 h, weighted and analyzed for DM and NDF content.

Evaluations for ammonia nitrogen ($N\text{-}NH_3$) concentration, medium pH and dry matter (DM) disappearance were carried out after 48 h of incubation. At the end of the incubation period, 2 mL of medium content (liquid and solid phase) were taken and stored at -20 ˚C until the next analysis.

DNA extraction

After thawing, samples were shaken and transferred to 1.5 ml micro tubes containing glass beads and vortexed twice for 2 min with incubation on ice between shakings. Tubes were centrifuged at 200 × g for 5 min at 4 ˚C for the sedimentation of feeds particles. The supernatants (200 µL) were transferred to a fresh 1.5 mL micro tubes and DNA extraction was performed using a genomic DNA Extraction Kit (AccuPrepTM, Bioneer Corporation) following the manufacturer′s instructions.

Real time-PCR

Fungi rDNA concentrations were measured using real time PCR relative to total bacteria amplification (ΔΔCt) and the SYBR Green PCR Master Mix Kit (SYBR Green I qPCR Master Mix, Syntol, Russia). The 16s rRNA gene-targeted primer sets used in the present study are described in Table 1. Templates (1 µL) were added to amplification reactions (25 µL) containing 0.6 µL of primer mixture containing 10 pmol of each primer, 11.5 µL of SYBR Green I qPCR Master Mix (Syntol) and 12 µL of deionized water. SYBR Green I qPCR. Master Mix contained KCl, Tris-HCL (pH 8.8), 6.25 m*M* $MgCl_2$, dNTP, Taq DNA polymerase, Tween, and SYBR Green I. A no-template (sterile distilled water) negative control was loaded on each plate run to screen for contamination and dimmer formation and to set the background fluorescence for plate normalization. Amplification and detection were performed using an applied biosystems (ABI) 7300 sequence detection system under the following conditions: initial denaturation at 95 ˚C for 5 min was followed by 40 cycles of denaturation at 95 ˚C for 15 s, annealing at 61 ˚C for 15 s, extension at 72 ˚C for 30 s, and then by the melting curve program (60-95 ˚C with a heating rate of 0.2 ˚C per second and a continuous fluorescence measurement). For total bacteria the threshold cycle of each standard dilution was determined during the exponential phase of amplification and regressed against the logarithm of known total bacterial. Total bacteria population size is reported as nano gram (ng) per µL of extracted DNA.

The copy number of total bacteria 16 S ribosomal RNA gene was determined as: Log10 copy number= Ct-(y-intercept/efficiency), where the formula parameters were derived from a standard curve of total bacteria. The population of fungi were expressed relative to the estimated abundance of total bacterial 16 S ribosomal RNA gene.

Chemical analysis

The chemical composition of the diet is reported in Table 2. DM content determined for each substrate by drying samples for 48 h in a 65 ˚C forced air oven (AOAC, 1990). Dry samples of the feed were maintained overnight at 550 ˚C in a furnace and organic matter (OM) was subsequently calculated as 100 minus the percentage ash (AOAC, 1990). Total nitrogen of each diet was determined by the kjeldahl method (AOAC, 1990), crud protein was calculated as N × 6.25. Ether extract was determined following the AOAC (1990).

The neutral detergent fiber (NDF) and acid detergent fiber (ADF) of the feed samples were analyzed by the detergent system using the sequential procedure of Van Soest *et al*. (1991).

For $N\text{-}NH_3$ concentration determination, a 5 mL of each sample was acidified with 5 mL of 0.2 N HCL and was measured by the kjeldahl method. The medium pH was measured at the end of incubation with a pH meter (Metrohm744, Switzerland).

The disappearance of DM was calculated as original weight of the DM minus weight of dry residue (after incubation) divided by weight of the original sample.

Table 1 PCR primers utilized for amplifying the target bacteria

Target species	Forward/reverse	Primer sequence	Reference
Total bacteria	F	GTGSTGCAYGGYTGTCGTCA	Maeda *et al*. (2003)
	R	ACGTCRTCCMCACCTTCCTC	
Fungi	F	GAGGAAGTAAAAGTCGTAACAAGGTTTC	Zhang *et al*. (2008)
	R	CAAATTCACAAAGGGTAGGATGATT	

Table 2 Dry matter and chemical composition of alfalfa hay, sugar beet pulp and barley grain

Composition (% of DM)	Alfalfa hay	Sugar beet pulp	Barley grain
Dry matter (DM)	90.1	85.2	92.3
Crude protein (CP)	14.2	12.4	9.4
Ash	7.2	9.5	3.1
Neutral detergent fiber	45.1	43.1	25.1
Acid detergent fiber	29.9	23.6	7.5
Ether extract	2.1	0.5	2.1

Statistical analyses

Statistical analysis of data from *in vitro* gas production test were made using SAS (1999) with the following model:

$$Y_{ijk} = \mu + A_i + \beta_j + T_k + e_{ijk}$$

Where:
Y: depended variable.
μ: overall mean.
A_i: effect of EO.
B_j: effect of feed sample.
T_k: effect of concentration.
e_{ijk}: residual error, were conducted in a 3 × 6 × 3 factorial design.

Statistical analysis of rumen fermentation characteristics were conducted as a randomized complete design using SAS program with the following model:

$$Y_{ij} = \mu + A_i + e_{ij}$$

Where:
Y: depended variable.
μ: overall mean.
A_i: effect of EO.
e_{ij}: residual error.

Evaluations of significance of difference between means of treatments and controls were determined at (P<0.05) using the Dunnett test.

RESULTS AND DISCUSSION

The effect of treatments on *in vitro* gas production parameters

The effects of EO on *in vitro* gas production parameters are shown in Table 3. Addition EO to AH showed that cinnamon significantly decreased c parameter at 200 μL (P<0.05), but it did not produce gas at 400 μL.

Also, supplementation of cumin significantly decreased gas production parameters and b parameter at 200 and 400 μL (P<0.05), respectively. Moreover, addition of garlic oil and fennel significantly increased c parameter at 200 μL (P<0.05) and black pepper significantly increased c parameter at both 200 and 400 μL (P<0.05) and monensin significantly increased both gas production parameters (P<0.05). In addition EO, BP showed that cinnamon and black pepper significantly decreased c parameter but increased b parameter at 200 μL, while, cinnamon did not produce gas at 400 μL. Moreover, cumin addition significantly decreased gas production parameters and b parameter at 200 and 400 μL (P<0.05), respectively. Also, fennel addition significantly decreased b parameter at both 200 and 400 μL (P<0.05), and garlic oil decreased b parameter at 200 μL (P<0.05), while, monensin significantly increased it. Addition evaluations for EO to BG showed that cinnamon and garlic oil additions significantly increased b parameter at 200 μL, while it did not produce gas at 400 μL. Also, black pepper addition and monensin significantly decreased c parameter but increased b parameter (P<0.05), while fennel decreased b parameter at 200 μL (P<0.05).

The effects of treatments on rumen fermentation characteristics on *in vitro* condition

The effects of treatments on disappearance of concentrations of DM, pH and N-NH_3 are shown in Table 4 after 48 h incubation, respectively. Additions of all EO to AH significantly increased pH of culture (P<0.05), but decreased disappearance of DM except in fennel. Black pepper and cinnamon significantly decreased N-NH_3 concentration (P<0.05).

In addition, addition of cinnamon and cumin significantly decreased disappearance of DM and N-NH_3 concentration of BP (P<0.05), while, cinnamon significantly increased pH of culture (P<0.05). Moreover, all treatments significantly decreased disappearance of DM of BG except fennel. Also, cinnamon and cumin significantly increased pH of culture but decreased N-NH_3 concentration (P<0.05).

Table 3 Effect of treatments on gas production of alfalfa hay, sugar beet pulp and barley grain

Treatments	Amount[1]	Parameters	
	Concentration (µL/g DM)	b (mL/0.3 g DM)	c (/h)
Alfalfa hay (AH)	-	49.3	0.07
AH + cinnamon	200	49.1	0.03*
AH + cumin	200	28.7*	0.03*
	400	20.4*	0.06
AH + fennel	200	44.7	0.17*
	400	45.6	0.09
AH + garlic	200	44.7	0.10*
	400	40.6	0.08
AH + black pepper	200	45.8	0.13*
	400	52.2	0.11*
AH + monensin[1]	-	60.9*	0.12*
Sugar beet pulp (SBP)	-	84.0	0.09
SBP + cinnamon	200	98.1*	0.05*
SBP + cumin	200	47.5*	0.03*
	400	44.5*	0.06
SBP + fennel	200	73.8*	0.10
	400	69.7*	0.01
SBP + garlic	200	71.5*	0.08
	400	75.0	0.07
SBP + black pepper	200	93.8*	0.01*
	400	80.9	0.07
SBP + monensin[1]	-	94.7*	0.07
Barley grain (BG)	-	98.7	0.04
BG + cinnamon	200	110.6*	0.04
BG + cumin	200	107.1	0.03
	400	104.8	0.02
BG + fennel	200	87.8*	0.04
	400	106.4	0.06
BG + garlic	200	113.9*	0.04
	400	108.4	0.04
BG + black pepper	200	108.6*	0.07*
	400	100.7	0.04
BG + monensin[1]	-	109.6*	0.07*
SEM	-	1.49	0.004
P-value	-	0.05	0.05

* (P<0.05).
SEM: standard error of the means.
[1] Monensin used as 5 µmol in medium.

The effects of treatments on ruminal anaerobic fungal population

Effects of treatments on ruminal anaerobic fungal population are shown in Figure 1. Results of the present study demonstrate difference between treatments for evaluations of ruminal fungal population. All treatments significantly decreased (P<0.05) both fungal population of AH and fungal population of BG in rumen except cumin.

In general, rumen microbial activity and gas production were affected by the use of EO. These results are consistent with those reported in Fraser *et al.* (2007) and Macheboeuf *et al.* (2008) in that cinnamon decreased gas production at incubation periods of 24 h and 16 h. Also, Jahani-Azizabadi *et al.* (2009) reported that addition of cumin powder (4% of incubated DM) on *in vitro* rumen microbial fermentation of alfalfa hay decreased production of gas and methane.

On the other hand, results of the present study did not conform to those reported in Hodjatpanah-Montazeri *et al.* (2015) in which cumin and cinnamon increased gas production 24 h post incubation. Other reports have demonstrated that garlic decreased gas production (Busquet *et al.* 2005; Kilic *et al.* 2011).

Furthermore, it has been reported that garlic oil has antimicrobial activity and a high dose of garlic could have a detrimental effect on ruminal fermentation (Feldberg *et al.* 1988). Cinnamon is a monophenolic compound that binds proteins and disturbs energy metabolism in cells (Wendakoon and Sakaguchi, 1995). Oussalah *et al.* (2006) also reported that cinnamon had an inhibitory effect on pseudomonas putida. The chemical structure determines the activity and antimicrobial effect of an EO (Dorman and Deans, 2000).

Table 4 Effect of treatments on rumen fermentation characteristics in *in vitro* condition of alfalfa hay, sugar beet pulp and barley grain

Treatments	Parameters[1]		
	disappearance of DM	pH	N-NH_3 (mg/100 mL)
Alfalfa hay (AH)	0.73	6.76	47.20
AH + cinnamon	0.41*	7.05*	29.70*
AH + cumin	0.55*	6.95*	48.97
AH + fennel	0.734	6.89*	51.14
AH + black pepper	0.57*	6.88*	26.82*
Beet pulp (BP)	0.88	6.63	30.86
SBP + cinnamon	0.47*	7.27*	22.58*
SBP + cumin	0.67*	6.66	22.87*
SBP + fennel	0.89	6.65	29.05
SBP + black pepper	0.91	6.67	29.86
Barley grain (BG)	0.90	6.56	30.60
BG + cinnamon	0.38*	7.03*	26.28*
BG + cumin	0.78*	6.9*	25.53*
BG + fennel	0.88	6.58	33.55
BG + black pepper	0.81*	6.68	29.19
SEM	0.022	0.02	2.73
P-value	0.05	0.05	0.05

* ($P<0.05$).
SEM: standard error of the means.

Various results have been reported on the effects of EO on ruminal pH. The findings of the present study show that cinnamon and cumin caused a significant ($P<0.05$) increase in pH of culture. Results presented here are consistent with those reported in Fraser *et al*. (2007) in which cinnamon addition increased the fermenter pH in a RUSITEC. Similar results were obtained by Busquet *et al*. (2006) in which addition of cinnamon oil increased fermenter pH an *in vitro* fermentation system. Moreover Hodjatpanah-Montazeri *et al*. (2015) showed the pH value of corn silage increased by cumin in *in vitro* rumen fermentation. Benchaar *et al*. (2007) reported an increase in ruminal pH when dairy cows received EO. In contrast to the effects observed in this study, other reports show that EO had no effect on pH (Yang *et al*. 2007). Whiles Jahani-Azizabadi *et al*. (2014) investigated EO of cumin at its 280 µL L^{-1} reduced pH. Increase in value pH may be related to the higher amount of ruminal NH_3 concentration in our experiment. Results of different studies for the impact of EO on ruminal N-NH_3 concentration are variable. In some studies, EO showed good potential to alter rumen microbial fermentation and ammonia producing bacteria in the rumen (McIntosh *et al*. 2003; Patra and Yu, 2014). The results of the current study on cinnamon and cumin impact showed a significant ($P<0.05$) decrease in N-NH_3 concentration. Several authors have reported a reducing effect on N-NH_3 concentration in the incubation medium by cinnamon (Jahani-Azizabadi *et al*. 2011; Jahani-Azizabadi *et al*. 2014). Similar results were obtained by Cardozo *et al*. (2005) when supplementing ocinnamon in an *in vitro* fermentation system fed a high-concentrate finishing diet.

In addition, Busquet *et al*. (2006) demonstrated that cinnamon inhibited N-NH_3 concentration at high concentration (3000 mg/L). Fraser *et al*. (2007) reported that cinnamon addition decreased N-NH_3 in a RUSITEC. Macheboeuf *et al*. (2008) reported that cinnamon decreased ammonia in an *in vitro* fermentation system. Ferme *et al*. (2004) showed that cinnamon addition to an *in vitro* rumen simulation system resulted in a reduction in *Prevotella* spp. a group of bacteria known to be involved in deamination, providing evidence of a mechanism of activity. These results suggest that cinnamon extract inhibited peptidolysis. The results of this study indicated that black pepper significantly ($P<0.05$) decreased N-NH_3 concentration of AH. These results are consistent with results reported in Cardozo *et al*. (2004) in which pepper addition decreased N-NH_3 concentration in a continuous culture. Busquet *et al*. (2006) demonstrated that capsicum oil (active component of black pepper) inhibited N-NH_3 concentration at high concentration (3000 mg/L). Cardozo *et al*. (2005) reported that capsaicin decreased N-NH_3 concentration in an *in vitro* fermentation system fed a diet of straw/concentrate 10:90. Although several studies have demonstrated antimicrobial activity of capsicum oil, the lack of the effect of capsicum oil compared with other EO could be related to its low content of oxygenated hydrocarbons. Some studies have observed that hydrocarbons monoterpenes are in general significantly less active than oxygenated monoterpenes (Cox *et al*. 2001). Decreased N-NH_3 concentration with EO suggests that these types of additives decrease deamination activity of bacteria that rely on peptides and AA as a source of N (Taghavi-Nezhad *et al*. 2014).

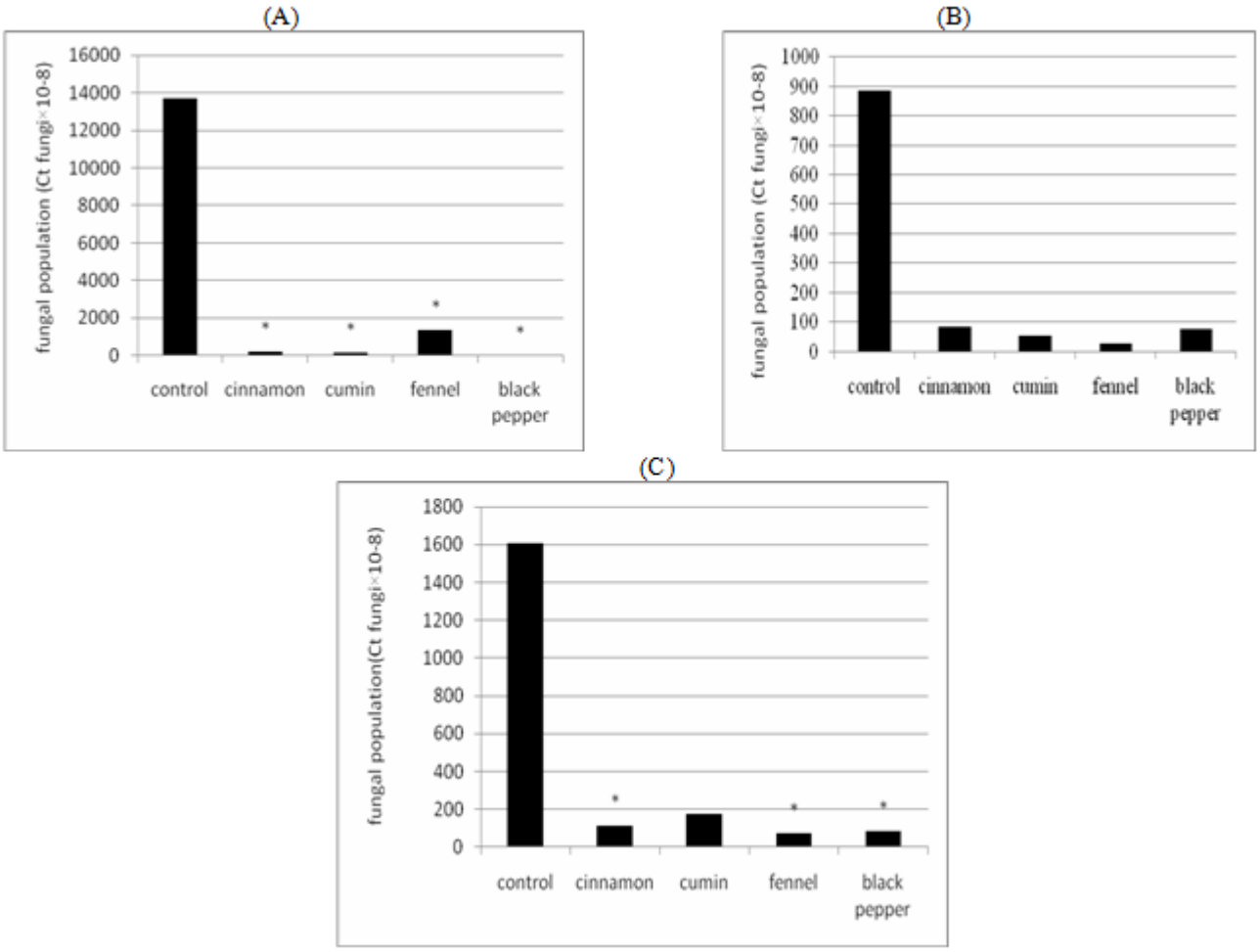

Figure 1 Effect of treatments on quantity of the fungal population existing in the *in vitro* culture relative to total bacteria population (Ct fungi×10-8) of alfalfa hay (A), sugar beet pulp (B), barley grain (C)
Asterisk differ significantly from the control (P<0.05)

Wallace *et al*. (2002) suggested that anti-microbial properties of EO can be exploited to modulate activity of rumen microbial populations by reducing degradation of dietary protein and thereby enhancing rumen N escape. Also, recent studies indicated that application of EO resulted in a significant reduction in protozoal population in the rumen (Patra and Yu, 2014; Khorrami *et al*. 2015; Lin *et al*. 2013). Rumen protozoa are responsible for a considerable amount of rumen ammonia due to predation of rumen bacteria and consumption of feed proteins (Bonhomme, 1990; Firkins *et al*. 2007). Therefore, a portion of observed reduction in ammonia concentration may be due to anti-protozoa effects of the EO (Lorenz *et al*. 2011). Perhaps the different effects of EO on feed samples reflect that there are different species responsible for digestion of the various proteins. Beside these differences among studies, results may have been affected by method of experimentation.

In the present study, cinnamon and cumin decreased disappearance of DM confirming the findings of some previous studies (Jahani-Azizabadi *et al*. 2011; Jahani-Azizabadi *et al*. 2014; Hodjatpanah-Montazeri *et al*. 2015). These findings are similar with those of Fraser *et al*. (2007) who observed a decrease in DM digestibility when cinnamon leaf oil was supplied at 500 mg/L in a RUSITEC. Because fiber disappearance was reduced by cinnamon, this can be lead to the conclusion that fungi were affected by cinnamon addition. However, the magnitude of the reduction in DM disappearance was greater for barley grain than for alfalfa. This implies that microbial populations involved in concentrate digestion were affected more by cinnamon than those involved in forage digestion. Reduction in DM disappearance might be nutritionally unfavorable for an animal but a decrease in concentration of N-NH_3 because of an increase in ruminal escape dietary protein can improve efficiency of the nitrogen use by the ruminants (Van Nevel and Demeyer, 1988).

Moreover, this study showed that EO resulted in a decrease in the rumen fungal population.

These results are consistent with those reported in McIntosh *et al*. (2003) showing that addition of a blend of EO inhibited *N. frontalis* fungal activity in the rumen with H_2 production at the concentration of 40 ppm. Similar results were obtained by Talebzadeh *et al*. (2013) in that fungal growth (*Neocalimastix* spp.) were inhibited and activity of fibrolytic fungal enzymes were reduced by adding ajowan EO. Kongmun *et al*. (2010) reported that ruminal anaerobic fungi population was not influenced by coconut oil and garlic powder. Although, garlic oil had fungicidal properties, as reported by Pai and Platt (2008). The antifungal effect of EO may also have contributed to decreased fiber digestibility. However very little information is available on the effect of EO on rumen fungal population. The results observed in the present trial suggest that careful selection of plant extracts may help to improve efficiency of rumen microbial fermentation. However, future research may help to establish the efficacy of EO as rumen microbial modulators.

CONCLUSION

The results obtained in the present study suggest that EO are able to modify rumen fermentation by changing gas production, protein metabolism or by causing disappearance of DM. In terms of protein metabolism, the mechanism of action may be related to inhibition of deamination, although the inhibition of peptidolysis has also been suggested for some EO. However, further research is necessary to determine optimal doses of EO, the potential for adaptation of rumen microflora to the actions of these additives, the fate of these products in an animals and the presence of residue in meat or milk, and effects on animal performance.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the technical and financial support of Ferdowsi University of Mashhad.

REFERENCES

AOAC. (1990). Official Methods of Analysis. Vol. I. 15th Ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Arroquy J.I., Cocran R.C., Nagarja T.G., Titgemeyer E.C. and Johnson D.E. (2005). Effect of type non-fiber carbohydrate on *in vitro* forage fiber digestion of low-quality grass hay. *Anim. Feed Sci. Technol*. **120,** 93-106.

Bakkali F., Averbeck S., Averbeck D. and Idaomar M. (2008). Biological effects of essential oils: a review. *Food Chem. Toxicol*. **46,** 446-475.

Balandrin M.F. and Klocke J.A. (1985). Natural plant chemicals: sources of industrial and medicinal materials. *Science*. **228,** 1154-1160.

Benchaar C., Calsamiglia S., Chaves A.V., Fraser G.R., Colombatto D., McAllister T.A. and Beauchemin K.A. (2008). A review of plant-derived essential oils in ruminant nutrition and production. *Anim. Feed Sci. Technol*. **145,** 209-228.

Benchaar C., Petit H.V., Berthiaume R., Ouellet D.R., Chiquette J. and Chouinard P.Y. (2007). Effects of essential oils on digestion, ruminal fermentation, rumen microbial populations, milk production, and milk composition in dairy cows fed alfalfa silage or corn silage. *J. Dairy Sci*. **90,** 886-897.

Bonhomme A. (1990). Rumen ciliates: their metabolism and relationships with bacteria and their hosts. *Anim. Feed Sci. Technol*. **30,** 203-216.

Busquet M., Calsamiglia S., Ferret A., Cardozo P.W. and Kamel C. (2005). Effects of cinnamaldehyde and garlic oil on rumen microbial fermentation in a dual flow continuous culture. *J. Dairy Sci*. **88,** 2508-2516.

Busquet M., Calsamiglia S., Ferret A. and Kamel C. (2006). Plant extracts affect *in vitro* rumen microbial fermentation. *J. Dairy Sci*. **89,** 761-771.

Calsamiglia S., Castillejos L. and Busquet M. (2006). Alternatives to antimicrobial growth promoters in cattle. Pp. 129-167 in Recent Advances in Animal Nutrition. P.C. Garnsworthy and J. Wiseman, Eds. Nottingham University Press, Nottingham, UK.

Cardozo P.W., Calsamiglia S., Ferret A. and Kamel C. (2004). Effects of natural plant extracts on protein degradation and fermentation profiles in continuous culture. *J. Anim. Sci*. **82,** 3230-3236.

Cardozo P.W., Calsamiglia S., Ferret A. and Kamel C. (2005). Screening for the effects of natural plant extracts at different pH on *in vitro* rumen microbial fermentation of a high-concentrate diet for beef cattle. *J. Anim. Sci*. **83,** 2572-2579.

Cox S.D., Mann C.M. and Markam J.L. (2001). Interaction between components of the essential oil of *Melaleuca alternifolia*. *J. Appl. Microbiol*. **91,** 492-497.

Dorman H.J.D. and Deans S.G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol*. **88,** 308-316.

European Commission. (2003). Regulation (EC) No. 1831/2003 of the European Parliament and of the Council of 22 September 2003 on Additives for Use in Animal Nutrition. Off. J. Eur. Union L L268/29.

Feldberg R.S., Chang S.C., Kotik A.N., Nadler M., Neuwirth Z., Sundstrom D.C. and Thompson N.H. (1988). *In vitro* mechanism of inhibition of bacterial cell growth by allicin. *Antimicrob. Agents Chem*. **32,** 1763-1768.

Ferme D., Banjac M., Calsamiglia S., Busquet M., Kamel C. and Avgustin G. (2004). The effects of plant extracts on microbial community structure in a rumen-simulating continuous-culture system as revealed by molecular profiling. *Folia Microbiol*. **49,** 151-155.

Fernandez M., Serrano E., Frutos P., Gir´aldez F.J., Mantec´on A.R. and Llach J.R. (1997). Efecto del aditivo Crina HC sobre la actividad degradativa ruminal en la especie ovina. *Inf. Tech. Econ. Agraria*. **18,** 160-162.

Firkins J.L., Yu Z. and Morrison M. (2007). Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. *J. Dairy Sci*. **90,** 1-16.

Fraser G.R., Chaves A.V., Wang Y., McAllister T.A., Beauchemin K.A. and Benchaar C. (2007). Assessment of the effects of cinnamon leaf oil on rumen microbial fermentation using two continuous culture systems. *J. Dairy Sci*. **90,** 2315-2328.

Greathead H. (2003). Plants and plant extracts for improving animal productivity. *Proc. Nutr. Soc*. **62,** 279-290.

Guenther E. (1948). The Essential Oils. Publisher D. Van Nostrand Company Inc., New York.

Helander I.M., Alakomi H.L., Latva-Kala K., Mattila-Sandholm T., Pol L., Smid E.J., Gorris L.G.M. and von Wright A. (1998). Characterization of the action of selected essential oil components on gram negative bacteria. *J. Agric. Food Chem*. **46,** 3590-3595.

Hodjatpanah-Montazeri A., Danesh-Mesgaran M., Vakili A.R. and Tahmasebi A.M. (2015). Effect of essential oils of various plants as microbial modifier to alter corn silage fermentation and *in vitro* methane production. *Iranian J. Appl. Anim. Sci*. **6(2),** 269-276.

Jahani-Azizabadi H., Danesh-Mesgaran M., Vakili A.R. and Heravi-Moussavi A.R. (2009). Screening the activity of medicinal plants or spices on *in vitro* ruminal methane production. *J. Dairy Sci*. **92,** 277-278.

Jahani-Azizabadi H., Danesh-Mesgaran M., Vakili A.R., Rezayazdi K. and Hashemi M. (2011). Effect of various semiarid native medicinal plant essential oils on rumen fermentation characteristics of a high forage diet using *in vitro* batch culture. *African J. Microbiol. Res.* **27,** 4812-4819.

Jahani-Azizabadi H., Danesh-Mesgaran M., Vakili A.R. and Rezayazdi K. (2014). Effect of some plant essential oils on *in vitro* ruminal methane production and on fermentation characteristics of a mid-forage diet. *J. Agric. Sci. Technol*. **16,** 1543-1554.

Khorrami B., Vakili A.R., Mesgaran M.D. and Klevenhusen F. (2015). Thyme and cinnamon essential oils: potential alternatives for monensin as a rumen modifier in beef production systems. *Anim. Feed Sci. Technol*. **200,** 8-16.

Kilic U., Boga M., Gorgulu M. and Şahan Z. (2011). The effects of different compounds in some essential oils on *in vitro* gas production. *J. Anim. Feed Sci*. **20,** 626-636.

Kongmun P., Wanapat M., Pakdee P. and Navanukraw C. (2010). Effect of coconut oil and garlic powder on *in vitro* fermentation using gas production technique. *Livest. Sci*. **127,** 38-44.

Lin B., Lu Y., Salem A.Z.M., Wang J.H., Lianga Q. and Liu J.X. (2013). Effects of essential oil combinations on sheep ruminal fermentation and digestibility of a diet with fumarate included. *Anim. Feed Sci. Technol*. **184,** 24-32.

Lorenz M.M., Karlsson L., Hetta M. and Uden P. (2011). Recycling of microbial N and estimation of protein degradation by *in vitro* gas production. *Anim. Feed Sci. Technol*. **170,** 111-116.

Macheboeuf D., Morgavi D.P., Papon Y., Mousset J.L. and Arturo-Schaan M. (2008). Dose response effects of essential oils on *in vitro* fermentation activity of the rumen microbial population. *J. Anim. Feed Sci*. **145,** 335-350.

Maeda H., Fujimoto C., Haruki Y., Maeda T., Kokeguchi S., Petelin M., Arai H. and Tanimoto I. (2003). Quantitative real-time PCR using TaqMan and SYBR green for *Actinobacillus actin Omycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, tetQ gene and total bacteria. *FEMS Immunol. Med. Microbiol*. **39,** 81-86.

McIntosh F.M., Williams P., Losa R., Wallace R.J., Beever D.A. and Newbold C.J. (2003). Effects of essential oils on ruminal microorganisms and their protein metabolism. *Appl. Environ. Microbiol*. **69,** 5011-5014.

Menke K.H. and Steingass H. (1988). Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Anim. Res. Dev*. **28,** 7-55.

Nanon A., Suksombat W. and Yang W.Z. (2014). Effects of essential oils supplementation on *in vitro* and *in situ* feed digestion in beef cattle. *Anim. Feed Sci. Technol*. **196,** 50-59.

Newbold C.J., McIntosh F.M., Williams P., Losa R. and Wallace R.J. (2004). Effects of a specific blend of essential oil compounds on rumen fermentation. *Anim. Feed Sci. Technol*. **114,** 105-112.

Oussalah M., Caillet S., Saucier L. and Lacroix M. (2006). Antimicrobial effects of selected plant essential oils on the growth of a *Pseudomonas putida* strain isolated from meat. *Meat Sci*. **73,** 236-244.

Pai S.T. and Platt M.W. (2008). Antifungal effects of *Allium sativum* (garlic) extract against the *Aspergillus* species involved in otomycosis. *Appl. Microbiol. Lett*. **20,** 14-18.

Patra A.K. and Yu Z. (2014). Effects of vanillin, quillaja saponin, and essential oils on *in vitro* fermentation and protein-degrading microorganisms of the rumen. *Appl. Microbiol. Biotechnol*. **98,** 897-905.

Reichling J., Schnitzler P., Suschke U. and Saller R. (2009). Essential oils of aromatic plants with antibacterial, antifungal, antiviral, and cytotoxic properties: an overview. *Forsch. Komplementmed*. **16(2),** 79-90.

SAS Institute. (1999). SAS®/STAT Software, Release 8.2. SAS Institute, Inc., Cary, NC. USA.

Taghavi-Nezhad M., Alipour D., Flythe M.D., Zamani P. and Khodakaramian G. (2014). The effect of essential oils of *Zataria multiflora* and *Mentha spicata* on the *in vitro* rumen fermentation, and growth and deaminative activity of amino acid-fermenting bacteria isolated from Mehraban sheep. *J. Anim. Prod. Sci*. **54(3),** 299-307.

Tajodini M., Moghbeli P., Saeedi H.R. and Effati M. (2014). The effect of medicinal plants as a feed additive in ruminant nutrition. *Iranian J. Appl. Anim. Sci*. **4(4),** 681-686.

Talebzadeh R. and Alipour D. (2013). The effect of ajowan (*Carum copticum*) essential oils on eukaryotic ruminal microorganisms of Mehraban sheep. *Iranian J. Microbiol*. **4,** 418-421.

Tamminga S. (1996). A review on environmental impacts of nutritional strategies in ruminants. *J. Anim. Sci*. **74,** 3112-3124.

Ultee A., Kets E.P. and Smid E.J. (1999). Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl. Environ. Microbiol*. **65,** 4606-4610.

Van Nevel C.J. and Demeyer D.I. (1988). Manipulation of rumen fermentation. Pp. 387-443 in The Rumen Microbial Ecosystm. P.N. Hobson, Ed. Elsevier Applied Science, New York.

Van Soest P.J. (1982). Nutritional Ecology of the Ruminant. Comstock, Cornell Univ. Press, New York, NY.

Van Soest P.J., Robertson J.B. and Lewis B.A. (1991). Methods

for dietary fiber, neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci*. **74,** 3583-3597.

Wallace R.J., McEwan N.R., Mclntosh F.M., Teferedegne B. and Newbold C.J. (2002). Natural products as manipulators of rumen fermentation. *Asian-Australasian J. Anim. Sci*. **15,** 1458-1468.

Wendakoon C.N. and Sakaguchi M. (1995). Inhibition of amino acid decarboxylaseactivity of *Enterobacter aerogenes* by active components in spices. *J. Food Prot*. **58,** 280-283.

Yang W.Z., Benchaar C., Ametaj B.N., Chaves A.V., He M.L. and McAllister T.A. (2007). Effects of garlic and juniper berry essential oils on ruminal fermentation and on the site and extent of digestion in lactating cows. *J. Dairy Sci*. **90,** 5671-5681.

Zhang C., Guo Y., Yuan Z., Wu Y., Wang J., Liu J. and Zhu W. (2008). Effect of octadeca carbon fatty acids on microbial fermentation, methanogenesis and microbial flora *in vitro*. *Anim. Feed Sci. Technol*. **146,** 259-269.