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In vitro Effect of Essential Oils on Rumen Fermentation and Microbial Nitrogen Yield of High Concentrate Dairy Cow Diet

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Present study was conducted to investigate the effect of including plant essential oils on *invitro* ruminal fermentation and microbial nitrogen synthesis of a dairy cow diet rich in concentrate. The treatments consisted of the diet alone (control; BD) as well as containing 50 and 100 μl L^{-1} essential oil of thyme (BDT), mint (BDM), savory (BDS), or a mixture of the essential oils at the rate of 1:1:1 (BD_{mix}). Essential oilsdecreased gas production at 24, 48 and 96 h of incubation compared with that of BD. However, mint at the rate of 50 or 100 μl L^{-1} resulted an increase in the microbial nitrogen when compared toBD, BDS andBDT. The nitrogen content of truly undegradedresidu (NDFN) content and NH3-N concentration were lower,while the dry matter digestibility was greater in the BD_{mix}, regardless of dosage levels, as compared with the control. The inclusion of a mixture of essential oils at 50 μl L^{-1} to the basal diet caused intensified dry matter disappearance,in comparison to other treatments. Results showed that the synergetic effects of essential oils together in a dairy cow diet of rich in concentrate can alter rumen microbial fermentation and improve microbial protein yield.

Keywords: Thyme, Mint, Savory, Essential oils, *Invitro* fermentation, Microbial nitrogen.

In recent years, antibiotics and antimicrobial compounds produced by microorganisms have played an important role in animal diets to increase their performances and decrease mortality (Benchaar*et al.,* 2008). However, public concern over routine use of antibiotics in animal nutrition has increased due to the potential development of antibiotic resistant in animals and humans (Benchaar*et al.,* 2006). As a result, alternative feed additives such as aromatic plants and their extracts were researched instead of antibiotics and they have gained interest as growth and health promoters (Benchaar*et al.,* 2006). Essential Oils (**EOs**) are volatile, lipophilic substances obtained from plant materials which are gained generally by steam distillation mechanical separation or solvent extraction (Wallace, 2002; Castillejos *et al.,* 2007; Hart *et al.,* 2008). Hart *et al.*,(2008), described that the concentration of EOs may vary depending on the stage of

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plant growth, different part of plant, amount of moisture received, light, and temperature. Essential oils are complex mixtures of secondary plant metabolites that demonstrate a broad spectrum of antimicrobial properties, particularly against gram positive bacteria (Burt, 2004). They are naturally occurring plant components and are considered safe for human and animal consumption (FDA, 2003; Benchaar *et al.,* 2006). These appear to be selective in their antifungal, antiviral, bactericidal, and bacteriostatic effects on microorganisms (Burt, 2004; Janssen *et al.,* 1986). Oh *et al.,*(1967) and then Nagy and Tengerdy(1967), were the first to investigate effects of EOs on *invitro* ruminal microbial fermentation and observed that EOs inhibited gas production, however the degree of inhibition was dependent on the chemical structure of the EOs. The research of Smith-Palmer *et al.,*(1998), determined that out of their EOs test articles, the oils of cinnamon and thyme exhibited the greatest inhibitory effects with bacteriostatic activity at a concentration of 0.075% or less against *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes, Salmonella enteritidis*, and *Campylobacter jejuni*. However, negative gram organisms in this study (and others) were slightly less susceptible to EOs than positive gram bacteria (Smith-Palmer *et al.,* 1998; Burt, 2004). Certain phenolic compounds such as thymol (thyme) could inhibit negative gram bacteria as well as positive gram bacteria. The mechanism by which most EOs are thought to exert their antibacterial effects is by disrupting cell wall structures due to their lipophilic character. The chemical components of EOs interrupt electron transport, ion gradients, protein translocation, phosphorylation steps, and other enzyme-dependent steps, causing the bacterium to lose chemi-osmotic control (Ultee and Kets, 1999; Cox *et al.,* 2000; Dorman and Deans, 2000) .A number of researches were induced by the well-documented antimicrobial effectiveness of EOs improve their feed efficiency and so lead to an increased production efficiency animal (Wallace, 2002). During the past few years, various studies have been conducted to determine the effects of herbal plant EOs on rumen microbial fermentation and dry matter disappearance (Hart *et al.,* 2008; Busquet*et al.,* 2006). It seems that a level of EOs used in the diet and the source of supplied them from herbal plants to be important as regards its positive effects on rumen microbial fermentation. Essential oils provide a valuable approach to manipulate ruminal fermentation to decrease methane and NH3-N concentration and increased the amount of microbial protein production (MP) by ruminant (Patra and Saxena, 2010; Menke and Steingass, 1988). Microbial protein production plays a pivotal role in ruminant nutrition (Srinivas and Krishnamoorthy, 2013).Various studies have observed contradictory resultswhen improving microbial protein (MP) by the inclusion of essential oil (EOs). Menke and Steingass(Menke and Steingass, 1988),noted that plant extracts increase the degradation of cell wall constituents, yield and efficiency of MP synthesis. However, Kumar*et al.,*(2011), observed no adverse impact of EOs on MP of buffaloes,with this conflict being possibly related to inherent detoxification ability of bacteria or neglecting the dosage and type of EOs. Furthermore, the stereochemical properties of EOs can vary and depend on the method of extraction (Scorzoni *et al.,* 2007). However, extraction products may also vary qualitatively and quantitatively in their composition (Angioni *et al.,* 2006). Therefore, the objective of the present experiment was to evaluate the *invitro* effects of various essential oils on rumen fermentation and microbial nitrogen synthesis of a dairy cow diet rich in concentrate.

MATERIALS AND METHODS

Herbal plants were collected from botanical garden of chaharmahall and bakhtiari province (shahrekord city, Iran). The essential oil content for each plant was obtained through steam distillation by Clevenger Method. A basal experimental diet (BD) used for batch cultures was a dairy cow diet rich in concentrate. Diet ingredients and chemical composition are presented in Table 1. The treatments included BD (control), BD + thyme (BDT), BD + mint (BDM), BD + savory (BDS) and BD plus a mixture of thyme, mint and savory at the rate of 1:1:1 (BD_{mix}), 6 replicates per each treatment of three runs. Two different doses were considered for each essential oil as 50 and 100 ìl L^{-1} of the total culture medium. The gas production procedure and microbial N assay were performed as described by Grings*et al.,*(2005). Rumen inoculum was collected from three rumen

fistulae Holstein lactating dairy cows (620 ± 10 kg of BW, 310 ± 8 DIM, mean ± SD) andrumen liquor was handled under a constant stream of CO_2,all containers used were pre-warmed at 39 ºC and filled with CO_2. The cows were offered a diet (g/kg DM) containing alfalfa hay (181), corn silage (289), wheat straw (31), barley grain (165), corn grain (114), soybean meal (124), wheat bran (93), vitamin and mineral premix (10). Ruminal content was immediately transferred to the laboratory, then blended and strained through four layers of cheesecloth to eliminate large feed particles and transferred to the laboratory in a pre-warmed thermos.A sample of 250 mg was weighed into a 125-ml serum bottles in 3 runs with 6 replicates. The filtrate was then mixed with carbonate buffer (containing ammonium bicarbonate at 4g/l) and sodium bicarbonate (35g/l in N-rich incubation medium and only sodium bicarbonate at 39.25g/l in N-low medium), macro-mineral solution (5.7g anhydrous Na_2HPO_4, 6.2g anhydrous KH_2PO_4 and 0.6g $MgSO_4 \cdot 7H_2O$ per liter), and deionized water in a ratio of 1:1:0.5:1.5 and 0.1 ml micro-mineral solution (13.2g $CaCl_2 \cdot 2H_2O$, 10.0g $MnCl_2 \cdot 4H_2O$, 1g $CoCl_2 \cdot 6H_2O$ and 8.0 g $FeCl_3 \cdot 6H_2O$ per 100ml)

Table 1. Ingredients (% DM) and nutrient composition of basal diet

Ingredients	%
Corn silage	20
Alfalfa hay	20
Corn grain	14
Barley grain	17.5
Soybean meal	11
Wheat bran	10
Canola meal	7
Mineral and vitamin premix[1]	0.5
Chemical composition (% DM)	
Crude protein	17.2
Neutral detergent fiber	32.1
Acid detergent fiber	19.2
Ether extract,	2.1
Non-fiber carbohydrate[2]	44. 4

[1]Supplied 195 g/kg of Ca, 21 g/kg of Mg, 2.2 g/kg of Mn, 0.3 g/kg of Zn, 0.3 g/kg of Cu, 0.12 g/kg of I, 0.1 g/kg of Co, 600,000 IU/kg of vitamin A, 200,000 IU/kg of vitamin D, and 0.2 g/kg of vitamin E, 0.025 g/ kg of Se.

[2]Calculated by difference 100 " (% NDF + % CP + % fat + % ash)

was added. The medium was then reduced by addition of 41.7ml reducing agent (40ml deionized water, 1ml 1N NaOH and 1g $Na_2S \cdot 9H_2O$) per liter. Twenty milliliters of medium were dispensed into a 125ml glass serum bottle whose top were stopped with rubber and aluminum cabs and placed in a water bath for 96 h at 39 ºC. Blank samples were also incubated simultaneously to make correction in gas production, if any, from the medium. Gas production was measured at 2, 4, 6, 8, 10, 12, 24, 48, 72 and 96 h of the incubation by inserting a 23 gauge (0.6mm) needle attached to a pressure transducer (model PX4200-015GI, Omega Engineering, Inc. Laval, Que., Canada) connected to a visual display (Data Track, Christchurch, UK) into the head space of serum bottles.The transducer was then removed leaving the needle in place to permit venting. Pressure values were corrected for the amount of substrate organic matter (OM) incubated and the gas released from negative controls. In order to prevent accumulation of produced gases, the gas in the head space of each bottle was released. After subtraction of gas production from blank bottles, data were fitted to the following exponential model, based on Orskov and McDonald,(1979) model without lag phase: G = b (1 " $e^{"ct}$), where G is the cumulative gas volume produced at time t, b is the asymptotic (or maximal) gas volume, i.e., G(t '! "), and c is the gas production rate.Then, half time of maximal gas production ($t_{1/2}$) was calculated and same incubation with similar dietary substrates was done to assess the microbial N production. Microbial N production at $t_{1/2}$ was indirectly estimated by differentially quantifying all N sources, except microbial N, and includes use of information on the N content of diet, truly un-degraded N (NDFN) in diet samples at $t_{1/2}$, and the change in NH3–N levels in the incubation medium between 0 hour of incubation and $t_{1/2}$ using the equations: Microbial N production at $t_{1/2}$ = dietary N + "NH3–N – NDFN at $t_{1/2}$. Where "NH3–N=NH3–N in 0 hour blanks " NH3–N in diet incubations at $t_{1/2}$. Dry matter degradability of samples at $t_{1/2}$ was measured by refluxing the incubation residue with neutral detergent (ND) solution for 1 hour.

Analytical methods

Dietary samples were oven dried at 60 ºC for 48h, then ground to pass through a 1-mm screen in a Wiley mill (standard model 4; Arthur

Table 2. Effect of including plant essential oils (applied at the rate of 50 or 100 ¼l/L of medium)in a high concentrate diet on asymptotic gas production (b), first-order fractional rate constant (c), gas production at 24, 48, 96 h and $t_{1/2.}$

	Treatments[1]											
Parameters	BD	BDT Concentrate of EO added to basal diet (µl l-1)		BDM Concentrate of EO added to basal diet (µl l-1)		BDS Concentrate of EO added to basal diet (µl l-1)		BD_{mix} Concentrate of EO added to basal diet (µl l-1)		SEM	P value	
											BD vs. BD + EOs	BD+ 50µl/L EO vs BD+ 100 µl/L EO
		50	100	50	100	50	100	50	100			
b	54.11	56.03	54.46	51.21	51.1	54.37	52.4	56.53	55.97	1.9	0.37	0.51
c	0.08	0.07	0.08	0.07	0.07	0.07	0.07	0.08	0.07	0.003	0.62	0.69
$T_{1/2}$	2.3	2.56	2.1	3.01	2.41	2.9	3.02	3.28	3.44	0.51	0.26	0.48
$Gas_{t1/2}$	20.10[a]	16.94[b]	17.60[b]	17.33[b]	18.02[b]	17.10[b]	17.51[b]	16.99[b]	17.88[b]	1.09	0.001	0.53
Gas_{24}	49.70[a]	21.12[b]	19.29[b]	20.18[b]	25.05[b]	28.66[b]	19.37[b]	18.19[b]	20.24[b]	4.2	0.001	0.47
Gas_{48}	54.60[a]	39.55[b]	38.38[b]	39.00[b]	41.12[b]	40.99[b]	39.12[b]	39.77[b]	43.16[b]	2.9	0.001	0.49
Gas_{96}	77.39[a]	67.40[b]	65.12[b]	68.23[b]	69.44[b]	69.20[b]	66.23[b]	68.74[b]	70.40[b]	2.04	0.001	0.53

1 control (BD), BD + thyme (BDT), BD + mint (BDM), BD + savory (BDS) and BD plus a mixture of thyme, mint and savory at the rate of 1:1:1 (BDmix)

Table 3. Effect of including plant essential oils (applied at the rate of 50 or 100 ?l/Lof medium)on Microbial N yield (MN), NDFN, NH3-N, DMD (%), the ratio of microbial nitrogen to dry matter digestibility (MN/DMD), and undigested nitrogen (UDN) at $t_{1/2}$

Para meters	BD	BDT Concentrate of EO added to basal diet (µl l⁻¹)		BDM Concentrate of EO added to basal diet (µl l⁻¹)		Treatments[1] BDS Concentrate of EO added to basal diet (µl l⁻¹)		BD_{mix} Concentrate of EO added to basal diet (µl l⁻¹)		SEM	P value BD vs. BD + EOs	BD+ 50µl/L EO vs BD+ 100 µl/L EO
		50	100	50	100	50	100	50	100			
MN (mg)	4.10^{e}	4.31^{cde}	4.28^{cde}	4.40^{bc}	4.38^{bc}	4.28^{cde}	4.16^{cde}	4.57^{a}	4.64^{a}	0.09	0.01	0.39
NDFN (mg)	1.70^{d}	1.91^{b}	1.67^{f}	2.00^{a}	1.99^{a}	1.83^{c}	1.75^{e}	1.67^{g}	1.68^{f}	0.02	0.01	0.02
NH3-N	1.43^{a}	0.88^{bc}	1.04^{b}	0.71^{c}	0.74^{c}	1.04^{b}	1.03^{b}	0.86^{bc}	0.74^{c}	0.01	0.01	0.04
DMD %	37.16^{d}	41.88^{bc}	42.11^{bc}	42.55^{bc}	42.67^{bc}	43.17^{bc}	41.38^{c}	45.48^{a}	45.22^{ab}	1.07	0.01	0.21
DMD (mg) diss.)	92.91^{d}	104.72^{bc}	105.28^{bc}	106.38^{bc}	106.69^{bc}	107.94^{bc}	103.47^{c}	113.72^{a}	110.55^{ab}	2.69	0.01	0.36
UDN (mg)	1.53^{ab}	1.61^{ab}	1.48^{ab}	1.62^{ab}	1.68^{a}	1.48^{ab}	1.61^{ab}	1.38^{b}	1.43^{b}	0.1	0.03	0.34
MN/ DMD	0.044	0.041	0.041	0.041	0.042	0.04	0.039	0.041	0.04	0.02	0.39	0.32

1 control (BD), BD + thyme (BDT), BD + mint (BDM), BD + savory (BDS) and BD plus a mixture of thyme, mint and savory at the rate of 1:1:1 (BDmix)

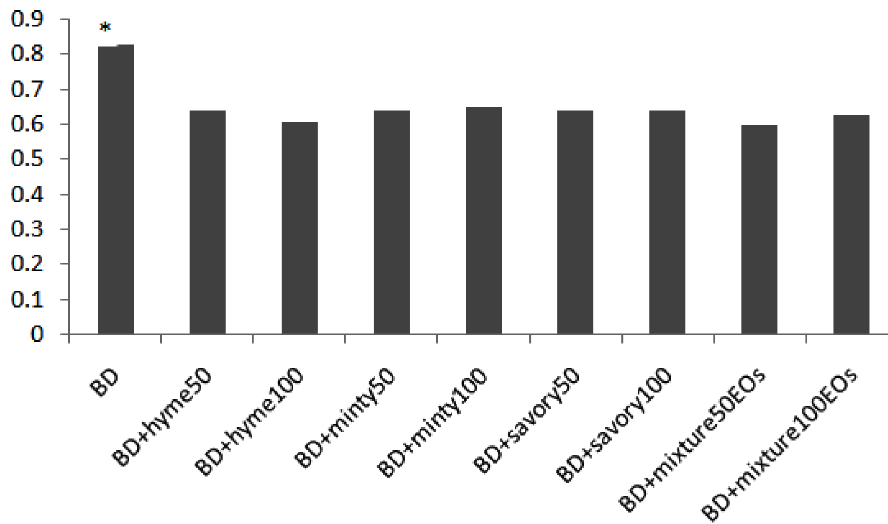

Fig. 1. The rates of gas production to dry matter dissaparance of diet based on various essential oils

H. Thomas Co., Philadelphia, PA) before chemical analyses. The measurement of diet nitrogen was conducted by Kjeldahl analysis (AOAC,1990). Crude protein was determined as nitrogen × 6.25. The fat content of the diet was determined using a Soxtec system HT6 apparatus according to AOAC,(1990). The concentration of NDF and ADF was determined as described by Van Soest*et al.,*(1991) without the use of sodium sulfite and heat-stable á-amylase. The NFC content of the diets was calculated as 1000 " (NDF + CP + ether extract + ash).

Statistical analysis

In order to assess the differences between dietary treatments, the data were analyzed in a completely randomized design using the general linear models procedure of SAS,(2003). In this analysis, mean values of various parameters of each diet (e.g. microbial N) were compared using the Duncan's multiple range tests. Contrasts were tested using the CONTRAST statement of SAS. Standard errors of means were calculated from the residual mean square in the analysis of variance. Standard errors of means were calculated from the residual mean square in analysis of variance. Significance was declared at P< 0.05.

RESULTS

***In vitro* gas production**

As illustrated in Table 2, the inclusion of EOs on diet, regardless of dosage levels, had no significant effect on asymptotic gas production (b), first-order fractional rate constant (c), and the time (h), when half of the asymptotic gas volume was produced ($t_{1/2}$).The gas volume at $t_{1/2}$ was the highest in BD and was the same across BDT, BDS, BDM and BD_{mix}at both 50 and 100 µl levels. Furthermore, gas production at 24, 48 and 96 h decreased with supplementing EOs, regardless of dosage levels and sort of plant EOs, as compared with that of control. However, the increase of each essential oil level from 50 µl to 100 µl did not have a significant effect onreduction of gas volume. Nevertheless, 50 µl of each essential oil had a minimal inhibiltory effect on gas production.

Microbial nitrogen production

Data of Microbial N yield (MN, mg), Nitrogen content of truly undegradedresidu (NDFN), NH3-N concentrate, dry matter digestibility,undigested nitrogen (UDN, mg) and the ratio of MN to DMD at $t_{1/2}$ are presented in Table 3. Microbial nitrogen was the highest in BD mixed withEOs,while it was the lowest in

BD without any essential oil supplementation. However, no detectable differences were observed between the two dosages (50 and 100 µl) ofEOs among treatments for microbial nitrogen yield (P>0.05). An increase in microbial nitrogen yield (MN) in the diet supplemented with a mixture of EOsis undoubtedly due to the synergetic effect of EOs when used together. The NDFN content in $BD_{mix}$was lower than that of BD plus thyme, mint and savory. Likewise, diets containing 100 µlof EOs had a lower NDFN than diets receiving 50 µl. The inclusion of essential oils to BD resulted in diminishedNH3-N concentration as compared with the control. TheBD plus mintessential oil, regardless of its level, had a lower NH3-N concentration than BD plus thyme and savoryessential oil.

DISSCUSION

The BD diet resulted in the greatest gas production as compared to other treatments. These findings might due to the inhibitory effect EOs on methane production, which is in line with Patra and Yu,(2012) findings who noted that total gas and methane production by the ruminal cultures decreased with inclusion of EOs in diet. Several studies have documented a reduction in methane production by EOs (Agarwal *et al.,* 2009; Chaves *et al.,* 2008). Macheboeuf*et al.,*(2008) reported that decrease in gas production up to 83% after addition of oregano to the incubation media was occured. Carvacerol and thymol caused a reduction in gas production (Benchaar*et al.,* 2007).Garcia-Gonzalez*et al.,*(2008) also indivated that the plant active compounds can reduce gas production through lessening methane production by affecting protozoa population. Methane production decreased in a batch culture when essential oils were added at 1 ìL/mL or at 70, 140 and 280 ppm (Jahani-Azizabadi*et al.,* 2014; Jahani-Azizabadi*et al.,* 2011). However, the *in-vivo* study of Beauchemin and McGinn,(2006) did not reveal any effect on methanogensis. The inconsistencies among results obtained in different studies may be attributed to the doses used, the main active compounds in plants, and the basal diet.

The decrease in NH3-N concentration with the addition of EOs was consistent with inhibition proteolysis, peptidolitic and deamination process of rumrn microorganisms (Castillejos*et al.,*2004). McIntosh *et al.,*(2003) demonstrated that a commercial blend of essential oil reduced the rate of amino acid deamination and inhibited the growth of a specific group of ammonia bacteria. Brochers,(1965) and Castillejos*et al.,*(2007) observed that an addition of thyme and oregano EOs to a medium containing rumen liquid resulted in an accumulation of amino acids nitrogen and a decrease in the ammonia nitrogen. Wallace *et al.,*(2002) noted that the antimicrobial properties of EOs affected rumen microbial activities by reducing the dietary protein degradation and deamination, thereby enhancing rumen N escape. On the other hand, dry matter digestibility was influenced by incorporation of EOs, where the effect was the greatest using a mixture of essential oils at both dosages, while it was the lowest in the control diet. We speculate that the lowest dry matter digestibility in the control diet was congruent with greater NDFN content. Undigested nitrogen content was lower in the diet containing a mixture of essential oils at both levels. The ratio of microbial nitrogen and total gas production to dry matter disappearance was the highest in BD when compared to BD_{mix}(Fig. 1).

CONCLUSION

Overall, the present results suggest that the plant essential oils used, especially when mixed EOs were used, resulted in a considerable effect on rumen microbial fermentation and microbial protein yield.Results obtained from the present study demonstrated that by using these EOs in ruminant, we may manipulate rumen fermentation rate by changing in microbial protein yield.

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