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Dose-Response Effects of Various Rumen Microbial Modifier Essential Oils on Protein Degradation Using *in vitro* Gas Production Technique

Research Article

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ABSTRACT

The aim of this study was to investigate the effects of various doses of essential oils (EO) of thyme (TEO) or cinnamon (CEO) on *in vitro* rumen degradation of protein of a commercial total mixed ratio (TMR) containing 16.4% crud protein (CP). Treatments were TMR without any of EOs (control); control plus 25 µL of TEO (TEO25); control plus 50 µL of TEO (TEO50); control plus 25 µL of CEO (CEO25) and control plus 50 µL of CEO (CEO50). Rumen fluid was collected before the morning feeding from two rumen fistulated dairy cows. Approximately, 90 mL of buffered rumen fluid (BRF), 400 mg of feed sample plus carbohydrates (maltose, xylose and starch) at four concentrations (100, 200, 300 and 400 mg) were added to screw-cap bottles. Gas production (mL) and ammonia nitrogen concentration (mg) in each bottle were measured at 4, 8, 12, 24 and 30 h post-incubation and *in vitro* crude protein degradation (IVDP) was calculated via a linear regression. Both TEO and CEO caused a significant reduction in IVDP values compared with those of control ($P<0.05$). The IVDP values for treatments of control, TEO50 and CEO50 after 30 h post incubation were 0.56, 0.33 and 0.48, respectively. Amount of the readily fermentable fraction and the potentially degradable fraction of the feed protein were affected significantly by treatments ($P<0.05$). Also, the effective crude protein degradability (EPD) was significantly affected by the EOs ($P<0.05$). These results suggest that the TEO and CEO might be used for manipulating the ruminal protein degradability.

KEY WORDS ammonia nitrogen, essential oils, feed protein, protein degradability.

INTRODUCTION

Feed proteins in ruminants are decomposed by rumen microorganisms into ammonia and branched-chain fatty acids. The ammonia is then used for de novo synthesis of amino acids by rumen microbes and excess amounts of it absorbed to blood stream trough rumen wall (Leng and Nolan, 1984). The excess ammonia is converted to urea with the cost of energy and some of this urea and non converted ammonia are excreted to environment (Lapierre *et al*. 2005). On the other hand, proteins are necessary for growth and lactation.

In low producing dairy cows, all of protein requirements can be supplied by microbial protein, but in high producing dairy cows the microbial protein is insufficient and by-passes feed protein is essential to meat protein requirements (Santos *et al*. 1998). For improving protein efficiency in the dairy cows, some of chemical agents such as monensin (an ionophore antibiotic) are using widely (Duffield *et al*. 2008), but the use of antibiotics in animal nutrition had some disadvantages such as antibiotic resistance (Lavine and Arrizabalaga, 2011). Therefore, we need to use some kind of effective and safe alternatives for the antibiotic

modifiers. In recent years, the nutritional effects of essential oils (EOs) were studied, frequently. An essential oil (EO) is a complex mixture of plant secondary metabolites that is extracted mainly by distillation (Benchaar *et al*. 2008). The EOs had some important roles in origin plants such as protection against invasive insects, bacteria, viruses and fungi and attracting pollinators (Bakkali *et al*. 2008). So far, a lot of EOs has been identified, which are very diverse in chemical composition and biologic effects (Van de Braak and Leijten, 1999). The EOs often has anti microbial effects against a wide range of bacteria, yeast and fungi (Reichling *et al*. 2009). Therefore, studying the nutritional effects of EOs in ruminant can lead to some new approaches for protection of proteins against ruminal fermentation. Both thyme (*Thymus vulgaris*) and cinnamon (*Cinnamomum cassia*) EOs showed some contradictory effects on CP fermentation in some *in vitro* and *in vivo* studies (Alsaht *et al*. 2014; Macheboeuf *et al*. 2008).

Main effective compounds in thyme and cinnamon EOs are thymol and cinnamaldehyde, respectively, which are classified as aromatic compounds (Bagamboula *et al*. 2004; Paranagama *et al*. 2001). Jahani-Azizabadi *et al*. (2011) announced that between 19 various EOs, the essential oil of cinnamon and thyme had the most effects on reducing *in vitro* release of ammonia in incubation medium. Between 45 various EOs, the CEO shown anti bacterial effects against both gram positive and gram negative bacteria and inhibitory effects against fungi and phages (Chao *et al*. 2000). Carvacrol, another major component that found in TEO showed anti bacterial effects associated with a change in the permeability of the cytoplasmic membrane for protons and potassium ions (Ultee *et al*. 1999). Recent studies also, showed that the use of TEO and CEO can to changes ruminal population and diversity of protozoa and bacteria (Patra and Yu, 2014; Khorrami *et al*. 2014; Talebzadeh *et al*. 2012; Agarwal *et al*. 2009; Ohene-Adjei *et al*. 2008). In these experiments, *in vitro* and *in vivo* application of TEO and CEO and some other EOs that contain thymol lead to a dose dependent reduction in the population of ruminal protozoa. Moreover, the number of some methanogenic and major protein degrading and amino acid-fermenting bacteria was affected by EOs. However, the effect of adding EOs on ruminal protein degradability has not been studied in details (Molero *et al*. 2004; Patra and Yu, 2014). There are a few studies that investigated the effects of EOs on ruminal protein degradability using *in situ* technique (Molero *et al*. 2004; Nanon *et al*. 2014) but, this technique has several shortcomings, including loss of fine feed particles through bags pores (Dewhurst *et al*. 1995) and the technique is inapplicable for soluble feed proteins (Hedqvist and Udén, 2006). On the other hand, *in vivo* studies are expensive, laborious and usually unable to eliminate the complex effects of animal and ruminal bacteria on rumen ammonia pool (Leng and Nolan, 1984). Karlsson *et al*. (2009) described a new *in vitro* gas production technique that facilitate rapid estimates of ruminal protein degradability during the time and eliminate complex effects of *de novo* synthesis of microbial proteins on protein degradability measurements. Therefore, the objective of this study was to evaluate the effects of various concentrations of TEO and CEO on *in vitro* protein degradation of a dairy cow ration using a new gas production technique.

MATERIALS AND METHODS

Feed sample, essential oils and experimental design

In this study the feed sample was a total mixed ratio (TMR) with 78.5% DM that composed of 26.89% alfalfa hay, 19.86% corn silage, 14.10% barley grain, 15.49% corn grain, 4.06% cottonseed meal, 12.23% soybean meal and 7.39% wheat bran.

Thyme was collected from the mountains located in north-east Iran and cinnamon barks were performed as commercially. Then, the EO content of both thyme and cinnamon (leaves and bark, respectively) were extracted with hydro distillation method using clevenger type apparatus. Five treatments in a complete randomized design were used. The treatments with three replicates were incubated simultaneously in three repeated runs. Each of the treatments was consisted of 400 mg of the feed sample with different doses of each EO including: the TMR without any of EOs (control); control plus 25 µL of TEO (TEO25); control plus 50 µL of TEO (TEO50); control plus 25 µL of CEO (CEO25) and control plus 50 µL of CEO (CEO50).

In vitro protein degradation

To determine *in vitro* protein degradation a new gas production technique as described by Karlsson *et al*. (2009) was used. All procedures were fully reported by Falahatizow *et al*. (2015). The rumen fluid was collected manually before the morning feeding from two rumen fistulated lactating Holstein dairy cows (640±38 kg, body weight, 250 DIM and 16 kg milk production) fed a TMR as already reported; animals had free access to both feed and fresh water. The Rumen fluid was filtered through four layers of cheese cloth and incubated with easily fermentable carbohydrates (3.5 g maltose, 1.8 g starch, 1.8 g xylose and 1.8 g pectin per liter of strained rumen fluid) for 3 h at 39 ˚C (pre-incubation). All handling of ruminal inoculum was under a constant stream of CO_2. After pre-incubation, the rumen fluid was buffered (BRF) using a buffered mineral solution (Menke and Steingass, 1988) in the ratio of 1:2. Then, 400 mg of the feed sample and four levels of carbohydrates including 100, 200, 300 and 400 mg comprised of

maltose, xylose and starch in ratio of 2:1:1 were added into a 250 mL screw-cap bottle (Falahatizow *et al.* 2015). Afterwards 90 mL of buffered rumen fluid (BRF) was added to each bottle. The EOs were added to the bottles by an automatic pipette, immediately before adding BRF. The oxygen was removed from the bottles headspace using continuous stream of CO_2. Incubation of the feed sample with graduated amounts of carbohydrates will lead to different amounts of gas production and different levels of ammonia-N.

This provides the possibility of establishing a linear regression between gas production (x-mL) and ammonia-N (y-mg). The incubations were performed in a water bath at 39 ˚C for 30 h. Blanks contained only BRF and treated in the same manner as done for the feed sample.

All incubations were simultaneously carried out in three repeats and three runs. At 4, 8, 12, 24 and 30 h post-incubation, gas production volume was recorded and liquid samples for ammonia nitrogen measurements were drawn from each bottle.

The gas pressure builds inside the bottle was measured manually by an electronic pressure transducer (Pressure Sensor, PSA-01, Autonics) and through a special duct for the measurement of the gas pressure. The gas pressure was converted into volume using an experimentally calibrated curve (Jahani-Azizabadi *et al.* 2011).

The liquid samples were drawn manually by a 5 mL plastic syringe and without opening the door of the screw-cap incubation bottles, through a separate special duct and a fixed fine plastic tube. Liquid samples were immediately cooled down using water-ice mixture and then centrifuged (3000×*g* at 5 ˚C for 10 minute) and supernatant was collected. All samples were stored in freezer at -20 ˚C until chemical analysis.

Chemical analysis

Feed sample was ground (1 mm particle diameter), then the chemical composition was determined as follows. Dry matter (DM) was determined by drying at 65 ˚C for 48 h using air-forced drying oven. Total nitrogen was determined using Kjeldahl method according to AOAC (1990) by a Kjeltec Analyzer Unit (Kjeltec 2300 Auto analyzer, Foss Tecator AB, Hoganas, Sweden). Ether extract was extracted with hexane solvent by Soxhlet extractor (Soxtec system HT, tacator, Sweden) according to AOAC (1990). Neutral detergent fiber (NDF) was determined according to Van Soest *et al.* (1991) without alpha amylase. Ammonia nitrogen concentration was measured in supernatant using a phenol-hypochlorite reaction (Weatherburn, 1967).

Biometric analysis

In vitro rumen degradation of protein (IVDP) values were calculated via estimated intercept of linear regression between GP (as main variable, x) and ammonia nitrogen (as dependent variable, y) and assumed that regression equations intercept (b_0) is indicator of total ammonia nitrogen that released at zero gas production as discussed by Raab *et al.* (1983). Then, b_0 and related ammonia nitrogen concentration from blanks were replaced in below equation (Raab *et al.* 1983) to calculate the amount of IVDP for each time point:

IVDP= (b_0-(NH_3-N from blanks)) / (total nitrogen of feed sample) [1]

The kinetic parameters of rumen crude protein degradation were estimated by fitting the IVDP values to the following equation (Ørskov and McDonald, 1979) using SAS software (SAS, 2002):

$$Y= a + b(1-e^{-ct}) \quad [2]$$

Where:
Y: degraded part of crude protein at time t.
a, b and c: soluble part, potentially degradable part and the constant rate of degradable b fraction of crude protein, respectively.

Effective crud protein degradability (EPD) value was calculated via the equation of Ørskov and McDonald (1979):

$$EPD= a + (b×c/k+c) \quad [3]$$

Where:
a, b and c: defined above and out flow rate (k) assumed to be 0.06/h.

The effects of different doses of the EOs on the IVDP values, kinetic parameters of CP degradation and EPD were statistically determined using the GLM procedure of SAS (SAS, 2002), using the model:

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

Where:
y: dependent variable.
μ: overall mean.
A_i: effect of the essential oil at each concentration level used.
e_{ij}: residual error.

Dunnett's test was employed to compare the means with those of the control (P<0.05).

RESULTS AND DISCUSSION

Chemical composition of the diet used was as follows: 16.4% crud protein, 6.1% Ether extract, 30.6% neutral detergent fiber, 18.6% acid detergent fiber and 6% ash. The concentration of ammonia nitrogen in the rumen fluid before pre-incubation was 7.33 and after treatment was declined to 3.69 mg/dL (SEM=0.90). The pre-incubation treatment caused to reduce ammonia nitrogen levels in rumen fluid inoculants considerably (P<0.001). The concentration of ammonia nitrogen in the rumen fluid after pre-incubation was on average 54% lower than those of the untreated rumen fluid.

Estimates of IVDP at various post-incubation times were significantly affected by different doses of the EOs (P<0.05, Table 1). Approximately, at each post-incubation (time) except 12 h, adding EOs reduced protein degradability. In general, IVDP values declined when the incubation time and the dose of EO were increased. Except at 4 h incubation, at the all times points of post-incubation, TEO50 had the lowest IVDP values. Also, in both doses, the effects of TEO on reducing IVDP were stronger than CEO. At the dose of 25 µL, CEO25 at 4 h and 12 h, and the TEO25 only at 4 h post-incubation had higher IVDP value than those of the control.

In vitro CP degradation parameters and EPD values are shown in the Table 2. Readily fermentable fraction of the feed protein (a) was reduced significantly by all of the treatments compared with those of the control (P<0.05). Amount of the readily fermentable fraction was reduced by the TEO25 more than other treatments. Potentially degradable fraction of the feed protein (b) was increased significantly by treatments except TEO50 (P<0.05). The b fraction was reduced numerically by TEO50. Values of EPD of the feed protein were decreased considerably by treatments (P<0.05). The reduction in EPD value for TEO50 was more than other treatments. Amounts of the EPD values were decreased whit increasing application dose of the EOs.

This experiment was conducted using an *in vitro* technique, which is innovated to eliminate complex effects about determination of CP degradation in the rumen (due to *de novo* synthesis of protein by rumen bacteria simultaneously with degradation of feed proteins) (Karlsson *et al.* 2009). A significant decrease in ammonia nitrogen after pre-incubation due to the use of ammonia and carbohydrates for microbial protein synthesis suggests that favorable microbial activity was existed in the rumen fluid immediately before the start of experiment. In this experiment reduction in IVDP values due to adding EOs to the incubation medium indicated that the application of both TEO and CEO might be effective in reducing protein degradability in the rumen.

These results confirmed the data indicated the effects of cinnamon and thyme on the decline of *in vitro* protein disappearance reported by Jahani-azizabadi *et al.* (2011). Several authors reported a reducing effect on ammonia concentration in the incubation medium by both cinnamon and thyme (Jahani-Azizabadi *et al.* 2011; Jahani-Azizabadi *et al.* 2014).

Ammonia is the main end product of the breakdown of proteins in the rumen (Krishnamoorthy *et al.* 1990) which monitored in the gas production technique used in the current study to estimate IVDP values. Therefore, the reductions in IVDP values that were observed in current study indicated a reducing effect by TEO and CEO on *in vitro* ammonia production. However, Newbold *et al.* (2004) showed that a blend of EOs including thymol decreased deamination of amino acid and degradation of proteins without considerable effect on ammonia production. The main reason for the effects of EOs on ammonia concentration refers to their effects on ammonia hyper producing bacteria (McIntosh *et al.* 2003).

Most of ammonia hyper producing bacteria are gram positive (Russell *et al.* 1988). Gram positive bacteria are more sensitive to EOs than gram negative bacteria (Burt, 2004). Some recent studies indicated that essential oils including TEO and CEO can to change the number of major protein degrading and amino acid-fermenting bacteria in the rumen (Patra and Yu, 2014; Khorrami *et al.* 2014). Patra and Yu (2014) reported that origanum oil (contain thymol as main component) showed greater reduction in ammonia concentration and bacterial population than other EOs in the *in vitro* incubations. They attributed these anti microbial and ammonia inhibitory effects of the origanum oil to thymol and its phenolic structure. Also, these recent studies indicated that application of the EOs resulted in a significant reduction in protozoal population in the rumen (Patra and Yu, 2014; Khorrami *et al.* 2014). 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 and IVDP estimates in the current study may be due to anti-protozoa effects of the TEO and CEO (Lorenz *et al.* 2011). In the present study both EOs decreased IVDP values during the beginning of post-incubation. However, at the final points of the incubation, reduction in IVDP values for those containing TEO were more than treatments containing CEO, especially at the dose of 50 µL of EO. Thymol is a monoterpene phenol compound and main constituent of TEO (32-46 percent of essential oil) and anti microbial effects of TEO are correlates with its thymol content (Porte and Godoy, 2008; Varga *et al.* 2015; Shabnum and Wagay, 2011).

Table 1 *In vitro* extend of rumen CP degradation over 30 h incubation of a typical lactating dairy cow ration in responses to various doses of essential oils using a gas production technique

Time/h	Treatments					
	Control	TEO25	TEO50	CEO25	CEO50	SEM
4	0.19	0.15*	0.18	0.21*	0.17	0.009
8	0.27	0.26*	0.20*	0.26	0.23	0.011
12	0.34	0.36	0.25*	0.38*	0.30*	0.012
24	0.51	0.48*	0.30*	0.42*	0.37*	0.012
30	0.56	0.50*	0.33*	0.54	0.48*	0.015

Control: a total mixed ratio (TMR) contain 16.4% crude protein (CP) without essential oils (EO); TEO25: control plus 25 µL of thyme essencial oil (TEO); TEO50: control plus 50 µL of TEO; CEO25: control plus 25 µL of cinnamon essential oil (CEO) and CEO50: control plus 50 µL of CEO.
SEM: standard error of the means.
* ($P<0.05$).

Table 2 *In vitro* degradation parameters and effective degradability of CP (EPD) of a typical lactating dairy cow ration in responses to various doses of essential oils using a gas production technique

Kinetic parameter	Treatments					
	Control	TEO25	TEO50	CEO25	CEO50	SEM
a	0.27	0.03*	0.14*	0.09*	0.11*	0.02
b	0.44	0.65*	0.35	0.7*	0.63*	0.06
c	0.039	0.056	0.028	0.040	0.028	0.01
EPD	0.42	0.34*	0.25*	0.36*	0.28*	0.02

Control: a total mixed ratio (TMR) contain 16.4% crude protein (CP) without essential oils (EO); TEO25: control plus 25 µL of thyme essencial oil (TEO); TEO50: control plus 50 µL of TEO; CEO25: control plus 25 µL of cinnamon essential oil (CEO) and CEO50: control plus 50 µL of CEO.
a: immediately degradable fraction of protein; *b*: potentially degradable fraction of protein and *c*: disappearance rate of *b* fraction.
SEM: standard error of the means.
* ($P<0.05$).

Carvacrol and thymol as the major component of the TEO, showed antimicrobial effects against both gram positive and gram negative bacteria by disintegrating the cell membrane of bacteria while, CEO did not disintegrate the cell membrane or deplete the intracellular ATP pool (Burt, 2004). Cinnamaldehyde is an aldehyde and main constituent component of the CEO (42-92 percent of essential oil) and inhibit bacterial growth mainly due to preventing of the action of protease enzymes (Chang *et al.* 2013; Koroch *et al.* 2007; Burt, 2004). Therefore, these different effects of the two EOs on IVDP values can be explained by different mode of action of them. Our results confirmed the findings of Jahani-azizabadi *et al.* (2014) who reported that TEO and CEO decreased *in vitro* CP disappearance and TEO had the most reducing effect on CP disappearance among seven EOs. Benchaar *et al.* (2007) conducted an *in vitro* study using EOs and their components.

They concluded that the TEO and CEO and their components had no effect on rumen ammonia concentration and protein degradability. Contradictory effects of EOs that observed in previous studies may be due to using diets with different CP concentrates which alter bacterial population and PH (Cardozo *et al.* 2005; Wallace *et al.* 2002; Vlaeminck *et al.* 2006). Molero *et al.* (2004) using *in situ* studies concluded that the ratio of forage to concentrate and rumen pH may to alter influence of the EOs on rumenal protein degradation. In addition, rumen fluids which used as inoculants for *in vitro* studies may affect results (Krishnamoorthy *et al.* 1990).

In our study, the TEO at the dose of 50 µL reduced the EPD value of feed protein considerably. The estimates of the EPD are influenced by the assumed rumen passage rate (k) and the kinetic parameters of degradation (formula No. 3).

Among the kinetic parameters of degradation, the immediately degradable fraction of protein (*a* fraction) has the most impact on the estimate of EPD. The EOs reduced the contribution of the immediately degradable fraction of protein (*a*) of the total CP and in contrast those increased the contribution of the potentially degradable fraction of feed CP (*b* fraction).

The rate of degradation of *b* (i.e. the *c* parameter) was not affected by Eos, therefore, this confirm that the main reason for observed reduction in IVDP and EPD values because of the application of Eos refer to their effects on degradability of the soluble proteins (*a*). Rodriguez *et al.* (2000) according to lipid content of the rumen liquid associated bacteria in comparison to solid associated bacteria indicated that gram positive bacteria are predominant population in liquid phase of the rumen content, which those are more sensitive to the EOs.

Nevertheless, long term *in vivo* studies about the efficiency of EOs in ruminant nutrition; don't report any considerable beneficial effects on nutritional efficiency (Benchaar *et al.* 2007; Alsaht *et al.* 2014). Therefore, more *in vitro* and *in vivo* studies at most similar conditions should be conducted to reveal actual effects of EOs on rumen metabolism.

CONCLUSION

The results of this study showed that the application of essential oils might alter the amounts of protein degradation in the rumen. Under the condition of the present study, both TEO and CEO caused to reduce ruminal CP degraded, which the observed decreases were enhanced with the increasing of the dose of the EOs. Both TEO and CEO decreased the immediately degradable fraction of the feed protein evaluated while increased the potentially degradable fraction. However, the degradation rate of the CP was not affected significantly. Effective protein degradability values were decreased by the increasing of the application doses of the EOs and the highest reduction in EPD was occurred when 50 µL of TEO were added to the incubation medium. These results suggest that both TEO and CEO have a potential to manipulate the ruminal protein degradability. However, more *in vitro* and *in vivo* studies at the same conditions are proposed to achieve practical recommendations for widespread use of the TEO and CEO in ruminant nutrition.

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