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Effects of Thyme Essential Oil and Disodium Fumarate on Ruminal Fermentation Characteristics, Microbial Population and Nutrient Flow in a Dual Flow Continuous Culture System

Research Article

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ABSTRACT

The aim of the present study was to investigate the effects of di-sodium fumarate (DSF) and thyme essential oil (TEO) solely and simultaneously on ruminal fermentation properties and microbial abundance. A dual-flow continuous culture system (DFCC) with eight 1400-mL fermenters was used in a period of 12 d that divided to 9 d for adaptation and 3 d for sampling. Fermenters were fed 100 g d^{-1} [dry matter (DM) basis] of a 50:50 alfalfa hay:concentrate ration. Treatments were no additive (control), 10 mM of DSF, 125 µL/L of TEO and simultaneous use of 10 mM of DSF, and 125 µL/L of TEO (SIMTF). Treatments had no effect on organic matter (OM) and neutral detergent fiber (NDF) disappearance, total and cellulolytic bacteria and protozoa abundance, large peptides and N-NH_3 concentration of the effluent, N-NH_3 and dietary N flow and molar proportions of acetate (C2), butyrate and isovalerate. DSF significantly increased crude protein (CP) degradation, the molar proportion of propionate (C3) and reduced C2:C3 ratio ($P<0.05$). TEO decreased ($P<0.05$) DM disappearance (-14.4%) and total volatile fatty acid (-19.4%) concentration, relative to the DSF. Relative to the control, small peptide plus amino acid N concentration was higher ($P<0.05$) in TEO treatment and DSF. SIMTF increased ($P<0.05$) the acid detergent fiber (ADF) disappearance and decreased the N-NH_3 concentration from zero to 4 h after feeding. Total N, non-ammonia N and bacterial N flow and efficiency of microbial CP synthesis increased with SIMTF. Findings demonstrated that feed efficiency and ruminal fermentation were improved by the simultaneous use of DSF and TEO.

KEY WORDS cellulolytic bacteria, essential oil, microbial crude protein, rumen fermentation.

INTRODUCTION

Intermediates in the succinate-propionate pathway such as organic acids can reduce the production of methane by acting as hydrogen acceptors (Martin, 1998; Lopez *et al.* 1999a; Newbold *et al.* 2005). Fumarate is a hydrogen sink and acting as C3 precursor in the rumen. Fumarate (salts and free acid form) and malate supplementation have been examined for manipulation and enhance the efficiency of the ruminal anaerobic fermentation process (Garcia-Martinez *et al.* 2005; Lin *et al.* 2013; Riede *et al.* 2013). Previous studies have reported that inclusion of fumarate in ruminant diets, decrease methane production, C2:C3 ratio and ammonia nitrogen concentration, and increase the number of cellulolytic bacteria and organic matter (OM) disappearance (Lopez *et al.* 1999b). Newbold *et al.* (2004) demonstrated that sodium fumarate had efficient effects on fiber digestion, dry matter degradation and hydrogen acceptation in batch culture and rumen simulation technique (RUSITEC) compared with free acids and sodium acrylate.

On the contrary, the effects of fumarate are unclear in *vivo* studies and presumably, it is due to the unknown of the optimum level of fumarate. Zhou *et al.* (2012) observed that the addition of 20 g/d di-sodium fumarate (DSF) in Hu sheep diet fed by a high-forage ration did not affect C3 proportion and C2:C3 ratio. Yang *et al.* (2012) indicated that the addition of 10 g/d DSF in goats fed according to maintenance requirements, increased C3 proportion, but not change C2:C3 ratio. Remling *et al.* (2011) reported that 100, 200 or 300 g/d of fumaric acid did not affect the performance of growing bulls fed with full forage diet.

There is some evidence that essential oil (EO) and some secondary compounds can reduce C2:C3 ratio, methane and N-NH_3 production through specific inhibition of ruminal gram-positive microbes that usually involvement in C2, methane and ammonia production (Szumacher and Cieslak, 2010). Thyme essential oil (TEO) as regards owning thymol, carvacrol, p-cymene and γ-terpinene have higher antibacterial properties (Ultee *et al.* 2002; Martinez *et al.* 2006). Researchers demonstrated that the use of a blend of essential oils includes thymol (Castillejos *et al.* 2006; Castillejos *et al.* 2007) and thyme EO (Jahani-Azizabadi *et al.* 2011) resulted in a decrease in C2:C3 ratio, methane and N-NH_3 concentration. The addition of thymol and eugenol at 5, 50 and 500 mg/L in DFCC system implied that 5 mg/L of thymol tended to decrease the proportion of C2, and increase the large peptide (LPep N) concentration without reducing total volatile fatty acid (VFA) concentration (Castillejos *et al.* 2006).

In our previous study, we examined the effect of different doses of TEO (from 100 to 400 µL/L) and DSF (from 8 to 12 mM) on *in vitro* batch culture rumen anaerobic fermentation characteristics. Baraz *et al.* (2018) demonstrated that 125 µL/L of TEO and 10 mM of DSF generally resulted improve in ruminal fermentation (decrease in methane, N-NH_3, C2:C3 ratio and nonsignificant decrease in total VFA and significant increase organic matter disappearance). But, it is still unclear, what is the effect of TEO and DSF solely and simultaneously in the DFCC system that is much nearer to the active dynamic of ruminant's rumen. In addition, when EO with high antimicrobial activity is applied for rumen manipulation and reduce methane production, the hydrogen produced from organic matter fermentation will be accumulated in rumen fluid (Lin *et al.* 2012; Lin *et al.* 2013), so, employ an alternative pathway for capturing of reducing equivalents can result to ultra-improve in the rumen anaerobic fermentation. Therefore, the objective of the present study was to evaluate the effect of TEO and DSF and their synergistic effects on rumen anaerobic microbial fermentation of a 50:50 alfalfa hay: concentrate diet in the DFCC system.

MATERIALS AND METHODS

Experimental design and treatments

A DFCC system with eight 1400-mL fermentors was used in a period of 12 d that divided into adaptation (9 d) and sampling (3 d) periods. On the first day to begin, 400 mL of strained rumen fluid and 1000 mL of pre-warmed McDougall's buffer (McDougall, 1948) were added to each fermenter. Fermentors were fed 100 g/d DM (in three equal meals, every 8 h) of a diet included a 50:50 alfalfa hay:concentrate. Chemical composition and ingredients of offered ration presented in Table 1. Rumen fluid was taken from 10 slaughtered rams that fed with a 60:40 forage: concentrate diet. Fermentor's temperature was maintained at 38.5 ˚C by a circulating water bath.

For maintaining the anaerobic conditions N_2 gas at 40 mL min^{-1} infused into fermenters. Artificial saliva which contains urea solution (0.4 g urea/L) for simulate ruminal N recycling, was continuously infused into the fermenters. Liquid and solid dilution rates maintained at 10 and 5 percent h^{-1}, respectively.

Treatments were; control without additive (CON), DSF at 10 mM (DSF; Disodium salt; Sigma Chemical Co., Poole, and Dorset, UK), TEO at 125 µL/L (TEO; MONIN Company, France) and 10 mM of DSF plus 125 µL/L of TEO (SIMTF). All treatments were incorporated directly into the contents of the fermentor at d 4 of the experiment before morning feeding. TEO was dissolved in ethanol (96%; 1:1 ethanol:TEO) before incorporating into fermenters. Fermenters selected for the CON group were also supplied 125 µL/L of ethanol per feeding.

Sample collection and preparing

During sampling days (the final 3 d), 20 mL of filtrated (filter pore size was 48 µm) fermentors contents were taken at zero, 1, 2, 3 and 4 h after the feeding (on the morning meal) to determine N-NH_3 concentration and pH. Moreover, at 4 h after the morning feeding, 1.5 mL of strained sample was removed and mixed with 375 µL of 25% orthophosphoric acid to determine VFA concentration and frozen at −20 ˚C. During sampling days, effluent collection vessels were maintained in cold water (4 ˚C) for the debarment of additional microbial fermentation.

All content of collection vessels was mixed for 1 min, then immediately strained through cheesecloth to eliminate solid and liquid parts, and solid residues were dried in the oven (55 ˚C for 48 hours) and used to estimate DM, CP, OM, NDF and ADF digestibility. Subsamples of the filtrate were used for the determination of total N, ammonia N, tungstic acid (TA) soluble N and trichloroacetic acid (TCA) soluble N.

Table 1 Ingredients and chemical composition of experimental diet

Composition	Amount
Ingredients, % of DM	
Alfalfa hay	50
Corn grain	16.5
Barley grain	18.5
Soybean meal	9.8
Wheat bran	4.5
Salt	0.2
Mineral and vitamin premix[1]	0.5
Chemical composition, g/kg DM	
Crude protein	154
Neutral detergent fiber	290
Acid detergent fiber	190
Ether extract	23
Ash	74
Non-fiber carbohydrates[2]	456

[1] Each kg of premix containing: Ca: 190 g; P: 90 g; Na: 50 g; Mg: 19 g; Cu: 3 g; Fe: 3 g; Mn: 2 g; Zn: 3 g; Co: 100 mg; I: 100 mg; Se: 1 mg; vitamin A: 500000 IU; vitamin D_3: 100000 IU; vitamin E: 100 mg and Antioxidant: 3 g.
[2] NFC= 100 - (NDF+CP+EE+Ash).

Samples for determining bacterial N were procured from the 2 sampling d, followed the procedure of (Makkar *et al.* 1982). The effluents (30 mL) were centrifuged in two-time at 3000 × g at 4 ˚C for 10 min to separate feed particles, in each time supernatants were separated and stored in another tube and pellets were washed with normal saline (0.9% NaCl) and centrifuged. Then the supernatants of previous steps were collected and centrifuged at 15000 × g for 20 min. The supernatants were discarded and the pellet (bacterial cells) dried in the oven (55 ˚C for 48 h) and used to determine bacterial N by the Kjeldahl procedure. The flows of total N, dietary N, bacterial N and non-ammonia N (NAN) were calculated as described by Stern and Hoover (1990).

On the final day, 2 mL of fermenters' contents were taken at 4 h after the morning feeding to the enumeration of total viable and cellulolytic bacteria by most probable number (MPN) procedure from 3 replicate (Oblinger and Koburger, 1975).

The medium samples were serially diluted (10 fold increments) in the liquid version of mediums in the Hungate tubes, 3 replicate per each dilution (Caldwell and Bryant, 1966). For cellulolytic bacteria, cellulose filters paper used as a single source of energy. The tubes were incubated at 38.6 ˚C for 14 d, and then growth was scored (positive or negative). For the enumeration of total viable and cellulolytic bacteria, was considered pH variation and disappearance of cellulose filter paper (Whatman® No. 7), respectively.

For protozoa counting, 7 mL of fermenters' contents were fixed in an equal volume of a 50% formalin solution (18.5% formaldehyde concentration). Protozoa were enumerated microscopically in a Sedgwick-Rafter counting chamber according to Dehority (1984) method.

Chemical analyses

VFA concentration was determined using gas chromatography (GC, Philips, pu 4410). Solid residues or diet samples were dried using an oven at 55 ˚C for 48 h and analyzed for DM, CP, and OM based on the methods recommended by AOAC (1995). The crude protein of diet and solid residues, total N of effluent, bacterial N and N-NH_3 concentration of the contents of the fermentor were determined using the Kjeldahl method (Kjeltec Vapodest 30s, Gerhardt).

The TCA- and TA-soluble N concentration of effluents were determined as described by Winter *et al.* (1964). The feed and effluent samples were dried (at 55 ˚C for 48 h), ground to pass through a 1 mm screen. The ashless NDF and ADF content of the diet and solid phase of effluents were determined using the method recommended by Van Soest *et al.* (1991) and AOAC (1995), respectively.

Calculations and statistical analysis

Results of the TCA and TA soluble N concentration of liquid phase of effluents were used for calculation of large peptide (LPep (mg/dL)= LPep= TCA soluble N - TA soluble N) and small peptide plus amino acid N (SPep+AAN (mg/dL)= SPep + AAN =TA soluble N-ammonia N).

All statistical analyses were conducted using generalized linear model (GLM) procedure of SAS (2001) with the following statistical model:

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

Where:
Y_{ij}: dependent variable.
μ: overall mean.
T_i: effect of the treatments at each concentration.
e_{ij}: residual error.

The results of N-NH_3 concentration and medium pH were analyzed as randomized block design. Tukey test was employed to compare the means (P<0.05).

RESULTS AND DISCUSSION

Nutrients disappearance and microbial abundance

The effects of treatments on the abundance of total viable and cellulolytic bacteria, protozoa and nutrients disappearance are presented in Table 2. Dry matter disappearance was significantly (P<0.05) decreased by TEO supplementation, relative to those of the DSF (596 *vs*. 696 g/kg, respectively). Relative to the control, DM, OM and NDF disappearance did not affect by treatments, although they were numerically lower in TEO. Supplementation of the TEO and DSF significantly increased (P<0.05) ADF disappearance, relative to the CON and TEO solely. Total viable bacteria, cellulolytic and protozoa abundance were not affected by treatments.

Crude protein degradation and N metabolism

Treatments had no effect on effluent N-NH_3 concentration at 4h after morning feeding (Table 3). Relative to the control, DSF resulted in an increase in CP degradation. But, the addition of TEO relative to the SIMTF, decreased (P<0.05) total and non-ammonia N concentration. The SIMFT treatment resulted in an increase (P<0.05) in bacteria N flow and efficiency of microbial crude protein synthesis (EMPS) (Table 3). The results of our study showed that LPep N concentration was not affected by the addition of treatments (Table 3). However, the concentration of SPep + AA N was higher (P<0.05) in TEO compared to the CON and DSF. The concentration of medium N-NH_3 was evaluated from zero to 4h post-feeding (Figure 1). Results showed that the simultaneous use of DSF and TEO (SIMTF) decreased N-NH_3 concentration at zero, 2 and 4 h after feeding, compared to the CON (P<0.05).

Volatile fatty acid concentration and medium pH

In the present study, the pH of fermentors was evaluated from 0 to 4 h of post-morning feeding (Figure 2). The inclusion of DSF in the diet resulted in an increase (P<0.05) in the minimum and average of medium pH, relative to TEO and CON (Table 4). In addition, the medium pH at all recording time in DSF, TEO and SIMTF was higher than CON (Figure 2). The findings of the present study showed that the addition of DSF and TEO solely and simultaneously did not affect (P>0.05) proportion of C2, C4 and isovalerate (Table 4). Adversely, relative to the CON and use of TEO significantly decreased total VFA concentration (-19.4%, P<0.05).

DSF and SIMTF treatments increased (P<0.05) the proportion of C3 (14.7 and 20.0%, respectively) and decreased C2:C3 ratio compared to those of the CON and TEO treatments (P<0.05).

Fumarate affects rumen fermentation as H acceptor and as a precursor for C3 (Newbold *et al.* 2005), on other hand TEO effects on rumen fermentation because it's antimicrobial activity (Ultee *et al.* 2002). Therefore, it appears that simultaneously use of DSF and TEO may lead to synergistic effects on rumen microbial anaerobic fermentation. The decrease in DM disappearance with TEO supplementation was in agreement with previous studies (Castillejos *et al.* 2007; Jahani-Azizabadi *et al.* 2014; Pirondini *et al.* 2015). For instance, Jahani-Azizabadi *et al.* (2014) reported a 21.6 and 66% decrease in DM and NDF disappearance with supplemented 280 µL/L of TEO, which was associated with inhibitory and antibacterial effects on rumen bacteria (Ultee *et al.* 2002). Since the fumarate is an intermediate in rumen microbial metabolism, it appears that the addition of fumarate at 10 mM in SIMTF may be removed some negative effect of TEO on DM disappearance (Table 2).

Relative to the control, we observed an increase (P>0.05) in cellulolytic bacteria with DSF (2.3%) and SIMTF (1.8%) supplementation. There has been some evidence that the use of fumarate leads to an increase in the abundance of cellulolytic and some fumarate-utilizing bacteria (Lopez *et al.* 1999b). It's assumed that the metabolism of fibrinolytic bacteria intermediate products (such as hydrogen) accelerate by fumarate-utilizing bacteria (Mao *et al.* 2007). On the other hand, an increase in ADF disappearance without significant change in abundance of cellulolytic bacteria, demonstrating that some obligate fiber fermenting bacteria that had a higher activity for fermentation of fiber fractions relatively increased. We observed that protozoa abundance did not affect with DSF and TEO. I agreeing to our findings, Lopez *et al.* (1999b) reported that fumarate was unable to changes in the protozoa population, likewise, Lin *et al.* (2012) observed that under *in vitro* condition, 500 mg/L of essential oil active compounds (EOAC) inhibited protozoa. Although, in the current study were observed a numerical decrease in the abundance of protozoa with the addition of TEO (P=0.12). It seems that a decrease in the protozoa population maybe one of the wide-range effects of essential oils on microbial activities.

Higher NAN in SIMTF and consequently increased bacterial N (0.97 g/d) flow relative to the CON (0.69 g/d) demonstrated that SIMTF had more efficiency in increase organic matter fermentability and microbial protein synthesis. Results showed that SIMTF treatment resulted in a significant increase in the EMPS compared with those of the CON (15.76 *vs*. 11.25 g N/kg of digested OM, P<0.05).

Table 2 Effects of di-sodium fumarate and thyme essential oil solely and simultaneously (SIMTF) on nutrients digestibilities and abundance of total and cellulolytic bacteria in a dual-flow continuous culture system

Item	Treatment				SEM	P-value
	CON	DSF	TEO	SIMTF		
Digestibilities, g/kg of DM						
Dry matter	639^{ab}	696^{a}	596^{b}	654^{ab}	13.5	0.050
Organic matter	662	684	646	667	11.3	0.713
Neutral detergent fiber	543	534	495	549	13.7	0.535
Acid detergent fiber	411^{b}	506^{ab}	424^{b}	547^{a}	17.4	0.004
Microbes, log10/mL of rumen fluid						
Total bacteria	10.18	10.23	10.02	10.14	0.039	0.307
Total cellulolytic	9.57	9.79	9.45	9.74	0.069	0.335
Total protozoa	4.57	4.58	4.53	4.55	0.007	0.134

CON: control; DSF: 10 mM di-sodium fumarate; TEO: 125 µL/L thyme essential oil and SIMTF: 10 mM DSF and 125 µL/L TEO.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 3 Effects of di-sodium fumarate and thyme essential oil solely and simultaneously (SIMTF) on the flow of N in a dual-flow continuous culture system

Item	Treatment				SEM	P-value
	CON	DSF	TEO	SIMTF		
NH_3 N, mg/dL	14.93	14.85	14.61	15.67	0.225	0.405
N flow, g/d						
Total	2.01^{ab}	2.04^{ab}	1.86^{b}	2.19^{a}	0.039	0.008
Ammonia	0.33	0.30	0.32	0.31	0.006	0.271
Non-ammonia	1.68^{ab}	1.74^{ab}	1.54^{b}	1.88^{a}	0.040	0.007
Dietary	0.99	0.83	1.00	0.91	0.097	0.165
Bacterial	0.69^{b}	0.73^{b}	0.72^{b}	0.97^{a}	0.037	< 0.01
Crude protein degradation, %	56.9^{b}	64.3^{a}	57.9^{ab}	59.8^{ab}	1.10	0.049
EMPS, g/kg of digested OM	11.25^{b}	11.58^{b}	12.07^{b}	15.76^{a}	0.887	< 0.01
N fraction, mg/dL						
LPep N	6.38	7.32	4.72	6.82	0.406	0.085
SPep + AA N	6.50^{b}	6.27^{b}	10.98^{a}	7.71^{ab}	0.730	0.022

CON: control; DSF: 10 mM di-sodium fumarate; TEO: 125 µL/L thyme essential oil and SIMTF: 10 mM DSF and 125 µL/L TEO.
EMPS: efficiency of microbial crude protein synthesis; LPep N: N from large peptides and SPep + AA N: N from small peptides and AA.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

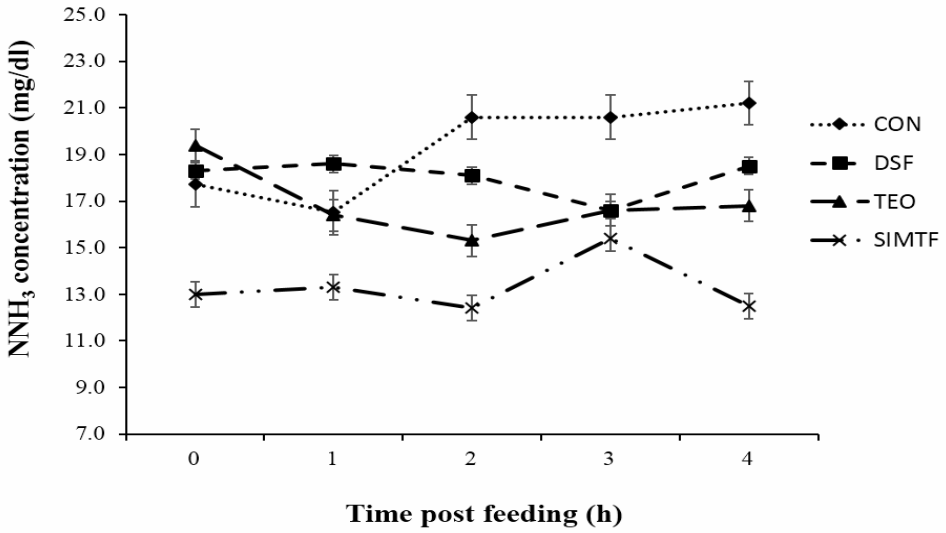

Figure 1 The effect of di-sodium fumarate and thyme essential oil solely and simultaneously (SIMTF) on medium NH_3-N fluctuation in a dual-flow continuous culture system

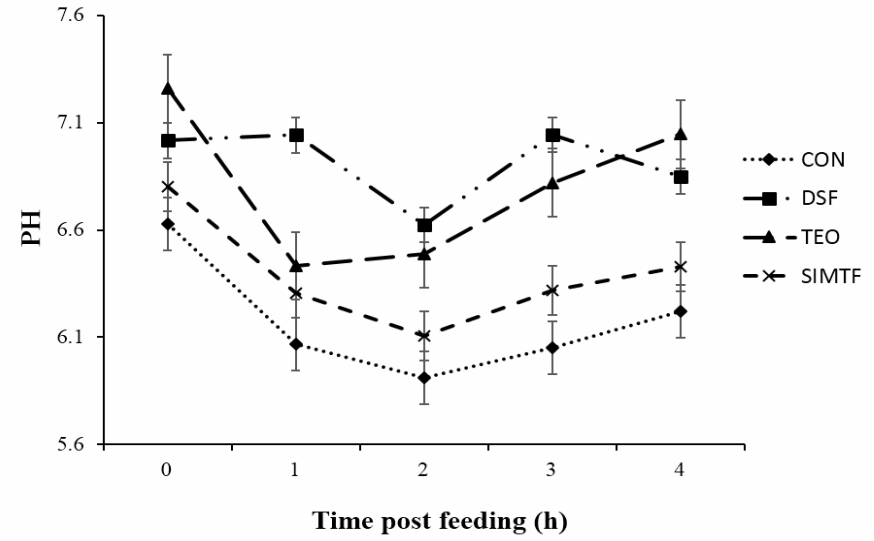

Figure 2 The effect of di-sodium fumarate and thyme essential oil solely and simultaneously (SIMTF) on medium pH fluctuation in a dual-flow continuous culture system

Table 4 Effects of di-sodium fumarate and thyme essential oil solely and simultaneously (SIMTF) on total and individual volatile fatty acid (VFA) concentration at 4 h post-feeding and pH in the dual-flow continuous culture system

Item	Treatment				SEM	P-value
	CON	DSF	TEO	SIMTF		
pH						
Maximum	6.65	7.20	7.25	6.90	0.119	0.271
Minimum	5.95^{b}	6.60^{a}	6.35^{b}	6.10^{b}	0.100	0.028
Average	6.17^{b}	6.91^{a}	6.81^{a}	6.39^{b}	0.117	0.005
Total VFA, mM	149.6^{a}	150.5^{a}	120.6^{b}	158.1^{a}	5.69	0.017
Individual, mol/100 mol						
Acetate	47.03	43.33	48.96	46.14	0.893	0.119
Propionate	26.66^{b}	30.58^{a}	25.97^{b}	32.0^{a}	0.969	< 0.01
Butyrate	15.39	17.96	15.62	14.19	0.716	0.355
Isovalerate	2.03	1.97	2.21	1.49	0.170	0.589
C2:C3 ‡	1.77^{a}	1.42^{b}	1.88^{a}	1.44^{b}	0.078	0.005

CON: control; DSF: 10 mM di-sodium fumarate; TEO: 125 µL/L thyme essential oil and SIMTF: 10 mM DSF and 125 µL/L TEO.
C2:C3: acetate:propionate ratio.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Relative to the CON, solely the use of TEO did not affect the N flow. Similarly, in the previous studies that evaluated the effect of supplementation of thymol (a main active component of thyme essential oil) in the DFCC system (Castillejos *et al.* 2006; Castillejos *et al.* 2007), observed that N-NH_3 concentration, N flows, EMPS and CP degradation were not affected by treatments. Supplying of DSF significantly increased CP degradation around 13% compared to those of the CON (P<0.05). Numerically this effect appeared with an accumulation of LPep N (P>0.05), which was higher in DSF relative to the CON treatment. The increase in CP degradation without an increase in N-NH_3 concentration suggested that DSF supplementation may result in an increase in ruminal efficiency use of dietary protein and a decrease in microbial deamination of amino acids.

The accumulation of SPep + AA N with TEO supplementation, suggests that peptidolysis stimulated or deamination was inhibited by TEO. In agreement with our findings, Castillejos *et al.* (2006) observed an increase in the concentration of LPep and SPep + AA N by thymol supplementation at 500 mg/L. Our findings showed that the simultaneous use of DSF and TEO (SIMTF) decreased the rate of N-NH_3 production from 0 to 4 h after morning feeding, relative to the CON (Figure 1). McIntosh *et al.* (2003) reported that a commercial blend of EO compounds containing thymol, through inhibiting the growth of ammonia hyper-producing bacteria, resulted in a significant decrease in the rate of amino acid deamination. Castillejos *et al.* (2007) reported that the addition of a commercial blend of EO (Crina®) resulted in a higher accumulation of SPep + AA N and a decline in the N-NH_3 concentration.

Similarly, several *in vitro* and *in situ* studies demonstrated that the concentration of N-NH_3 (Jahani-Azizabadi *et al.* 2011) was lower (-16 to -44%) and deaminase inhibited (Newbold *et al.* 2004) by the addition of TEO and Crina® in the diet, respectively. Moreover, there are a few data on the impacts of DSF and TEO on ruminal fermentation in DFCC, but evaluated the effects of some EOAC (contained thymol) along with monosodium fumarate in *vitro* studies (Lin *et al.* 2012; Lin *et al.* 2013). Consistent with our findings, Lin *et al.* (2012) and Lin *et al.* (2013) observed that the addition of 200 or 500 mg/L of a blend of EO with 0, 5, 10 or 15 mM of monosodium fumarate, after 24 h of incubation decreased the N-NH_3 concentration relative to the control and EO solely.

Higher medium pH at 0 to 4 h of morning feeding with supplementation of DSF, TEO, and SIMTF observed in the present study, confirms findings of previous *in vitro* batch culture and RUSITEC studies (Callaway and Martin, 1996; Asanuma *et al.* 1999; Carro and Ranilla, 2003; Newbold *et al.* 2005). In the ruminal fermentation process, conversion of hexose to VFAs results in an overall net release of reducing equivalents. In the rumen anaerobic condition, H_2 is used to reduce fumarate, therefore fumarate as H_2 acceptor resulted in a decrease in the availability of H_2 in the internal environment of the rumen, which was associated with the inhibitory effect of fumarate on methane production (Callaway and Martin, 1996). In addition, at least a part of higher medium pH in DSF, TEO and SIMTF treatments compared to those of the control, related to their buffering capacity, decrease in overall fermentation and VFA production and their synergistic effects, respectively.

Previous evaluation of the short and long-term effects of some EO showed that high dose (5000 or 500 mg/L) of thymol strongly decreased total VFA concentration in the DFCC system and *in vitro* batch culture (Castillejos *et al.* 2006). Similarly, Pirondini *et al.* (2015) reported that the supplementation of the medium with thymol or TEO (500 mg/L) after 24 h of incubation significantly decreased total VFA concentration, relative to the control. It seems that reducing in total VFA concentration is consistent with the known antimicrobial properties of TEO on a wide range of gram-positive and -negative bacteria in the rumen (Ultee *et al.* 2002).

Increase in C3 and a decrease in C2:C3 ratio, with DSF and SIMTF supplementation, confirm previous findings in the batch culture system (Lopez *et al.* 1999b; Carro and Ranilla, 2003; Garcia-Martinez *et al.* 2005). Propionate is considering a valuable energy fuel for the ruminants, and this is a highly desirable effect on animal performance and efficient use of feed energy. In contrast to our observation, Lopez *et al.* (1999b) reported an 11.6 and 24.6% increase in the molar proportion of C2 at 5 and 10 mM of sodium fumarate in a 75: 25 forage: concentrate diet. However, Garcia-Martinez *et al.* (2005) also observed a 7.7% increase in C3 proportion at 8 mM of DSF in a medium-forage diet. At least, a part of inconsistencies in the studies results may be related to the basal diets (medium forage *vs.* higher forage diets).

Similarly, Lin *et al.* (2013) observed that the simultaneous use of monosodium fumarate and a blend of EO resulted in a significant increase in the molar proportion of C3 and a decrease in C2:C3 ratio. Fumarate can be converted to C3 (48% of supplemented fumarate) and C2 (20% of fumarate) via different pathways (Ungerfeld *et al.* 2007).

CONCLUSION

In the present study, use of DSF along with TEO resulted in higher modulatory effects on total VFA, the proportion of C3 and C2:C3 ratio compared to TEO treatment ($P<0.05$). Relative to the CON, our findings demonstrated a numerical decline in lipogenic (C2 and butyrate) precursors and significant enhance (20%) in glucogenic (C3) precursor with the simultaneous addition of DSF and TEO. Probably, this glucogenic impact can improve effectiveness production of liver glucose, glucose provides to the mammary gland and lactose and milk production in the high-producing dairy cow.

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