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# Effects of Thyme Essential Oil and Disodium Fumarate Alone or in Combination on Performance, Blood Metabolites, Ruminal Fermentation and Microbial Communities in Holstein Dairy Cows

**Research Article**

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## ABSTRACT

Nine Holstein dairy cows (630±50 kg) were used in a completely randomized design with repeated measures (two 21 d periods) to investigate the effects of disodium fumarate (DSF, 160 g/d), thyme essential oil (TEO, 9 mL/d) and simultaneous use of TEO and DSF (SIMTF, 160 g/d DSF and 9 mL/d TEO) on performance, apparent digestibility, blood metabolites, rumen microbial communities and fermentation characteristics. Dry matter intake and apparent digestibility (except for TEO), milk production and composition were not affected by the treatments. Relative to the control (CON, no additive), organic matter apparent digestibility tended to decrease by TEO supplementation (578 *vs*. 599 g/kg). Serum urea nitrogen concentration was lower in TEO than CON (9.8 *vs*. 10.9 mg/dL). Also, DSF supplementation significantly increased the molar proportion of propionate and the glucogenic: non-glucogenic ratio of volatile fatty acids (VFA) and decreased the molar proportion of butyrate. Supplementation of DSF and SIMTF resulted in a significantly decrease in the acetate: propionate ratio. Relative to the CON, DSF and SIMTF supplementation significantly increased serum glucose concentration. TEO supplementation decreased rumen fluid ammonia nitrogen and increased large peptides and small peptides plus amino acid nitrogen concentration. Rumen total and cellulolytic bacteria and protozoa abundance were not affected by treatments (except for total bacteria that decreased by TEO). Results of the present study demonstrated that the simultaneous use of DSF and TEO relative to DSF alone could not have synergistic effects on performance, ruminal fermentation and serum metabolites of dairy cow. DSF can be used as a ruminal glucogenic precursor and resulted in an increase in the serum concentration of glucose in dairy cows.

**KEY WORDS** bacterial population, chewing activities, essential oil, glucogenic precursor, serum urea nitrogen.

## INTRODUCTION

Propionate is an important fuel source for ruminants. In the rumen some microbes produce propionate from degradation of fermentable carbohydrates. According to the stoichiometry of anaerobic fermentation of carbohydrates, the enhance propionate production compared with other volatile fatty acids (VFAs) can improve the utilization of metabolic hydrogen in the rumen and energy use efficiency (Newbold *et al*. 2005). Several nutritional strategies, such as the use of growth-promoter antibiotics, yeast extracts, organic acids (fumarate, malate, acrylate), plant extracts like as essential oils (EO), saponins, condensed tannins, and probiotics (Newbold *et al*. 2005; Buddle *et al*. 2011), has been conducted for increase the ruminal protein and energy efficiency use. Fumarate is a hydrogen acceptor, in which acts as propionate precursor by the rumen microbes. Fumarate and malate (salts and free acid form) supplementa-

tion have been examined for rumen manipulation and enhance *in vitro* and *in vivo* efficiency of rumen fermentation process (Lin *et al*. 2013; Riede *et al*. 2013). Effects of fumarate were unclear in *in vivo* studies, and presumably it is due to the unknown optimum level of fumarate. Zhou *et al*. (2012) reported that supplementation of 20 g/d of disodium fumarate (DSF) in Hu sheep fed on high-forage diets did not affect propionate proportion and acetate/propionate ratio. Yang *et al*. (2012) indicated that the addition of 10 g/d DSF to the goat's diet increased propionate proportion. Thyme EO (TEO) and their main active components (thymol, carvacrol) have higher antibacterial properties compared to other plants EO (Ultee *et al*. 2002). The use of a specific blend of essential oils includes thymol (Castillejos *et al*. 2006) or TEO (Jahani-Azizabadi *et al*. 2014) resulted in a decrease in acetate: propionate ratio, methane production, and ammonia nitrogen ($NH_3$-N) concentration. Some studies reported an adverse effect of TEO on *in vitro* dry matter disappearance (Jahani-Azizabadi *et al*. 2014). We hypothesized that the use of TEO as an anti-methanogenesis factor and DSF as a hydrogen acceptor will have synergistic effects resulteing increase in the rumen fermentation efficiency and animal performance. In our previous study, we examined the effect of TEO (125 µL/L) and DSF (10 mM) in a dual flow continuous culture system and our findings showed that synergistic effects of TEO and DSF resulted in a beneficial, modifying the VFAs concentration without adverse effects on nutrient digestibility. Several studies investigated the impact of a commercial blend of EO consisting in TEO or thymol on rumen fermentation and performance of lactating dairy cattle (Tassoul and Shaver, 2009), but based on our information there is not evidences about the effects of TEO and DSF solely and in combination on performance of lactating dairy cattle. Therefore, the aim of the present study was to investigate the effects of TEO and DSF, alone and in combination, on rumen fermentation characteristics, total-tract apparent digestibility, blood metabolites, and performance of mid-lactating Holstein dairy cows.

## MATERIALS AND METHODS

### Animals, experimental design, and treatments

Nine mid-lactating Holstein dairy cows (averaging 163±49 days in milk (DIM); 650±50 kg BW and 28±2.4 kg/d milk yield (Mean±SE), at the beginning of the experiment) from dairy filed of University of Kurdistan (Iran), were used in a completely randomized design with repeated measures (two 21d periods; each period contains 14 d for adaptation and 7 d for sample collections). Cows were housed in the tie-stall barns with free access to water.

Cows were fed adlibitum (at 06:00, 14:00 and 22:00 h) with a total mixed ration (TMR) and milked three times daily (05:00, 13:00 and 21:00 h). The experiments were approved by the Institutional Ethics Committee of University of Kurdistan, Sanandaj, Iran (No. A9532). The diet was formulated to meet (NRC, 2001) recommendation for mid-lactating Holstein cows with average 28 kg of milk production (Table 1). Holstein dairy cows were divided into 4 groups balanced for milk production, DIM and number of lactation, then randomly allocated to one of the following treatments. Treatments were; control (CON, no additive), DSF at 160 g/d (DSF; disodium salt produced in chemistry lab), TEO at 9 mL/d (TEO; MONIN company, France) and simultaneous use of TEO and DSF (SIMTF). Treatments were mixed with a part of daily concentrate (150 g) for each cow and then divided in three equal parts and added to ration of each meal.

### Sample collection

In each experimental period, during sampling days (the final 7 d of each period) the weight of TMR offered, orts and milk yield were recorded daily. Consecutively, samples of feces at three days of each period (d 14, 15 and 16) were taken 2 and 4 h after the morning feeding. The samples used for determination of the milk composition were taken at sampling periods. Moreover, 30 mL of milk sample was collected and stored at -20 ˚C for determination of milk urea nitrogen (MUN) concentration. Ruminal content samples (by stomach tube) and blood samples from the jugular vein of cows were obtained on d 21 of the experiment at 4 h after morning feeding. Ruminal contents were used for pH, N-$NH_3$, tungstic acid (TA) soluble N, trichloroacetic acid (TCA) soluble N, VFA, enumeration of total viable and cellulolytic bacteria and protozoa counting. Blood serum was separated after 4h staying at 4 ˚C, and then stored in micro-tube at -20 ˚C until analysis.

### Chemical analyses

The feed, orts, and feces samples were oven dried at 55 ˚C for 72 h, then ground to pass through a 1 mm screen and used to determine dry matter (DM, method #934.01), organic matter (OM) and crude protein(CP) (method #924.05) concentration according to the standard recommended procedures (AOAC, 1995). Also, the ash-less neutral detergent fiber (NDF) and acid detergent fiber (ADF) (method#973.18) content of the feed, orts, and fecal samples were determined using the method recommended by Van Soest *et al*. (1991) and AOAC (2005), respectively.

Milk samples were analyzed for protein, fat, lactose and solid non-fat by milkoscan apparatus (Milkana Kam 98-2A, Bulteh).

**Table 1** Ingredients and chemical composition of basal diet fed as a total mixed ration

| Ingredient (g/kg DM) | Content | Chemical composition (g/kg DM) | Content |
|---|---|---|---|
| Alfalfa hay | 219.0 | Crude protein | 162.0 |
| Maize silage | 211.0 | Ether extract | 43.0 |
| Maize grain, ground | 142.5 | Organic matter | 923.0 |
| Barley grain, ground | 168.2 | Ash | 77.0 |
| Soybean meal | 102.6 | Neutral detergent fiber | 284.0 |
| Wheat bran | 60.4 | Acid detergent fiber | 191.0 |
| Cottonseed meal | 28.5 | Non-fibre carbohydrate[2] | 434.0 |
| Calcium salts of palmitate | 17.1 | | |
| Fish meal | 28.5 | | |
| Vitamin-mineral premix[1] | 15.36 | | |
| Salt | 3.99 | | |
| Mycotoxin binder | 2.85 | | |

[1] Composition per each kg, according to manufacturer information: vitamin A: 500000 IU; vitamin $D_3$: 100000 IU; vitamin E: 100 mg; Antioxidant: 3 g; Calcium: 190 g; Phosphorus: 90 g; Sodium: 50 g; Magnesium: 19 g; Copper: 3 g; Iron: 3 g; Manganese: 2 g; Zinc: 3 g; Cobalt: 100 mg; Iodine: 100 mg and Selenium: 1 mg.
[2] NFC= 100 - (NDF+CP+EE+Ash) according to NRC (2001).

Analysis of MUN was performed as described by Butler *et al*. (1996). Serum urea nitrogen (SUN), glucose and triglyceride concentration were determined using Pars Azmun special kits (Pars Azmun, Karaj, Iran) and beta-hydroxy butyrate (BHBA) and non-esterified fatty acid (NEFA) determined using Randox special kit (Randox, Ardmore, UK) by spectrophotometry apparatus (JASCO, V-570, Tokyo, Japan) according to manufacturer information. The TCA and TA-soluble N concentration of rumen fluid were determined as described by Winter *et al*. (1964). A 10 mL sample of strained rumen fluid was added to 2.5 mL of 10% sodium tungstate (10 g/100 mL distilled water) and 2.5 mL of 1.07 N sulfuric acids. Samples were stored at 5 ˚C for 4 h and then centrifuged at 9000 × g for 15 min. The supernatant was collected and frozen until analyzed for TA soluble N by the Kjeldahl method. To determine TCA soluble N, 2.5 mL of 50% TCA solution (50 g trichloroacetic acid per 100 mL distilled water) were added to 10 mL of strained rumen fluid. After 4 h staying at 5 ˚C, samples were centrifuged at 9000 × g for 15 min. The supernatant was frozen until analyzed for TCA soluble N by the Kjeldahl procedure.

**Volatile fatty acid analyses**

To determine VFA concentration 1.5 mL of strained (filter pore size was 48 µm) sample was removed and mixed with 375 µL of 25% orthophosphoric acid and frozen at −20 ˚C until analyzed by gas chromatography (GC, Philips, pu4410). Oven initial and final temperatures were 55 and 195 ˚C, respectively, and detector and injector temperatures were set at 250 ˚C. Before injection into GC, samples were centrifuged at 10000 ×g for 4 minutes.

**Enumeration of microbes**

Two ml of whole rumen content was taken 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 at the end of incubation, growth was scored (positive or negative). For enumeration of total viable and cellulolytic bacteria, were considered pH variation and disappearance of cellulose filter paper (Whatman® No. 7), respectively. For protozoa counting, 7 ml of whole rumen content was fixed in an equal volume of a 50% Formalin solution (18.5% concentration of formaldehyde). Then, two drops of brilliant green dye was added to 1 ml of rumen fluid and stored overnight at the laboratory temperature and subsequently diluted in 9 ml of 30% glycerol solution. Protozoa were enumerated microscopically in a Sedgwick-Rafter counting chamber according to the method proposed by Dehority (1984).

Total-tract apparent digestibility and chewing activity

Eating and ruminating behaviors were measured visually for cows in treatment groups over a 24 h period described by Colenbrander *et al*. (1991). Eating and ruminating activities were noted every 5 min, and each activity was presumed to the entire 5 min interval. To estimate time spend for ruminating or eating per kilogram of DM, NDF, or ADF intake, the average intake for the experimental period was used. The sum of time spend eating and rumination were used for calculate total chewing.

Total-tract apparent digestibility of DM, OM, NDF, ADF, and CP was estimated using acid insoluble ash (AIA) as an internal marker (Van Keulen and Young, 1977). For this purpose, 2 g of dry sample (feces, feed or ort) was taken and poured into the weighted crucible. Then, samples were burned at 550 ˚C for 4 h.

The resulting ashes were poured in 200 mL erlenmeyer contain 50 mL HCL 2N, and were heated for 10 min to reached boiling point. Then, the samples were filtered on ash-less filter paper (Whatman® No. 7), afterward the residue along with filter paper were burned at 550 ˚C for 2 h. The percentage of AIA in samples (feces, feed and ort) was calculated using the following equation:

% AIA= 100 × [(wt. of crucible+ash) – wt. of crucible] / wt. of samples]

Total-tract apparent digestibility of a nutrient was calculated using the following equation:

% AD= 100 - [100 × (% AIA in feed/% AIA in feces) × (% nutrient in feces / % nutrient in feed]

**Calculations and statistical analysis**

The amount of non-fiber carbohydrate (NFC) in ration was estimated from the equation as described by NRC (2001).

NFC= 100 - (NDF+CP+EE+Ash)

The following equation is used to calculation the 4% fat-corrected milk (FCM):

% 4 FCM (kg/d)= 0.4 × milk yield (kg/d) + 15 × (milk fat (%)/100) × milk yield (kg/d)

The following equation is used to calculation the energy-corrected milk (ECM):

ECM (kg/d)= milk yield (kg/d) × [(38.3×milk fat (kg/d)+24.2×milk protein (kg/d)+16.54×milk lactose (kg/d)+20.7) / 3.14]

Glucogenic/non-glucogenic ratio of VFAs (GNR) was calculated using the following equation (Ørskov, 1975):

$GNR= (Val+isoVal+C_3) / (isoVal+(2\times C_4)+C_2)$

Where:
Val: valeric acid.
isoVal: isovaleric acid.
$C_3$: propionic acid.
$C_4$: butyric acid.
$C_2$: acid acetic.

The results of the TCA- and TA-soluble N were used for calculation of the large peptide N (LPepN) and small peptide plus amino acid N (SPep+AAN) concentration (Licitra *et al*. 1996):

LPepN (mg/100 mL)= TCA soluble N - TA soluble N
SPep + AAN (mg/100 mL)= TA soluble N - ammonia N

All data were statistically analyzed using MIXED procedure of SAS, (2002) (version 9.1; SAS Institute Inc., Cary, North Carolina, USA). The period was considered as a random effect. The statistical model was:

$$y_{ijk}= \mu + \tau_i + \delta_{ij} + t_k + (\tau^*t)_{ik} + e_{ijk}$$

Where:
$y_{ijk}$: observation for dependent variables.
μ: overall mean.
$\tau_i$: impact of treatment *i* (*i*=1-4).
$t_k$: impact of period k (k=1-2).
$(\tau^*t)_{ik}$: impacts of interaction between treatment i and period k.
$\delta_{ij}$: random error with the mean 0 and variance $\sigma^2_\delta$, the variance between animals (j=1-8) within treatment, moreover, it is equal to the covariance between repeated measurements within animals.
$e_{ijk}$: random error with the mean 0 and variance $\sigma^2$, the variance between measurements within animals.

The results of microbial counting were analyzed by GLM procedure as a completely randomized design, and Tukey test was employed to compare the means (P<0.05). Results are presented as least square means (LSM) ± standard error (SE).

## RESULTS AND DISCUSSION

Effects of dietary DSF (160 g/d) and TEO (9 mL/d) supplementation, alone and in combination, on total-tract apparent digestibility and nutrient intake in mid-lactation dairy cows are shown in Table 2. There were no significant differences between treatments for DM, OM, CP, NDF and ADF intake, and total tract apparent digestibility of nutrients (except for OM). Total tract apparent digestibility of OM for cows fed 9 mL/d TEO was lower than the control group (P<0.05). The inclusion of TEO, DSF and SIMTF did not have significant effects on DMI and eating, rumination (min/kg of DM, ADF, and NDF), total chewing and resting (min/d) time of mid-lactation dairy cows (Table 3). Several studies have been demonstrated that supplementation of EOs altered *in vitro* and *in situ* nutrients disappearance (Jahani-Azizabadi *et al*. 2014; Newbold *et al*. 2005). The discrepancy between our observation and previous studies could be due to experimental conditions (*in vitro vs. in vivo,* ruminal *vs.* total tract investigation), type of EOs and dose of supplementation.

Table 2 Nutrient intake and total-tract apparent digestibility in dairy cows fed diets containing disodium fumarate (DSF: 160 g/d), thyme essential oil (TEO: 9 mL/d) and simultaneous use of disodium fumarate and thyme essential oil (SIMFT)

| Item | Treatment | | | | P-value | | |
|---|---|---|---|---|---|---|---|
| | Control (no additive) | DSF | TEO | SIMFT | T | P | T × P |
| **Intake (kg/d)** | | | | | | | |
| Dry matter | 20.0±0.3 | 18.9±0.3 | 19.1±0.5 | 19.1±1.7 | 0.72 | 0.37 | 0.96 |
| Organic matter | 18.5±0.2 | 17.5±0.3 | 17.7±0.4 | 17.6±0.4 | 0.71 | 0.18 | 0.96 |
| Crude protein | 3.2±0.04 | 3.1±0.06 | 3.1±0.06 | 3.0±0.07 | 0.73 | 0.16 | 0.92 |
| NDF | 5.6±0.08 | 5.2±0.09 | 5.2±0.2 | 5.1±0.2 | 0.73 | 0.04 | 0.87 |
| ADF | 4.4±0.08 | 4.1±0.09 | 4.1±0.08 | 4.2±1.0 | 0.72 | 0.67 | 0.97 |
| **Total-tract apparent digestibility (g/kg)** | | | | | | | |
| Dry matter | 523±14 | 518±4 | 518±12 | 530±8 | 0.39 | 0.07 | 0.38 |
| Organic matter | 599±8$^{a}$ | 588±15$^{ab}$ | 578±18$^{b}$ | 594±11$^{ab}$ | 0.03 | 0.01 | 0.61 |
| Crude protein | 586±7 | 601±17 | 587±14 | 611±5 | 0.36 | 0.92 | 0.64 |
| Neutral detergent fiber (NDF) | 519±11 | 521±18 | 506±11 | 550±17 | 0.47 | 0.02 | 0.61 |
| Acid detergent fiber (ADF) | 475±18 | 475±17 | 451±19 | 479±17 | 0.77 | 0.81 | 0.69 |

P: period and T: treatment.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Table 3 Eating and rumination behaviors in dairy cows fed diets containing disodium fumarate (DSF: 160 g/d), thyme essential oil (TEO: 9 ml/day), alone and in combination (SIMFT)

| Item | Treatment | | | | P-value | | |
|---|---|---|---|---|---|---|---|
| | Control (no additive) | DSF | TEO | SIMFT | T | P | T × P |
| **Eating** | | | | | | | |
| Min/day | 220±18 | 242±17 | 261±21 | 248±17 | 0.58 | < 0.01 | 0.08 |
| Min/kg DMI | 11.0±0.96 | 12.9±1.15 | 13.7±1.29 | 13.1±1.00 | 0.59 | < 0.01 | 0.06 |
| Min/kg NDF | 39.5±4.05 | 47.7±5.59 | 50.9±6.87 | 48.7±0.97 | 0.62 | < 0.01 | 0.32 |
| Min/kg ADF | 3.8 ±0.23 | 47.7±0.17 | 50.9±0.22 | 48.7±4.67 | 0.62 | < 0.01 | 0.32 |
| **Rumination** | | | | | | | |
| Min/day | 330±17.6 | 322±13.9 | 302±21.2 | 325±17.9 | 0.52 | 0.09 | 0.74 |
| Min/kg DMI | 16.5±1.01 | 17.1±1.11 | 15.9±1.34 | 17.1±0.99 | 0.80 | 0.08 | 0.84 |
| Min/kg NDF | 59.2±4.72 | 63.3±6.17 | 58.8±7.45 | 63.4±3.28 | 0.87 | 0.05 | 0.91 |
| Min/kg ADF | 86.6±5.16 | 90.3±5.91 | 84.1±6.84 | 90.4±3.35 | 0.83 | 0.11 | 0.85 |
| **Total chewing** | | | | | | | |
| Min/day | 550±34 | 556±30 | 563±39 | 573±27 | 0.96 | 0.02 | 0.33 |
| Min/kg DMI | 27.5±1.94 | 30.0±2.25 | 29.6±2.49 | 30.2±1.57 | 0.85 | 0.01 | 0.47 |
| Resting, min/day | 710±34 | 695±30 | 696±39 | 686±27 | 0.95 | 0.01 | 0.33 |

DMI: dry matter intake; NDF: neutral detergent fiber and ADF: acid detergent fiber.
P: period and T: treatment.

Impacts of TEO on total-tract apparent digestibility in the present study relative to the *in vitro* and *in sacco* studies demonstrated that lower ruminal nutrient degradation compensated by post-ruminal digestion. In agreement with results of the present study, several *in vivo* research reported that the use of Crina® (contain thymol as active compound of TEO) in dairy cows (Tassoul and Shaver, 2009) and a mixture of EOs (containing 50% thyme EO) in sheep (Khateri *et al*. 2017) had no significant effect on nutrient digestibility. Relative to the control, decrease in OM digestibility with TEO supplementation (-35 g/kg; P<0.05) was associated with antibacterial and inhibitory effects of TEO on a wide range of ruminal bacteria (Ultee *et al*. 2002).

It seems that high dose of TEO (9 mL/d cow) used in the present study compared to other *in vivo* study causing long-term inhibitory effects of TEO on ruminal fermentation.

With attention to reported *in vitro* decrease in nutrient digestion by supplementation of plant EOs, we expected a change in DMI, feeding behaviors and chewing activity in cows treated with high dose of TEO. In contrast with this hypothesis, DMI and eating, rumination and chewing time was not affected by the addition of TEO. Inconsistency between the results could be due to rumen fermentation that is more daynamic process relative to the *in vitro* batch culture fermentation. Hence, the length of time that rumen microbiota are exposed to EO (e.g. short time *in vitro* studies *vs*. long time *in vivo* study) and microbial adaptation

and degrading of EO main compounds can lead to this observation. In the present study inclusion of DSF, alone and in combination with TEO, did not have significant effect on eating behavior and apparent nutrients digestibility. In the agreement, some of the previous studies reported no significant effects of fumarate (free or di-sodium salt) on DM, NDF and ADF digestibility in steers (Bayaru *et al*. 2001) and dairy cows (Kolver and Aspin, 2006). In contrast, Yu *et al*. (2010) observed that the addition of DSF (10 g/kg DM) increased CP and cellulose digestion in dairy goats. In agreement with our findings, there is some evidence that the use of fumarate resulted in an increase in the abundance of cellulolytic and some fumarate-utilizing bacteria (Zhou *et al*. 2012). It is assumed that fumarate-utilizing bacteria accelerate the metabolism of the intermediate products (such as hydrogen) of fibrolytic bacteria (Mao *et al*. 2007).

Results of the present study showed that milk yield, 4% FCM, ECM, milk composition, MUN concentration and feed efficiency was not affected by the use of TEO and DSF, alone and in combination, in mid-lactation cows (Table 4). Relative to the control, milk yield and feed efficiency for milk yield (milk yield:DMI ratio) were numerically (P>0.05) increased with DSF supplementation. Our observation demonstrated that milk production and milk composition of mid lactation dairy cows, was not affected by the inclusion of TEO (9 mL/d) and DSF (160 g/d) alone and in combination. A few studies investigated the effect of TEO on the performance of dairy cows. Most conducted studies was used of the commercial mixtures of EO (such as Crina®). Several studies evaluated the effects of different concentration of Crina® on the performance of dairy cattle. Benchaar *et al*. (2006) reported that supplementation of 2 g/d Crina® and 350 mg/d monensin in dairy cows did not affected DMI, milk production and composition. DMI, milk yield, FCM, milk composition and fatty acids profile of milk fat was not significantly affected by 750 mg/d (Benchaar *et al*. 2007) and 1.2 g/d (Tassoul and Shaver, 2009) Crina® supplementation in dairy cows. It seems that, response of dairy cows from supplementation of plant EO is dependent to lactation stages, type, and levels of EOs and diet composition.

As well as, a little information exists about the effect of fumarate (salt form) on milk production and composition of dairy cow. Kolver and Aspin (2006) reported that supplementation of 50 g/kg DM of DSF in dairy cows fed high-quality pasture did not affected the milk production and composition. Consistent with our findings, Van Zijderveld *et al*. (2011) observed that DMI, milk production and milk composition of lactating cows not influenced by calcium fumarate supplementation (25 g/kg dry matter).

Remling *et al*. (2014) reported that 300 or 600 g/d fumaric acid supplementation in lactating cows did not have significant effects on milk fat, protein, and lactose production (kg/d) but decreased the milk fat content relative to those of the control group. Variable responses in the performance of dairy cows to fumarate supplementation could be due to the form of fumarate (free or salty), amount of daily fumarate suplementation, feed quality and diet composition (forage and cereal type, forage: concentrate ratio) and physiological aspects (Kolver and Aspin, 2006; Mao *et al*. 2007; Zhou *et al*. 2012).

Results of the present study showed that the addition of DSF and TEO, alone and in combination, did not affect ruminal pH, total VFA and the molar proportion of acetate, valerate, and isovalerate (Table 5). Relative to the control the use of DSF resulted in an increase (P=0.05) in the molar proportion of propionate (25%) and decrease (P<0.05) in the molar proportion of butyrate (-24%). Addition of DSF solely or in combination with TEO (SIMTF) resulted in a decrease in the acetate: propionate ratio compared to the control and TEO treatments (P<0.05). The $NH_3$-N and LP-N concentration numerically affected (P>0.05) by the addition of DSF and TEO, alone and in combination. Relative to the control ruminal SP + AA - N concentration increased (P=0.05) when TEO was added (21.2 *vs*. 11.7 mg/dL). The use of TEO and SIMTF resulted in an increase in the LP-N relative to the CON treatment (14.0 and 17.1 *vs*. 12.4 mg/dL).

Decreasing effect of DSF supplementation on ruminal $NH_3$-N concentration confirms findings of other *in vivo* studies (Yu *et al*. 2010; Zhou *et al*. 2012; Remling *et al*. 2014). Fumarate supplementation could cause excitation in abundance of some species of cellulolytic and fumarate-utilizing bacteria (Newbold *et al*. 2004) which might increase in ruminal $NH_3$-N utilization. On the other hand, the result of the present study demonstrated the non-significant enhance in total cellulolytic bacteria by DSF supplementation, relative to the control group (10.47 *vs*. 9.61 $\log_{10}$/mL). Decline in MUN concentration (P>0.05) with supplementation of TEO alone and in combination with DSF (SIMFT) it seems to be in coordinate with decrease (P>0.05) in ruminal concentration of N-$NH_3$. Decreasing in ruminal N-$NH_3$ concentration might increase escape of dietary protein from ruminal degradation and improve the efficiency use of nitrogen in ruminant (Van Nevel and Demeyer, 1989).

The accumulation of SP+AA-N (P<0.05) and LP-N (P>0.05) in the rumen fluid of cows receiving TEO (9 mL/d) suggests that amino acids deamination and peptidolytic activity was inhibited by TEO supplementation, respectively.

**Table 4** Milk yield, milk composition and feed efficiency in dairy cows fed diets containing disodium fumarate (DSF: 160 g/day), thyme essential oil (TEO: 9 mL/day) and simultaneous use of disodium fumarate and thyme essential oil (SIMFT)

| Item | Treatment | | | | P-value | | |
|---|---|---|---|---|---|---|---|
| | Control (no additive) | DSF | TEO | SIMFT | T | P | T × P |
| **Milk yield, kg/day** | | | | | | | |
| Actual | 28.5±0.39 | 29.2±0.34 | 28.4±0.17 | 28.6±0.4 | 0.94 | 0.11 | 0.92 |
| 4% fat-corrected milk | 26.0±0.52 | 27.2±0.24 | 25.8±0.23 | 27.0±0.44 | 0.83 | 0.16 | 0.78 |
| Energy-corrected milk | 25.5±0.49 | 26.6±0.24 | 25.6±0.21 | 26.1±0.50 | 0.94 | 0.14 | 0.16 |
| Protein | 0.88±0.009 | 0.91±0.011 | 0.89±0.002 | 0.90±0.015 | 0.86 | 0.01 | 0.06 |
| Fat | 0.96±0.022 | 1.03±0.012 | 0.95±0.019 | 1.02±0.018 | 0.73 | 0.08 | 0.20 |
| Lactose | 1.24±0.11 | 1.28±0.12 | 1.29±0.04 | 1.27±0.06 | 0.99 | 0.09 | 0.33 |
| Solid non fat | 2.33±0.022 | 2.40±0.016 | 2.35±0.007 | 2.37±0.024 | 0.62 | 0.01 | 0.01 |
| **Milk composition, %** | | | | | | | |
| Protein | 3.12±0.02 | 3.14±0.01 | 3.14±0.01 | 3.15±0.02 | 0.97 | 0.01 | 0.11 |
| Fat | 3.41±0.06 | 3.57±0.05 | 3.40±0.07 | 3.62±0.07 | 0.84 | 0.01 | 0.27 |
| Lactose | 4.41±0.39 | 4.41±0.38 | 4.57±0.12 | 4.21±0.37 | 0.94 | 0.11 | 0.49 |
| Solid non fat | 8.27±0.06 | 8.31±0.03 | 8.33±0.02 | 8.33±0.04 | 0.98 | 0.01 | 0.10 |
| Milk urinary nitrogen, mg/dL | 13.4±0.41 | 13.9±0.39 | 12.4±0.69 | 12.0±0.43 | 0.35 | 0.95 | 0.45 |
| **Feed efficiency, kg/kg** | | | | | | | |
| Milk:DMI (dry matter intake) | 1.42±0.02 | 1.55±0.03 | 1.49±0.01 | 1.51±0.03 | 0.78 | 0.54 | 0.86 |
| Fat-corrected milk:dry matter intake (FCM:DMI) | 1.31±0.03 | 1.44±0.02 | 1.35±0.02 | 1.42±0.04 | 0.79 | 0.03 | 0.39 |
| energy-corrected milk:dry matter intake (ECM:DMI) | 1.32±0.02 | 1.42±0.01 | 1.43±0.01 | 1.40±0.03 | 0.76 | 0.35 | 0.46 |

P: period and T: treatment.

**Table 5** Ruminal fermentation characteristics in dairy cows fed diets containing disodium fumarate (DSF: 160 g/day), thyme essential oil (TEO: 9 mL/day) and simultaneous use of disodium fumarate and thyme essential oil (SIMFT)

| Item | Treatment | | | | P-value | | |
|---|---|---|---|---|---|---|---|
| | Control (no additive) | DSF | TEO | SIMFT | T | P | T × P |
| pH | 6.48±0.08 | 6.74±0.03 | 6.58±0.04 | 6.67±0.03 | 0.49 | 0.79 | 0.03 |
| Total volatile fatty acid, mM | 102.7±5.54 | 92.3±3.58 | 90.3±3.22 | 102.0±7.17 | 0.22 | 0.18 | 0.34 |
| **Individual, mol/100 mol** | | | | | | | |
| Acetate | 56.4±1.11 | 55.7±0.89 | 55.4±1.48 | 56.0±1.01 | 0.97 | < 0.01 | 0.64 |
| Propionate | 16.7±0.93$^{b}$ | 20.9±0.90$^{a}$ | 17.2±0.69$^{b}$ | 18.3±0.36$^{ab}$ | 0.05 | 0.03 | 0.42 |
| Butyrate | 20.5±1.11$^{a}$ | 15.6±0.48$^{b}$ | 20.8±1.03$^{a}$ | 19.7±0.97$^{a}$ | 0.04 | < 0.01 | 0.27 |
| Valerate | 3.8±0.23 | 4.4±0.17 | 4.3±0.22 | 4.0±0.18 | 0.21 | 0.03 | 0.08 |
| Isovalerate | 3.4±0.23 | 3.5±0.09 | 3.4±0.03 | 2.9±0.25 | 0.30 | 0.22 | 0.16 |
| Acetate:propionate | 3.4±0.24$^{a}$ | 2.7±0.14$^{b}$ | 3.4±0.38$^{a}$ | 2.9±0.10$^{b}$ | 0.02 | 0.02 | 0.23 |
| Glucogenic:non-glucogenic ratio | 0.24±0.01$^{b}$ | 0.32±0.01$^{a}$ | 0.25±0.02$^{b}$ | 0.26±0.01$^{b}$ | 0.02 | 0.26 | 0.49 |
| **Nitrogen fraction[3], mg/dL** | | | | | | | |
| $NH_3$-N | 13.2±0.72 | 12.5±0.76 | 10.6±1.03 | 11.1±0.86 | 0.64 | 0.18 | 0.58 |
| LP-N | 12.4±1.15 | 11.2±1.13 | 14.0±1.04 | 17.1±2.28 | 0.52 | 0.06 | 0.69 |
| SP+AA-N | 11.7±0.58$^{b}$ | 11.4±0.65$^{b}$ | 21.2±1.65$^{a}$ | 15.3±1.68$^{ab}$ | 0.05 | 0.02 | 0.04 |
| **Microbes, $log_{10}$/mL** | | | | | | | |
| Bacteria | 11.09±0.05$^{ab}$ | 11.13±0.04$^{ab}$ | 10.84±0.07$^{b}$ | 11.24±0.03$^{a}$ | 0.05 | - | - |
| Cellulolytic | 9.61±0.053 | 10.47±0.24 | 9.70±0.042 | 10.45±0.22 | 0.12 | - | - |
| Protozoa | 4.75±0.012 | 4.70±0.015 | 4.68±0.013 | 4.70±0.015 | 0.66 | - | - |

P: period; T: treatment; $NH_3$-N: ammonia nitrogen; LP-N: nitrogen from large peptides and SP+AA-N: nitrogen 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).

In the agreement with our findings, Castillejos *et al.* (2006) reported that thymol supplementation at 500 mg/L, increased the concentration of LP-N and SP+AA-N in the dual-flow continuous culture system. Fumarate is a hydrogen acceptor and key intermediates in the succinate-propionate pathway.

In this pathway fumarate used by *Selenomonas ruminantium* to form succinate and propionate (Martin, 1998).

Previous *in vitro* studies shown that fumurate converted to propionate and decrease methane production and acetate: propionate ratio (Mao *et al.* 2007).

**Table 6** Serum metabolites of dairy cows fed diets containing disodium fumarate (DSF: 160 g/d), thyme essential oil (TEO: 9 mL/d) and simultaneous use of disodium fumarate and thyme essential oil (SIMFT)

| Item | Treatment | | | | P-value | | |
|---|---|---|---|---|---|---|---|
| | Control (no additive) | DSF | TEO | SIMFT | T | P | T × P |
| Glucose (mg/100 mL) | 48.7±1.36 [b] | 52.9±1.15[a] | 50.1±2.18[ab] | 54.0±1.03[a] | 0.04 | 0.42 | 0.95 |
| TG (mg/100 mL) | 18.0±2.39 | 16.9±2.51 | 17.9±1.46 | 16.0±0.84 | 0.95 | 0.72 | 0.94 |
| Urea (mg/100 mL) | 10.9±0.32[a] | 10.6±0.21[ab] | 9.8±0.34[b] | 10.2±0.31[ab] | 0.03 | 0.47 | 0.14 |
| BHBA (mM) | 0.68±0.07 | 0.71±0.18 | 0.58±0.01 | 0.70±0.12 | 0.34 | 0.16 | 0.48 |
| NEFA (mM) | 0.45±0.08 | 0.42±0.16 | 0.40±0.01 | 0.57±0.06 | 0.85 | 0.24 | 0.34 |

P: period; T: treatment; TG: triglyceride; BHBA: beta-hydroxybutyric acid and NEFA: non-esterified fatty acids.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Although, in previous *in vivo* studies proportion of propionate rising with fumarate supplementation in sheep (Zhou *et al*. 2012) and goat (Yang *et al*. 2012), however any change was observed in the acetate:propionate ratio. For an explanation of this dissension, Ungerfeld *et al*. (2007) indicated that fumarate could be converting to propionate (48% of supplemented fumarate) and acetate (20% of supplemented fumarate) via different pathways. Propionate is the main precursor for glucose synthesis in the ruminant liver (Ungerfeld *et al*. 2007).

Cows receiving 9 mL/d TEO had lower blood urea-N compared with control group (P<0.05; 9.8 *vs*. 10.9 mg/dL). However, the addition of DSF and SIMFT does not have significant effect on blood urea-N concentration (P>0.05). Results of the present study showed that DSF and TEO supplementation, alone and in combination, had no effects on serum BHBA, TG and NEFA concentration. Increase in ruminal propionate proportion with DSF and SIMTF inclosion in dairy cows ration, resulted in an increase in serum glucose concentration in these groups. Some studies reported that fumarate (free acid or salt form) supplementation enhanced serum glucose concentration of steers (Bayaru *et al*. 2001) and dairy goat (Yu *et al*. 2010). Furthermore, Remling *et al*. (2014) demonstrated that the addition of 300 or 600 g/kg DM fumaric acid had no significant effects on serum glucose concentration in dairy cows. Probably, the result inconsistent between studies because of fumarate form (free acids or salty form), basal diet and animal physiological status.

Changes in propionate and butyrate with DSF and SIMFT supplementation increased the GNR (Table 5). In the outcome of the increased GNR values, higher glucose concentration observed in cows receiving DSF and SIMTF. Increasing glucose (8-10%) did not affected milk lactose content and milk yield. Presumably, glucose uptake in the mammary gland enhancing from pre-calving period to the peak of milk production and thereupon gently reduces following the decline of milk production in mid- and late-lactation (Nielsen *et al*. 2001).

Accordingly, the glucogenic impact of DSF can improve the glucose supply to the mammary gland and increase in lactose and milk production in the fresh and high-producing dairy cow.

In this study increase in ruminal SP + AA - N and LP-N and a decrease in $NH_3$-N concentration for cows receiving TEO, was consistent with reductions in blood urea-N. There is a high correlation between ruminal $NH_3$-N concentration and milk and blood urea N (Powell *et al*. 2011). Results of previous studies have shown that 20 g/kg DM of fumaric acids reduced plasma urea-N at 2 and 5 h after morning feeding in Holstein steers were fed sorghum silage (Bayaru *et al*. 2001). In another hand, Yu *et al*. (2010) reported that blood urea-N at 3 h after feeding was not affected by 6 g/d fumaric aids supplementation in dairy does. Apparently, the results contradiction is due to the difference in basal diet and physiological status of animals (early *vs*. mid or late lactation).

No significant changes in serum NEFA and TG concentration with TEO and DSF inclusion confirm previous studies. Khateri *et al*. (2017) reported that a mixture of EOs (containing 50% thyme EO) in sheep diet had no significant effects on serum BHBA and TG concentration. Kolver and Aspin (2006) reported that supplementation of 50 g/kg dry matter of DSF in dairy cows fed high-quality pasture did not affect NEFA concentration. Mainly source of longer-chain fatty acids in milk fat is derived from the blood NEFA and lipoproteins (Palmquist, 2006). Decrease in ruminal lipogenic VFA and no significant change in milk fat content in dairy cows fed with DSF, probably due to higher serum NEFA concentration. Presumabley serum NEFA has been used as milk fat precursors and kept milk fat percent (Palmquist, 2006).

## CONCLUSION

In conclusion, diets containing di-sodium fumarate (160 g/d) and thyme essential oil (9 mL/d) improved the efficie-

ncy of fermentation. Use of DSF can be enhancing ruminal glucogenic precursors and serum glucose concentration; this glucogenic impact can improve glucose provides to the mammary gland, lactose and milk production in dairy cow. Despite the negative effect on feedstuff organic matter digestibility, thyme essential oil improved nitrogen metabolism in dairy cows without harmful effects on nutritional behaviors. In addition, results of the present study demonstrated that simultaneous use of DSF and TEO relative to DSF alone could not have synergistic effects on dairy cow performance. Overall, these experiments provided support for a positive effect of DSF treatment on serum glucose concentration, and this should be further explored on fresh and high producing dairy cow's performance.

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