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Simultaneous use of thyme essential oil and disodium fumarate can improve *in vitro* ruminal microbial fermentation characteristics

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Abstract

Two trials were conducted to investigate the effects of disodium fumarate (DSF; 0.00, 8.00, 10.00 and 12.00 mM) and thyme essential oil (TEO; 0.00, 100.00, 200.00, 300.00 and 400.00 µL L^{-1}) solely and simultaneously (10.00 mM DSF along with 100.00, 200.00, 300.00 and 400 µL L^{-1} TEO) on *in vitro* ruminal fermentation of a 50:50 alfalfa hay to concentrate diet. The DSF and TEO did not affect crude protein disappearance, gas production, microbial crude protein synthesis and hydrogen recovery. The DSF addition linearly increased partitioning factor (PF) and molar proportion of propionate and decreased acetate: propionate ratio and methane production. Moreover, 100.00 µL L^{-1} of TEO decreased ammonia nitrogen, total volatile fatty acids concentration and methane production and increased PF compared to the control. Results of the present study demonstrated that simultaneous use of DSF and TEO can cause a further decrease in methane production and linearly increase in the molar proportion of propionate and efficiency of feed use compared to DSF and TEO solely.

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استفاده همزمان از اسانس آویشن و دی-سدیم فومارات می‌تواند خصوصیات تخمیر میکروبی شکمبه‌ای را در شرایط برون تنی بهبود بخشد

چکیده

دو آزمایش به منظور بررسی آثار دی سدیم فومارات (DSF؛ ۰/۰۰، ۸/۰۰، ۱۰/۰۰ و ۱۲/۰۰ میلی‌مول) و اسانس آویشن (TEO؛ ۰/۰۰، ۱۰۰/۰۰، ۲۰۰/۰۰، ۳۰۰/۰۰ و ۴۰۰/۰۰ میکرولیتر بر لیتر) به تنهایی و به صورت همزمان (۱۰/۰۰میلی مول DSF به همراه ۱۰۰/۰۰، ۲۰۰/۰۰، ۳۰۰/۰۰ و ۴۰۰/۰۰ میکرولیتر TEO) بر تخمیر شکمبه ای جیره حاوی ۵۰:۵۰ علوفه به کنسانتره در شرایط برون تنی انجام پذیرفت . DSF و TEO ناپدید شدن پروتئین خام، تولید گاز، سنتز پروتئین خام میکروبی و بازیافت هیدروژن را تحت تاثیر قرار نداد. افزودن DSF به طور خطی شاخص تفکیک (PF) و نسبت مولی پروپیونات را افزایش و نسبت استات به پروپیونات و تولید متان را کاهش داد. همچنین ، ۱۰۰ میکرولیتر بر لیتر TEO غلظت نیتروژن آمونیاکی، غلظت کل اسیدهای چرب فرار و تولید متان را کاهش و PF را در مقایسه با شاهد افزایش داد. نتایج این مطالعه نشان داد که نسبت به استفاده DSF و TEO به تنهایی ، استفاده همزمان از DSF و TEO می تواند موجب کاهش بیشتری در تولید متان و افزایش خطی نسبت مولی پروپیونات و بازده استفاده از خوراک شود.

واژه های کلیدی: اسیدهای چرب فرار، بازیافت هیدروژن، نیتروژن آمونیاکی

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Introduction

Recently, numerous studies have been conducted to determine the effects of medicinal plants essential oil (EO) and extract as alternatives for growth-promoter antibiotics on ruminal fermentation wastes reduction and nutrients use efficiency improvement.[1-3] Previously, positive effects of thyme EO (TEO) on rumen microbial fermentation have been reported.[4,5] Studies have showed that use of a specific blend of EO including thymol and thyme EO can result in a decrease in N-NH_3 concentration and acetate:propionate ratio.[5,6]

Fumarate is a hydrogen acceptor and acts as a propionate precursor in the rumen. It can be converted to succinate and propionate through reduction and decarboxylation reactions, respectively.[1]

Hydrogen (H_2) is a major substrate for methane (CH_4) formation; therefore, methanogenesis can be reduced via hydrogen acceptors addition to ruminal fermentation process.[7] Several studies have reported a decrease in methane production and rumen fluid N-NH_3 concentration[8-10] and an increase in molar ratio of propionate and acetate,[8,9] number of cellulolytic bacteria[11] and organic matter disappearance [10] following fumarate supplementation.

Hence, it appears that simultaneous use of disodium fumarate (DSF) and TEO may lead to synergistic effects on rumen fermentation. The aim of this study was to evaluate the effects of DSF and TEO solely and simultaneously on *in vitro* ruminal fermentation of a 50:50 forage: concentrate diet.

Materials and Methods

Experimental design. The experimental diet was a 50:50 alfalfa hay:concentrate diet [crude protein (CP): 15.50%, neutral detergent fiber: 29.20%, acid detergent fiber: 23% and non-fiber carbohydrate: 44.80%, dry matter (DM) basis] which was ground to pass through 1.50 mm screen. Rumen content was obtained from two adult rumen–fistulated (in dorsal sac of rumen) Kurdish sheep (30.00 ± 2.50 kg, body weight) before morning feeding. Sheep were fistulated by procedure described by Hecker[12] and three months later were used in this study. The experiments were approved by the Institutional Ethics Committee of University of Kurdistan, Sanandaj, Iran (No. A9532). Animals were fed twice daily with 0.50 kg of alfalfa hay and 0.50 kg of concentrate. The ruminal content was immediately strained through four layers of cheesecloth. In an anaerobic condition, 50 mL of buffered rumen fluid [ratio of buffer to rumen fluid was 2:1 and buffer was prepared as proposed by McDougall[13] was dispensed into a 125-mL serum bottle containing 0.50 g DM of the experimental diet. This study was included in 2 trials. Trial 1 evaluated the effects of different doses of DSF including 0.00, 8.00, 10.00 and 12.00 mmol L^{-1} (Sigma, St. Louis, USA) and TEO including 100.00, 200.00, 300.00 and 400.00 µL L^{-1} (Monin Company, Bourges, France) on *in vitro* ruminal fermentation characteristics (n = 6, runs = 2). In trial 2, the effects of concurrent using of selected dose of DSF (10.00 mM) plus 100.00, 200.00, 300.00 and 400.00 µL L^{-1} of TEO (T_{100}, T_{200}, T_{300} and T_{400}, respectively) on *in vitro* ruminal fermentation characteristics (n = 6, runs = 2) were analyzed. Bottles were sealed with rubber stoppers and aluminum caps and then placed in a shaking water bath for 24 hr at 38.60 ˚C. Head space gas pressure was recorded using a pressure transducer at 8, 16 and 24 hr of the incubation. Gas pressure was converted into volume using an experimentally calibrated curve [y (mL) = 6.59X + 0.241; R^2 = 0.97; x = gas pressure]. Following 24 hr of incubation, the bottle contents were filtered (pore size: 48 µm) and a 5 mL sample of each bottle filtrate was taken and acidified with 5 mL of 0.20 N HCl for ammonia nitrogen (N-NH_3) concentration determination and 1.50 mL of each was added to 375 µL of 20.00% orthophosphoric acid for volatile fatty acids (VFAs) concentration determination. The solid residues were oven-dried (55 ˚C for 48 hr) and used for *in vitro* dry matter (IDMD), organic matter (IOMD) and crude protein (ICPD) disappearances estimations.

Chemical analysis. Incubated or non-incubated samples were analyzed for DM (Method: 967.03), CP (Method: 976.05) and organic matter (Method: 942.05) by standard procedures.[14] The nitrogen concentration of samples and N-NH_3 concentration (Method: 976.05) of the medium were determined using Kjeldahl method (Kjeltec 2300, Foss Tecator AB, Hoganas, Sweden).[14] The VFAs concentration was determined using gas chromatography (PU 4410; Philips Unicam, Amsterdam, Netherlands).

Calculations and statistical analysis. Following 24 hr of incubation, partitioning factor (PF) was estimated as the ratio of truly degraded substrate to the mL gas produced.[15] Microbial crude protein synthesis (MCPS) was estimated according to equation recommended by Blummel and Becker.[14] Methane production was calculated from molar proportion of acetate, propionate and butyrate according to equation proposed by Bauchop.[16] Hydrogen recovery was estimated according to method proposed by Demeyer.[17] Orthogonal polynomial contrasts were performed to determine linear and quadratic effects of treatments. Data were analyzed as completely randomized design using PROC GLM of SAS (version 8.10; SAS Institute, Cary, USA). Tukey's test was employed to compare means.

Results

Trial 1. Compared to the control, the addition of DSF did not have any significant influence on IDMD, ICPD, gas production, MCPS, total VFAs, molar proportions of butyrate, valeric and isovaleric and hydrogen recovery

rate (Table 1). The N-NH_3 concentration was decreased (p = 0.009) at 12.00 mM DSF treatment. Compared to the control, DSF resulted in a linear increase in IDOM, proportion of propionate and PF (p < 0.01) and decrease in proportion of acetate (p = 0.026). Use of DSF at 8.00, 10.00 and 12.00 mM doses linearly decreased (p < 0.05) methane production compared to the control (-15.60, -11.80 and -13.50%, respectively). It should be noted that there were no differences between 8.00, 10.00 and 12.00 mM of DSF effects.

Compared to the control, the addition of 400.00 µL L^{-1} of TEO resulted in a decrease in IDMD (p < 0.05). A linear decrease was observed in IDMD with TEO concentration increase (p < 0.01). There were no significant differences in ICPD, gas production and MCPS (Table 2). The addition of 100.00 µL L^{-1} of TEO significantly decreased the N-NH_3 concentration and increased PF (p < 0.05). All concentrations of TEO (except 400.00 µL L^{-1}) resulted in an increase in IOMD. Supplementation of TEO quadratically increased IOMD, PF and molar proportion

Table 1. Effect of disodium fumarate on *in vitro* ruminal fermentation characteristics of a 50:50 alfalfa hay:concentrate diet after 24 hr of incubation.

Item*	Disodium fumarate (mM)					Effects	
	0.00	8.00	10.00	12.00	SEM	Linear contrasts	Quadratic contrasts
IDMD (%)	68.10	66.60	68.00	66.90	0.77	0.751	0.911
ICPD (%)	60.60	62.40	64.60	62.00	1.68	0.666	0.524
IOMD (%)	54.60^{a}	78.80^{b}	77.50^{b}	79.30^{b}	1.60	< 0.01	0.002
N-NH_3 (mg dL^{-1})	25.10^{a}	17.20^{ab}	17.50^{ab}	14.60^{b}	1.18	0.009	0.309
Gas	119.90	112.60	107.20	109.90	2.72	0.164	0.373
PF	2.00^{a}	3.53^{b}	3.42^{b}	3.50^{b}	0.122	0.001	0.009
MCP	162.70	174.90	178.40	159.40	4.46	0.905	0.216
Total VFAs (mM)	115.80	94.90	101.60	88.70	5.36	0.173	0.728
Individual (mol per 100 mol)							
Acetate	53.03	47.14	47.45	46.98	0.734	0.026	0.102
Propionate	18.85^{a}	25.06^{b}	23.25^{b}	24.02^{b}	0.420	0.006	0.012
Butyrate	26.88	26.32	27.48	27.27	0.759	0.739	0.910
Isovalerate	0.29	0.42	0.33	0.28	0.029	0.663	0.154
Valerate	1.54	1.21	1.49	1.43	0.079	0.989	0.409
Acetate:propionate	2.82^{a}	1.88^{b}	2.05^{b}	1.97^{b}	0.051	< 0.01	0.004
H_2 recovery (%)	84.72	92.21	89.58	87.01	2.32	0.843	0.335
Methane	29.43^{a}	24.85^{b}	25.95^{b}	25.45^{b}	0.299	0.020	0.055

^{ab} Means within a row with different letters are significantly different (p < 0.05).
* IDMD, ICPD and IOMD; *in vitro* DM, CP and OM disappearance, respectively. PF: partitioning factor (mg mL^{-1}), MCP: microbial crude protein (mg per g incubated DM), methane (mmol per 100 mol VFAs), Gas (mL per 0.50 mg DM).

Table 2. Effect of thyme essential oil on *in vitro* ruminal fermentation characteristics of a 50:50 alfalfa hay:concentrate after 24 hr of incubation.

Item*	Thyme essential oil (µL L^{-1})						Effects	
	0.00	100.00	200.00	300.00	400.00	SEM	Linear contrasts	Quadratic contrasts
IDMD (%)	68.10^{a}	67.30^{ab}	64.30^{ab}	62.20^{ab}	60.40^{b}	0.79	< 0.01	0.860
ICPD (%)	60.60	62.90	61.10	57.70	52.30	1.49	0.045	0.179
IOMD (%)	54.60^{a}	80.60^{b}	72.50^{b}	71.40^{b}	68.60^{ab}	1.77	0.155	0.002
N-NH_3 (mg dL^{-1})	25.10^{a}	17.50^{b}	21.50^{ab}	18.90^{ab}	23.00^{ab}	0.85	0.635	0.017
Gas	119.90	110.00	107.50	109.10	119.40	2.93	0.929	0.082
PF	2.00^{a}	3.38^{b}	3.19^{ab}	2.90^{ab}	2.88^{ab}	0.12	0.189	0.015
MCP	162.70	179.90	138.60	152.50	136.60	7.63	0.279	0.886
Total VFAs (mM)	115.80^{a}	81.00^{b}	100.60^{ab}	112.40^{a}	115.40^{a}	2.86	0.175	0.028
Individual (mol per 100 mol)								
Acetate	53.03	47.17	48.44	48.99	51.74	0.691	0.880	0.017
Propionate	18.85	20.96	20.07	21.56	20.62	0.496	0.264	0.385
Butyrate	26.88	30.05	29.42	24.81	25.42	0.772	0.166	0.186
Isovalerate	0.29	0.38	0.55	0.43	0.35	0.051	0.662	0.172
Valerate	1.54	1.44	1.52	1.93	1.87	0.064	0.029	0.466
Acetate:propionate	2.82	2.29	2.43	2.49	2.51	0.059	0.336	0.064
H_2 recovery (%)	84.72	96.49	89.05	83.82	82.62	1.310	0.111	0.072
Methane	29.43^{a}	26.54^{b}	28.05^{ab}	27.09^{ab}	27.78^{ab}	0.264	0.170	0.058

^{ab} Means within a row with different letters are significantly different (p < 0.05).
* IDMD, ICPD and IOMD; *in vitro* DM, CP and OM disappearance, respectively. PF: partitioning factor (mg mL^{-1}), MCP: microbial crude protein (mg per g incubated DM), methane (mmol per 100 mol VFAs), Gas (mL per 0.50 mg DM).

of valerate ($p < 0.05$) and decreased molar proportion of acetate, acetate: propionate ratio, N-NH_3 concentration ($p < 0.05$), hydrogen recovery rate ($p = 0.072$) and methane production ($p = 0.058$). The TEO at 100.00 µL L^{-1} level reduced total VFAs in comparison with control and 300.00 and 400.00 µL doses. Total VFAs concentration was quadratically affected by the TEO addition ($p = 0.028$).

Trial 2. In comparison with control, IDMD, N-NH_3 concentration, total VFAs, molar proportion of isovalerate and hydrogen recovery were unaffected in the treatments (Table 3). Inclusion of T_{200} resulted in an increase in ICPD ($p < 0.05$). The T_{300} and T_{400} significantly decreased gas production after 24 hr of incubation (– 6.50 and – 9.30%, respectively). Moreover, the addition of DSF_{10} with different doses of TEO quadratically decreased gas production ($p = 0.002$). Supplementation of DSF_{10} with different levels of TEO decreased ($p < 0.01$) molar proportions of acetate and butyrate, acetate: propionate ratio and methane production and significantly increased the molar proportion of propionate ($p < 0.01$) in comparison with control (Table 3).

Table 3. Effects of disodium fumarate (10.00 mM) with different doses of thyme essential oil (TEO) on *in vitro* ruminal microbial fermentation of a 50:50 alfalfa hay:concentrate diet after 24 hr of incubation.

Item*	Treatments**					SEM	Effects	
	C	T_{100}	T_{200}	T_{300}	T_{400}		Linear contrasts	Quadratic contrasts
IDMD (%)	72.00	75.90	75.60	73.10	75.90	0.58	0.248	0.391
ICPD (%)	74.30^{a}	77.00^{ab}	80.10^{b}	74.30^{a}	77.80^{ab}	0.52	0.272	0.112
IOMD (%)	82.20	79.50	79.50	80.90	80.10	1.18	0.745	0.624
N-NH_3 (mg dL^{-1})	13.10	11.70	9.50	10.50	11.10	0.62	0.251	0.180
Gas	123.80^{a}	118.90^{ab}	120.40^{ab}	115.80^{b}	126.60^{a}	0.86	0.688	0.002
PF	3.14	3.15	3.17	3.37	3.01	0.06	0.923	0.329
MCP	114.10	111.40	111.80	130.90	100.40	6.79	0.878	0.544
Total VFAs (mM)	88.80	85.50	84.50	94.40	93.30	1.08	0.071	0.076
Individual (mol per 100 mol)								
Acetate	48.28^{a}	45.23^{b}	45.83^{ab}	45.19^{b}	45.91^{ab}	0.238	0.004	0.034
Propionate	21.57^{a}	28.78^{b}	27.79^{bc}	25.73^{c}	26.71^{bc}	0.269	< 0.01	0.042
Butyrate	18.83^{a}	15.57^{b}	15.88^{b}	16.88^{b}	16.81^{b}	0.205	< 0.01	0.201
Isovalerate	6.52	7.38	7.30	8.64	7.22	0.386	0.544	0.261
Valerate	3.19	3.04^{a}	3.19^{ab}	3.56^{b}	3.34^{ab}	0.050	0.226	0.016
Acetate:propionate	2.24^{a}	1.72^{b}	1.76^{bc}	1.65^{c}	1.57^{bc}	0.017	0.001	< 0.01
H_2 recovery (%)	82.03	82.23	82.29	81.16	82.42	0.430	0.932	0.687
Methane	23.32^{a}	18.67^{b}	19.33^{b}	20.01^{b}	20.04^{b}	0.182	< 0.01	0.013

* IDMD, ICPD and IOMD; *in vitro* DM, CP and OM disappearance, respectively. PF: partitioning factor (mg mL^{-1}), MCP: microbial crude protein (mg per g incubated DM), Methane (mmol per 100 mol VFAs), Gas (mL per 0.50 mg DM)
** C: Control (no additive), T_{100}, T_{200}, T_{300} and T_{400}; 10 mM DSF plus 100, 200, 300 and 400 µL TEO, respectively.
^{ab} Means within a row with different letters are significantly different ($p < 0.05$).

Discussion

In contrast with our findings, *in vitro* comparison between DSF and other sodium salts of organic acids showed that DSF addition results in an increase in DM disappearance in high-forage diet.[1] It has been reported that DM disappearance of forage feeds increases when 7.00 mM of DSF is supplemented.[18] The effect of DSF on DM disappearance is not clear and varies with diet. The present results confirm previous findings suggesting that addition of 8.00 mM of DSF tends to decrease N-NH_3 concentration.[10] It has also been shown that addition of 7.35 mM of DSF does not affect N-NH_3 concentration in the semi-continuous culture system.[1] Probably, N-NH_3 amount reduction in the present study can be attributed to greater NH_3 utilization by rumen microorganisms and/or deamination activity reduction of hyper-ammonia producing bacteria.[19] The increase in PF demonstrated that DSF addition tends to improve fermentation efficiency.

As a consequence of these changes, the acetate: propionate ratio was decreased linearly as the concentration of fumarate increased confirming previous findings in batch culture system.[8,18] Fumarate can be converted to propionate and acetate via different pathways. Increase in molar proportion of propionate and no change in proportion of acetate in the present study may be due to acetate expenses for conversion to propionate.[11] In contrast with our findings, several studies have reported that fumarate supplementation can result in an increase in the acetate proportion.[11] At least, part of these inconsistencies may be due to differences in ingredients content and basal diets analysis.

In ruminal fermentation process, hexose conversion to VFAs results in an overall net release of reducing power. Hydrogen is used to reduce fumarate in the rumen and this decreases the H_2 availability for methanogen archaea that leading to CH_4 production fall. Reduction in CH_4 production by fumarate supplementation has been found in most of the previous *in vitro* studies.[8,11]

It seems that the effect of fumarate on CH_4 production may largely depend on the type of fermented substrate as fumarate can be more efficient in CH_4 production reduction in forage-based diets than high-concentrate ones.[8]

It is well-recognized that phenolic compounds such as thymol possess antibacterial and inhibitory effects on ruminal bacteria due to having hydroxyl group.[20] The present results confirm previous findings reporting that TEO addition reduces IDMD, ICPD, gas production and N-NH_3 concentration.[6,24] It seems that N-NH_3 concentration decrease with the TEO addition was associated with proteolysis, peptidolysis, deamination process and hyper-ammonia producing bacteria growth inhibition.[19,21] In the present study, linear decrease in ICPD might be due to inhibition of ruminal bacteria growth and ruminal fermentation by available phenolic compounds of TEO. Ruminal ICPD and N-NH_3 concentrations reduction might increase ruminal passage of dietary protein and enhance the efficiency of nitrogen utilization in ruminants.[22]

Generally, supplementation of TEO or thymol has caused either a decrease or no change in total VFAs concentration and methane production in most previous studies.[2,4,23,24] The present results confirm the previous findings reporting that addition of 500.00 mg L^{-1} of TEO reduces gas production (-17.40%), total VFAs (-25.80%) and proportion of propionate (-14.10%) and increases proportion of butyrate (+59.50%) and acetate: propionate ratio (+12.60%) and has no effect on proportion of acetate.[4] In the present study, 100.00 µL L^{-1} of TEO decreased (-9.80%) methane production compared to the control, although there was a concomitant decrease in total VFAs. It is well-demonstrated that antimicrobial activity of TEO can inhibit methanogenesis in rumen.[20] Also, 12.80% and 83.50% decreases in methane production with supplementation of 500 mg L^{-1} of TEO and 16.70 mg L^{-1} of thymol were found in a 24 hr *in vitro* batch culture, respectively.[4]

According to the results of current and previous studies, it is recognized that DSF at 10.00 mM level (DSF_{10}) can improve ruminal fermentation characteristics. Therefore, DSF_{10} was selected as a better dose to evaluate the effects of simultaneous use of DSF and different doses of TEO on ruminal fermentation characteristics. In trial 1, TEO at 400.00 µL L^{-1} level decreased IDMD (-11.30%), but in this trial, T_{400} did not affect the IDMD. Since fumarate is an intermediate in rumen microbial metabolism, it appears that DSF_{10} addition might remove some negative effects of TEO on IDMD. In contrast with our findings, it has been reported that addition of 200.00 mg L^{-1} of a blend comprising some essential oils active compounds (EOAC; containing thymol) with 0.00, 5.00, 10.00 or 15.00 mM of monosodium fumarate decreased N-NH_3 concentration compared to the control and EOAC solely.[23] The increase in *in vitro* ruminal ICPD without an increase in N-NH_3 concentration showed that ruminal efficiency of nitrogen usage increased and deamination relatively decreased. In this study, compared to control, simultaneous use of DSF and TEO (T_{300}) caused gas production reduction (-6.50%). It has been observed that gas production decreases (about 13.60 to 17.10%) by 200 mg L^{-1} of EOAC with or without fumarate, but no difference was observed among the different levels of fumarate.[5] In addition, it has also been reported that simultaneous use of fumarate and EOAC results in a significant increase in molar proportion of propionate and decrease in acetate: propionate ratio.[5] Our results revealed that simultaneous use of DSF_{10} and TEO can lead to glucogenic precursors increase. It is recognized that an enhance ratio of glucogenic (propionate) to lipogenic (acetate plus butyrate) VFAs in the rumen can improve liver glucose production, glucose supply for the mammary gland and lactose and milk production in high-producing dairy cows.[25] Results of the present study suggested that simultaneous addition of DSF_{10} and TEO results in a further decrease in methane production (from 14.00 to 20.00%) in comparison with alone DSF and TEO. Also, use of T_{100} linearly increased the molar proportion of propionate (33.40%) compared to DSF_{10} (27.90%). Hydrogen recovery was not affected by treatments in trial 1 and trial 2. However, hydrogen recovery in the DSF and TEO groups was higher than control and DSF along TEO groups. It shows that methanogenesis was further inhibited by DSF along TEO, while the treatments could not take all of the excess hydrogen for VFAs production.[5, 23]

In conclusion, the results of present study demonstrated that simultaneous use of DSF and TEO can cause a further decrease in methane production and acetate:propionate ratio compared to DSF and TEO solely. However, future studies are required to investigate the effects of simultaneous use of DSF and TEO in *in vivo* conditions, especially for dry and fresh cows.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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