Effects of eucalyptus oil and anise oil supplementation on rumen fermentation characteristics, methane emission, and digestibility in sheep[1]

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ABSTRACT: The objective of this study was to evaluate antimethanogenic activity of eucalyptus oil (**EUC**) and anise oil (**ANI**) in vitro and in vivo using sheep as a model. In vitro study was conducted using batch culture technique, each of EUC and ANI were added at 0, 50, 100, 200, or 400 mg/L of fermentation media with substrate containing 60% corn-based concentrate and 40% hay (DM basis). Total gas production (**GP**) linearly ($P < 0.01$) decreased with increasing ANI, whereas the GP was not affected with EUC addition. Supplementation of ANI and EUC linearly ($P < 0.01$) decreased total methane production and methane proportion in total gas. Total VFA and ammonia-nitrogen (NH_3-N) concentration linearly ($P < 0.01$) decreased with increasing ANI supplementation. For the in vivo study, a replicated 3 × 3 Latin square design was carried out using six ruminal cannulated Du Han hybrid sheep (BW, 64.5 ± 8.56 kg) with 22 d of periods. Three treatments were control diet (consisted of 60% corn-based concentrate and 40% Chinese wildrye hay), EUC (control diet supplemented with 0.5 g EUC/d per head), and ANI (control diet supplemented with 0.5 g ANI/d per head). Each period consisted of 14 d for adaption and 8 d for sampling and data collection. Supplementation of EUC and ANI had no effects on feed intake and apparent nutrient digestibility. Ruminal NH_3-N concentration was greater with EUC ($P < 0.01$) and ANI ($P = 0.03$) than control. Urinal allantoin output was less ($P < 0.05$) in sheep fed EUC and ANI than control animals. Methane emission was less ($P = 0.03$) in sheep fed ANI than sheep fed EUC, and a tendency of decrease for an eduction in this parameter was found for sheep fed with ANI ($P = 0.08$) compared to control. The in vitro results indicated a reduction of methane production with both EUC and ANI but in a dose-dependant manner. Supplementation of ANI tended to reduce ruminal methane production without adversely affecting rumen fermentation characteristics, nutrient intake, and digestibility, suggesting potential inhibition of ruminal methane emission in sheep supplemented with ANI.

Key words: anise oil, eucalyptus oil, methane, rumen fermentation, sheep

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INTRODUCTION

The increasing concerns by using antimicrobials in livestock production on the transmission and the proliferation of resistant bacteria via the food chain and the enteric methane emission are currently two major challenges facing by livestock

industry (Johnson and Johnson, 1995; Hristov et al., 2013). The use of natural compounds especially plant essential oil (**EO**) to explore as alternatives to in-feed antimicrobials was increasing in the attention of animal scientists during last decades due to their strong antimicrobial activities against a wide range of microorganisms (Sivropoulou et al., 1996). Numerous studies have shown that EO have the ability to affect rumen microbial activity (Oh et al., 2017) and reduce methane production in ruminants (Benchaar and Greathead, 2011).

Anise oil (**ANI**) is the extract of anise (*Pimpinella anisum L.*), and anethole (90.1%) is the major active component in ANI (Cardozo et al., 2006) which exhibits a broad spectrum of antimicrobial activity (Singh et al., 2008). Eucalyptus oil (**EUC**) is a complex mixture of a variety of monoterpenes, sesquiterpenes, and aromatic phenols (Batish et al., 2008). The EUC has been known as antibacterial, antifungicidal, and antiseptic in nature (Salari et al., 2006; Batish et al., 2008). Few studies, however, were reported for adding EUC and ANI in the diet of ruminants and their activities in inhibiting methane emission, especially with sheep. Therefore, the objective of this study was to evaluate the supplementation of EUA and ANI on rumen fermentation, methane emission, and nutrients digestibility in sheep. A batch culture study was conducted prior to in vivo study to assess the dose response of EUC and ANI by measuring gas production (**GP**) and fermentation characteristics, results were used to determine the dose of these two EOs to be used in vivo.

MATERIALS AND METHODS

The EUC and ANI that were used in this study were provided by Nanjing Vincero International Trading Co. Ltd. (Nanjing, China) with purity of 80% and 89%, respectively.

In Vitro Experiment

The in vitro incubation was conducted according to Menke and Steingass (1988) using calibrated glass syringes (Model Fortuna, Häberle Labortechnik, Germany). Rumen fluid was collected from four donor ruminally cannulated sheep fed with a diet containing 60% corn-based concentrate and 40% Chinese wildrye hay (DM basis; Table 1). The syringes were filled with 30 mL of medium comprising 10 mL of rumen fluid and 20 mL of buffer solution as described previously (Menke and Steingass, 1988). Substrate was the same as the diet fed to donor animals, ground through 0.5-mm sieve, with 200 mg (DM basis) weighed into 100-mL glass syringes fitted with plungers. The various amount of EUC or ANI were dissolved in ethanol and were added into 30 mL of inoculum to reach the doses at 0, 50, 100, 200, and 400 mg EO/L of the media. The incubation lasted for 24 h, and the total GP were read by the scale of syringes. After incubation, glass syringes were put in ice to stop the fermentation and the media pH was measured immediately using a portable pH meter (Starter 300; Ohaus Instruments Co. Ltd., Shanghai, China). The fermentation media were transferred to a 50 mL centrifuge tube. Then, the fermentation media was centrifuged at 800 × *g* for 15 min. Five milliliters of supernatant sample was added with 1 mL 25% (wt/vol) HPO_3 and stored at −20 °C to measure VFA concentration using gas chromatography (GC-8A; Shimadzu Corp., Kyoto, Japan) described by Hu et al. (2005). Another 5 mL supernatant sample was added with 1 mL of 1% H_2SO_4 (wt/vol) to determine ammonia-nitrogen (NH_3-N) concentration using the phenol-sodium hypochlorite colorimetric

Table 1. Ingredients and nutrient composition (% of DM, unless otherwise specified) of basal diet used for the sheep

Item	Content
Ingredients,%	
Corn grain[1]	42.6
Soybean meal	14.6
Chinese wildrye hay[2]	40.0
Limestone	0.80
$CaHPO_4$	0.50
NaCl	0.50
Premix[3]	1.00
Nutrients composition	
DM	88.2
OM	92.5
CP	13.1
EE	1.70
NDF	32.8
ADF	19.9
Ca	0.66
P	0.34
ME,[4] MJ/kg	9.30

[1]Composition was 85.7% DM, 98.5% OM, 8.4% CP, 9.4% NDF, 3.5% ADF, 1.9% EE, 0.09% Ca, and 0.28% P based on three samples composited by period.

[2]Composition was 90.6% DM, 92.1% OM, 8.0% CP, 68.8% NDF, 44.0% ADF, 1.8% EE, 0.39% Ca, and 0.09% P based on three samples composited by period.

[3]The premix provided the following per kg of diets: Cu 15.0 mg, Fe 100.0 mg, Mn 60.0 mg, Zn 100.0 mg, I 0.9 mg, Se 0.3 mg, Co 0.2 mg, VA 16 000 IU, VD_3 4 000 IU, VE 100 IU.

[4]Estimated based on small ruminant NRC (2007).

method described by Broderick and Kang (1980). The distilled water was added to the precipitate and mixed, then centrifuged at 800 × *g* for 15 min to discard the supernatant. This procedure was repeated twice. Then the precipitate was oven-dried at 105 °C for 12 h for the calculation of in vitro DM digestibility (**DMD**). The in vitro methane concentration in total gas was determined using TP-2060T gas chromatograph (Chromatographic conditions: Thermal conductivity detector, TDX-01 packed column, 1 mm × 3 mm × 2 mm, inlet temperature 150 °C, column temperature 120 °C, detector temperature 150 °C with carrier gas helium, flow rate 50 mL/min, injection volume 0.1 mL), and methane production was calculated according to the GP and methane content. The batch culture was repeated at different day.

In Vivo Experiment

Animals, experimental design, and diet. The experiment was conducted at the Animal Research Center of Beijing University of Agriculture and all experimental procedures were approved by the Animal Care Committee, Beijing University of Agriculture (Beijing, China). Six ruminally cannulated Du Han hybrid sheep (BW: 64.5 ± 8.56 kg) were randomly allocated to one of three dietary treatments in a replicated 3 × 3 Latin square design balanced for carry-over effects. Each period consisted of 14 d for adaptation, followed by 8 d for sampling and data collection. The basal experimental diet was the same as used in experiment 1. The treatments were control (i.e., basal diet without EO), control diet supplemented with 0.5 g EUC/d per head or with 0.5 g ANI/d per head. The dosage of EO used in sheep was estimated based on our in vitro results and assuming 5 L of rumen contents of sheep (Purser and Moir, 1966) and 8%/h of passage rate out of the rumen (Chaji et al., 2010).

The dietary ingredients and nutrient composition of the basal diet are presented in Table 1. The sheep were housed in individual tie-stalls and fed ad libitum and free access to water. The amount of the feed supplied to ensure at least 5% refusal. Feed offered and refused were recorded daily for each sheep for the entire experiment. The feed was sampled once a week to determine DM contents, and the samples were oven-dried at 55 °C for 48 h and ground through a 1-mm screen (standard model 4, Arthur Thomas Co., Philadelphia, PA) for subsequent chemical analysis.

Intake, total digestibility, urine, and fecal collection. Feed intake (kg/d) of each sheep was calculated as the difference between the feed offered and refused of each day. Sheep were placed in digestion crates for 3 d of adaptation (days 12 to 14) and 4 d of total collection of feces and urine (days 15 to 18). Feces were collected from the tray drawer carefully, homogenized individually, and weighed daily. The fecal samples were subsampled (approximately 10% of the total amount), and 10 mL of HCl (10%) was added to prevent ammonia loss. The feed and fecal samples were dried in a forced-air oven at 55 °C for 48 h and ground through a 0.5-mm screen using a Cyclotec mill (Tecator 1093; Tecator AB, Höganäs, Sweden) before analysis. Analytic DM content was determined at 105 °C for 5 h (Wang et al., 2014), and nutrient content of the samples were measured according to AOAC (1990) for OM (method 942.05), CP (method 988.05), and ADF (method 973.18). The NDF content was determined following the method of Van Soest et al. (1991) using heat-stable α-amylase without sodium sulfite. Total urine was collected from the bladder of each sheep using indwelling balloon catheters (26 French, 75-mL balloon; C. R. Bard Inc., Covington, GA), and directed through tubing into 5-L plastic buckets containing a sufficient quantity of acid (~100 mL 10% H_2SO_4) to keep pH < 2.5. Two sets of urine sample (about 1% of the total volume) were collected with five times dilution, and stored at −20 °C for analysis of allantoin, uric acid, and Xan + hypoxanthine. Total purine derivatives (**PD**) excreted (mmol/d) were estimated as the sum of uric acid, allantoin, and Xan + hypoxanthine and were used as an indirect measurement of microbial protein synthesis (Chen and Gomes, 1992). Nutrient digestibility in the total digestive tract was determined by total collection of feces during the 4-d period.

Ruminal pH and fermentation characteristics. Ruminal contents were collected on day 19, samples were taken at 0, 1, 3, 6, and 9 h after morning feeding. Approximately 30 mL ruminal contents were obtained from multiple sites within the rumen and strained through nylon mesh (pore size 355 μm). The pH in the ruminal fluid was immediately measured using a portable pH meter (Starter 300; Ohaus Instruments Co. Ltd., Shanghai, China). Subsamples (5 mL) were preserved with 1 mL of 25% (wt/vol) HPO_3 and 1 mL of 1% H_2SO_4 for determining VFA and NH_3-N concentrations, respectively, as described in the in vitro experiment. The samples were stored frozen at −20 °C until analyzed. In addition, 1 mL of ruminal fluid was preserved with 5 mL of methyl green-formalin-saline

solution and stored in darkness at room temperature for enumeration of protozoa. Samples for protozoa counting including *Entodinium*, *Diplodinium*, *Isotricha*, and *Ophryosolex* were measured at room temperature and in the dark using a Neubauer-Improved Bright-Line counting cell (Hausser Scientific, Horsham, PA) by microscopy (Ogimoto and Imai, 1981). Each sample was counted in duplicate and results were accepted with coefficient of variation <10%.

Methane emission measurement of sheep. Sheep were transferred to an open-circuit respiration chamber to measure the methane emission on days 20 to 22, and animals were fed using individual trough and water sink. The sheep were adapted to the Chamber on day 20, and the methane emission was measured for 48 h on days 21 to 22. The chamber design and associated analytical equipment were detailed by Grandl et al. (2016). In brief, the sheep were put into a chamber with 8.3 m^3 of volume and the chamber was air-conditioned to maintain a temperature of 18 °C and a relative humidity of 55% at air pressure of −60 Pa. Airflows were set to 250 L/min (Promethion FG-250 flow generators, Sable Systems Europe GmbH, Berlin, Germany). Concentrations of CH_4 and CO_2 were determined using a gas analyzer (Promethion GA-4, Sable Systems). The gas analyzers were calibrated automatically before each measurement using pure N_2 (99.999%) and a mixed gas (0.5% CO_2, 0.1% CH_4, in N_2 as carrier). Individual CH_4 emission was calculated as the average of the 2 d of measurement from each sheep.

Statistical Analyses

For the in vitro experiment, statistical analyses were carried out using PROC MIXED procedure of SAS software (version 9.0; SAS Inst. Inc., Cary, NC) with model including dose of EO as fixed effects and batch as random effects. Orthogonal polynomial contrasts were performed to examine linear and quadratic dose responses of EUC and ANI. For the in vivo experiment, the mixed model included the fixed effects of dietary treatment and the random effects of square, sheep within square, and period within square. Day (or time within day) was considered a repeated measure for variables measured over time. For the repeated measures, various covariance structures were tried with the final choice depending on low values for the Akaike's information criteria. The PDIFF option adjusted by the Tukey method was included in the least squares means (**LSMEANS**) statement to account for multiple comparisons among treatments. The results were reported as LSMEANS. Significance was declared at $P \leq 0.05$. Trends were discussed at $0.05 < P \leq 0.10$.

RESULTS

In Vitro Rumen Fermentation and Methane Production

Gas production linearly ($P < 0.01$) decreased with ANI, but it was not affected with EUC addition (Table 2). Supplementation of ANI or EUC linearly ($P < 0.01$) decreased total methane production and methane proportion in the total GP. Adding EUC linearly ($P < 0.01$) decreased in vitro DMD but adding ANI changed DMD at a quadratic response ($P = 0.04$). Adding ANI linearly ($P < 0.01$) decreased methane production (mL/g of digestible DM) but not with EUC addition. Total VFA and NH_3-N concentration linearly ($P < 0.01$) decreased with increasing adding ANI. The NH_3-N linearly ($P = 0.03$) increased with increasing dosages of EUC. Increasing ANI addition quadratically changed the molar proportion of acetate ($P < 0.01$), propionate ($P < 0.01$), and isovalerate ($P = 0.02$), as well as the ratio of acetate to propionate ($P < 0.01$). With adding EUC, quadratic response ($P < 0.01$) was also noticed on the molar proportion of propionate and butyrate, as well as the ratio of acetate to propionate ($P < 0.01$). Supplementation of ANI linearly ($P < 0.01$) decreased the proportion of butyrate and isobutyrate, and the molar proportion of isobutyrate ($P < 0.01$) and isovalerate ($P < 0.01$) were linearly decreased by increasing EUC addition.

Intake and Apparent Digestibility

Intake of DM and other nutrients was not affected by adding either EUC or ANI compared with control (Table 3). No treatment effect was found in the apparent digestibility of DM, OM, CP, and NDF in the total digestive tract, but the digestibility of ADF tended ($P < 0.10$) to be less with ANI than control or EUC supplementation.

Ruminal pH and Fermentation Characteristics

Ruminal pH, ruminal fermentation characteristics, and protozoa counts are presented

Table 2. Dose–response effect of eucalyptus oil (EUC) and anise oil (ANI) on in vitro gas production (GP), DM digestibility (DMD), and rumen fermentation characteristics after 24 h of incubation

		Treatment,[1] mg/L						*P* value	
Items		0	50	100	200	400	SEM	L[2]	Q[2]
GP, mL	EUC	51.6	46.2	56.4	53.5	49.0	3.40	0.82	0.26
	ANI	51.6	53.2	50.6	46.3	40.9	1.44	<0.01	0.74
DMD, %	EUC	21.3	19.6	19.7	19.5	15.8	0.54	<0.01	0.32
	ANI	21.3	17.7	17.9	15.9	13.0	0.54	<0.01	0.04
CH_4, mL	EUC	11.1	11.8	12.6	11.0	9.1	0.62	<0.01	0.08
	ANI	11.1	10.7	10.5	8.1	5.8	0.50	<0.01	1.00
CH_4, % of GP	EUC	21.6	22.5	22.3	20.6	18.6	1.01	0.02	0.46
	ANI	21.6	20.2	20.8	17.6	14.1	1.07	<0.01	0.86
CH_4, mL/g of digestible DM	EUC	261	302	320	283	288	15.7	0.87	0.19
	ANI	261	303	295	255	222	14.8	<0.01	0.17
pH	EUC	6.71	6.69	6.74	6.71	6.68	0.032	0.51	0.56
	ANI	6.71	6.61	6.62	6.66	6.59	0.024	0.03	0.67
NH_3-N, m*M*	EUC	27.7	25.2	23.5	26.0	37.3	5.37	0.03	0.10
	ANI	27.7	27.9	26.7	23.2	14.9	4.68	<0.01	0.43
Total VFA, m*M*	EUC	77.7	87.9	84.5	86.5	80.7	5.54	0.92	0.27
	ANI	77.7	67.5	72.3	61.6	58.2	2.46	<0.01	0.13
Molar proportion, m*M*/100 m*M*									
Acetate	EUC	67.0	67.0	67.3	67.4	65.5	0.60	0.08	0.15
	ANI	67.0	66.7	66.2	65.8	69.8	0.43	<0.01	<0.01
Propionate	EUC	16.7	16.4	16.4	16.4	22.7	0.40	<0.01	<0.01
	ANI	16.7	17.1	17.5	20.5	17.9	0.35	<0.01	<0.01
Butyrate	EUC	14.1	14.5	14.2	13.6	10.2	0.31	<0.01	<0.01
	ANI	14.1	14.1	14.1	12.0	11.0	0.226	<0.01	0.58
Isobutyrate	EUC	0.57	0.58	0.56	0.51	0.24	0.044	<0.01	0.06
	ANI	0.57	0.55	0.56	0.33	0.22	0.032	<0.01	0.69
Valerate	EUC	0.48	0.48	0.48	0.49	0.53	0.016	0.05	0.31
	ANI	0.48	0.45	0.49	0.53	0.50	0.019	0.13	0.28
Isovalerate	EUC	1.11	1.14	1.05	1.05	0.78	0.053	<0.01	0.18
	ANI	1.11	1.10	1.14	0.87	0.54	0.031	<0.01	0.02
Acetate:propionate	EUC	4.01	4.10	4.10	3.97	2.89	0.101	<0.01	<0.01
	ANI	4.01	3.91	3.79	3.21	3.90	0.089	0.13	<0.01

[1]The 0.5 mL EUC and ANI mixture (EUC and ANI dissolved in ethanol with different proportion) were added to 30 mL of medium respectively to up to 0, 50, 100, 200, and 400 mg/L of incubation media as five treatments.

[2]L, Q = linear or quadratic effect of different EUC and ANI addition concentration (0, 50, 100, 200, and 400 mg/L of incubation media).

in Table 4. No significant differences in ruminal mean pH and total VFA concentration were detected among the three diets. Molar proportion of acetate, isobutyrate, valerate, isovalerate did not differ. Ratio of acetate to propionate was similar among the treatments. There were sampling time effects on the molar proportion of some individual VFA but no interaction between treatment and sampling time was observed. No significant differences in the total protozoa counts were observed among the treatments. The *Ophryosolex* proportion in total protozoa showed a tendency ($P = 0.08$) to be less in sheep fed ANI compared to EUC. Ammonia-nitrogen concentration was greater in sheep fed diet supplemented with EUC ($P < 0.01$) or ANI ($P = 0.03$) than sheep fed control diet, and a sampling time effect was also noticed.

Urinary PD Excretion and Methane Emission

Urinary excretion of PD and daily methane emission data are shown in Table 5. Urinary allantoin production was less in sheep fed EUC ($P = 0.04$) and ANI ($P = 0.02$) than sheep fed control diet, but no difference in allantoin production was found between EUC and ANI. There was no difference in urinary production of uric acid and Xan + hypoxanthine among treatments. The amount of urinary PD excretion was less ($P = 0.03$) with ANI compared to the sheep fed control diet. Methane emission, expressed as daily production, tended

($P = 0.08$) to be less in sheep fed with ANI than in sheep fed with EUC or control diet. However, no significant differences in methane production were found when the methane emission was expressed based on unit of metabolic BW, DMI, or digestible DMI among the treatments.

Table 3. Effects of eucalyptus oil (EUC) and anise oil (ANI) on nutrients intake and apparent digestibility in the total digestive tract of sheep

	Treatment[1]				
Items	Control	EUC	ANI	SEM	*P* value
Intake, g/d					
DM	1,940	1,828	1,698	188.9	0.20
OM	1,794	1,690	1,570	174.7	0.20
CP	255	240	223	24.8	0.21
NDF	637	600	557	62.0	0.20
ADF	386	363	338	37.6	0.21
Digestibility, %					
DM	63.9	64.5	60.5	3.07	0.27
OM	66.5	67.1	63.3	2.90	0.32
CP	66.6	67.8	64.0	2.85	0.53
NDF	39.1	38.4	35.3	4.27	0.81
ADF	34.5	34.3	29.4	4.16	0.67

[1]Three treatments: control (fed a control diet, consisted of corn concentrate, soybean meal, and Chinese wildrye hay with concentrate:forage = 60:40), EUC treatment (control diet supplemented with 0.5 g/d/head EUC), ANI treatment (control diet supplemented with 0.5 g/d/head ANI).

DISCUSSION

Supplementation of EUC and ANI has been reported inconsistent effects on rumen fermentation from in vitro or in vivo studies (Calsamiglia et al., 2007). For instance, it has been reported anethol and ANI (2.2 mg/L) decreased total VFA concentration in continuous culture (Busquet et al., 2005). In contrast, the ruminal total VFA concentration was not affected when growing heifers was supplemented with ANI in a high-concentrate diet (90% concentrate; Cardozo et al., 2006). The dose of EO, type of substrate, or experimental conditions might be the key factors leading to the inconsistent results. Additionally, although studies using ANI or EUC were conducted to evaluate their effects on ruminal fermentation such as pH and VFA concentration, few studies using animals, especially sheep, were conducted to investigate their effects on methane production. Sheep production represents a great portion in livestock industry in China and many other places in the world. Thus, the contribution of sheep production to methane production is significant (Broucek, 2014). The present study was novel and significant scientifically and publically because the study was focusing on both developing alternatives to in-feed antimicrobials and mitigation of methane emission. Additionally, an advanced open-circuit respiration chamber was

Table 4. Fermentation characteristics and protozoa concentration in the rumen fluid of sheep fed a diet containing eucalyptus oil (EUC) and anise oil (ANI)

	Treatment[1]				*P* value		
Items	control	EUC	ANI	SEM	Treat	Time	Treat × time
pH	5.87	6.00	6.07	0.130	0.57	0.35	0.84
NH_3-N, mM	15.4[b]	19.4[a]	17.7[a]	1.21	<0.01	<0.01	0.43
Total VFA, mM	84.5	80.0	80.0	4.94	0.72	0.89	0.18
Molar proportion, mM/100 mM							
Acetate	60.8	61.7	61.0	2.14	0.92	0.58	0.77
Propionate	23.7	18.6	22.8	3.08	0.20	0.02	0.90
Butyrate	13.1	17.0	13.2	2.07	0.09	0.02	0.95
Isobutyrate	0.51	0.76	0.85	0.219	0.50	0.01	0.95
Valerate	0.74	0.71	0.75	0.076	0.84	0.25	0.70
Isovalerate	1.03	1.33	1.39	0.317	0.58	0.01	0.89
Acetate:propionate	2.68	3.47	3.12	0.476	0.29	0.06	0.84
Total protozoa, $\times 10^5$/mL	6.00	5.80	4.50	1.170	0.41	0.10	0.80
% of total							
Entodinium	82.8	82.9	77.3	3.56	0.21	0.62	0.97
Diplodinium	10.6	10.3	13.4	1.53	0.30	0.48	0.50
Isotricha	4.24	8.95	8.01	1.563	0.62	0.44	0.45
Ophryosolex	2.36	3.85	1.29	1.274	0.21	0.45	0.71

[a,b]Means in the same row not bearing a common superscript letter are significantly different ($P < 0.05$).

[1]Three treatments: control (fed a control diet, consisted of corn concentrate, soybean meal, and Chinese wildrye hay with concentrate:forage = 60:40), EUC treatment (control diet supplemented with 0.5 g/d/head EUC), ANI treatment (control diet supplemented with 0.5 g/d/head ANI).

Table 5. Effects of eucalyptus oil (EUC) and anise oil (ANI) on urinary excretion of purine derivatives (PD) and methane emission in sheep

	Treatment[1]				
Items	Control	EUC	ANI	SEM	P value
Urine PD, mmol/d					
Allantoin	14.08[a]	10.38[b]	9.73[b]	2.074	0.04
Uric acid	0.72	0.58	0.60	0.106	0.38
Xan + hypoxanthine	3.07	2.75	2.27	0.338	0.40
Total PD[2]	17.9	13.7	13.0	2.39	0.06
$BW^{0.75}$, kg	23.5	24.1	23.4	1.41	0.16
CH_4					
L/d	52.0	54.0	45.7	6.58	0.08
L/kg $BW^{0.75}$/d	2.22	2.22	1.97	0.267	0.23
L/kg of DMI	26.6	29.1	28.5	2.81	0.74
L/kg of digestible DMI	41.8	45.5	46.5	3.84	0.66

[a,b]Means in the same row not bearing a common superscript letter are significantly different ($P < 0.05$).

[1]Three treatments: control (fed a control diet, consisted of corn concentrate, soybean meal, and Chinese wildrye hay with concentrate:forage = 60:40), EUC treatment (control diet supplemented with 0.5 g/d/head EUC), ANI treatment (control diet supplemented with 0.5 g/d/head ANI).

[2]Purine derivatives (PD) were estimated as the sum of allantoin, uric acid, and Xan + hypoxanthine.

used to measure the methane emission which provided direct determination of methane production measurement.

In Vitro Experiment

In the present study, increasing in vitro dose of EUC or ANI linearly decreased methanogenesis, with a maximum methane reduction (mL/g of substrate) of 18% and 48% at 400 mg EUC and ANI/L, respectively. Our results are consistent with Kumar et al. (2009) and Patra and Yu (2012) who reported methane inhibition by adding EUC. It was reported that the methane production decreased linearly in the study of Kumar et al. (2009) which was due to the changed rumen fermentation and linearly decreased GP. It was reported the linearly decreased abundance of archaea, protozoa, and major cellulolytic bacteria (i.e., *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus*) was accompanied with the linearly decreased methane production for the incubation of EUC (Patra and Yu, 2012), indicating that these ruminal microbes might be associated with methane production. Thus, the decreased methane emission in the present study might be attributed to the similar mechanism such as the effects on protozoa or cellulolytic bacteria. A low dose of anethole up to 20 mg/L of medium also caused an in vitro inhibition of methane (Chaves et al. 2008). It was reported that EUC modified in vitro rumen fermentation in sheep, mainly by reducing GP and protein deamination (Attia et al., 2016). Although the methane emission as proportion of total GP linearly decreased with both EUC and ANI supplementation, the linear reduction in methane emission was only observed with ANI when it was expressed as unit of digestible DM. It suggests that methane reduction efficiency or feed efficiency has potentially improved with increasing dosages of ANI but it is not necessarily with EUC. The inhibition of enteric methane production was reported via two pathways including an inhibition of H_2 production or a shift in H_2 allocation (Guyader et al., 2015). Adding EUC or ANI changed rumen fermentation pattern into more propionate production explained the reduction of methane production by shifting H_2 allocation. The current study did not measure the H_2 production. Thus, another pathway of inhibition of enteric methane production by inhibition of H_2 production should be verified by further studies.

It was reported that addition of ANI decreased in vitro total VFA concentration, NH_3-N concentration, and the proportions of acetate and propionate (Busquet et al., 2005; Busquet et al., 2006; Cardozo et al., 2006). The inclusion of the EUC in an in vitro trial decreased the total VFA and NH_3-N concentration, methane emission, and GP, even at 0.5 mL/L concentration (Mukharji and Srivastava, 2015). Those results are mostly consistent with the addition of ANI and EUC in the current study except for the increased proportion of propionate. The quadratic response of the ratio of acetate to propionate to the dosages of ANI and EUC was mainly due to the increased propionate proportion. It was found that the highest propionate proportion was achieved at the highest dose (400 mg/L) for EUC but at 200 mg/L for ANI. These results suggest that adding either EUC or ANI favored to propionate production in the rumen but their activity is in the dose-dependent manner.

The in vitro results were used as reference to determine the EO dose to be evaluated in sheep study. The selection criteria were based on the methane production (mL/g digestible DM), DMD, and VFA production to meet our research objectives. It is clear that for both EO, the highest dose (400 mg/L) was not considered because the DMD were considerably reduced at the 400 mg/L. For EUC, the methane production (mL/g digestible DM) was not dose-dependent on EO, therefore, the dose of 50 mg/L was selected as the GP and

VFA concentration were not different from 50 to 200 mg/L. For ANI, the low dose of 50 mg/L was used in sheep since the DMD was considerably decreased starting with the low dose. Thus, the 0.5 g/d/head was used for the in vivo study.

In Vivo Experiment

The absence of EUC or ANI supplementation effects on rumen pH in sheep in the present study is in agreement with previous reports. Supplementation of EO had no effects on ruminal pH in beef cattle fed with mixed EO (Beauchemin and McGinn, 2006) or in dairy cows fed garlic and juniper berry EO (Yang et al., 2007). Till now, the research using sheep for evaluating the effects of EUC and ANI on rumen pH and rumen fermentation is scarce, and the results with the effects of EO on ruminal VFA production are inconsistent (Cardozo et al., 2006; Bodas et al., 2012). Cardozo et al. (2006) reported no ANI supplementation effect on the total rumen VFA concentration, but decreasing acetate-to-propionate ratio in growing heifers. Whereas, the present total VFA concentration and individual VFA profiles were unchanged by adding EUC or ANI comparing with control. In the present study, 0.5 g/d of ANI was supplemented to sheep (BW = 64.5 kg) showed the different results compared with Cardozo et al. (2006) which used 2 g/d of ANI to Holstein heifers (average BW = 450 kg) under a 90:10 concentrate:barley straw diet. This discrepancy might be attributed to the different efficient roles of ANI between heifers and sheep, but more studies need to be done to clarify it. It has been reported that EO supplementation had the ability to enhance rumen fermentation efficiency by decreasing acetate-to-propionate ratio, thus it favors to improve production efficiency (Khiaosa-ard and Zebeli, 2013). The increased rumen VFA production with EO might be achieved when the undissociated hydrophobic form of the EO active molecules is more active against the cell membrane of rumen microbes (Cardozo et al., 2006; Spanghero et al., 2008). Thus, the further study should be conducted to clarify the effects of EUC or ANI on specific ruminal microbial communities such as rumen cellulolytic bacteria (Bodas et al., 2012).

Feed intake and apparent digestibility were unchanged in the current study by supplementing either EUC, which are in agreement with the results from previous study by supplementing EUC to untreated rice straw in swamp buffaloes (Thao et al., 2014). Yang et al. (2007) also reported no change in intakes of DM, OM, NDF, and ADF by dietary supplementation of garlic oil or juniper berry oil (2 g/d) in lactating dairy cows. However, inconsistent effects of EO addition on DMI were reported by other studies: feeding 2 or 4 g/d of EO mixture increased DMI by growing beef cattle (Benchaar et al., 2006), but adding a mixture of cinnamaldehyde (0.6 g/d) and eugenol (0.3 g/d) oils decreased DMI in Holstein heifers (Cardozo et al., 2006). From these last two studies, it appeared that the response of DMI to EO was not associated with the dose of EO rather source of EO, diets or type of animals. The dose of EUC and ANI at 0.5 g/d applied in the current study was relatively higher compared with the previous studies using large cattle. However, no adverse effects on feed intake and DMD seemed to contrast to our in vitro results that DMD linearly decreased with increasing EO supplementation, suggesting the EO dose used in this study might be lower than necessary dose in sheep. The lowest level of EO used in vitro was selected for in sheep study because one of reasons is that the level of feed additives required in vitro to be active is often suggested higher than in vivo needed. However, failure to have significant effect might be due to lower level of EO used in sheep. In other word, we may speculate that sheep may need higher dose of EO than cattle to be effective.

It was commonly reported EO inhibit ruminal proteolytic activity, thus reduced in vitro ruminal NH_3-N concentration with ANI is consistent with other reports (Calsamiglia et al., 2007; Sallam et al., 2009). However, the greater ruminal NH_3-N concentration in sheep supplemented with EUC and ANI of the present study was somewhat contrasted to the generally known inhibition of proteolytic activity by EO. It was reported that the roles of inhibiting ruminal microbial activity for uptake NH_3-N was greater than the inhibiting role of ruminal proteolytic activity (Calsamiglia et al., 2007). Thus, the NH_3-N concentration was increased by the relative stronger inhibiting activity of ruminal microbial uptake NH_3-N. The decreased microbial N synthesis was often accompanied with greater NH_3-N concentration in the rumen (Broderick and Kang, 1980). The less urinary total PD excretion by sheep fed ANI indicated less microbial N synthesis (Chen et al., 1992). Thus, the greater ruminal NH_3-N might have resulted from less ruminal microbial protein production in sheep fed ANI, and thus less NH_3-N consumption. In addition, it was reported antimicrobial properties of natural plant extracts may provide an alternative way to manipulate ruminal fermentation for the improvement

of protein utilization in ruminants (Bodas et al., 2012). Thus, the greater NH_3-N was consisted with less total urinary PD in sheep fed ANI or EUC, suggesting the supplementation of these two EO especially for ANI adversely affect ruminal microbial protein synthesis in sheep.

Most studies found that methane inhibition by plant extract in in vitro conditions might not be translated into similar effects in in vivo conditions (Kamra et al., 2012; Guyader et al., 2017). This might happen due to improper selection of the dose of these metabolites in the ration of animals, different fermentation environment, or the adaption roles of additives by long-time feeding (Kamra et al., 2012; Guyader et al., 2017). Thao et al. (2014) suggested that feeding EUC could modify the rumen fermentation in reducing methane production with either untreated or 3% urea-treated rice straw in swamp buffaloes. The supplementation EUC was reported to inhibit methane production at different supplementation dose (Tatsuoka et al., 2008; Sallam et al., 2009; Patra and Yu, 2012). In addition, Sallam et al. (2009) reported that low dose of EUC addition has potential role in methane mitigation, and thus beneficial for improving nutrient utilization. The inhibition of methane production by EO in the rumen was reported due to specifically targeting the methanogens (Morgavi et al., 2010). Furthermore, the EUC was reported to inhibit methane emission by decreasing the number of ruminal protozoa (Patra, 2010; Oh et al., 2017), but the protozoa counts were not affected by EO in this study. Newbold et al. (2004) reported that ruminal protozoa counts were not affected when sheep were fed 110 mg/d of a mixture of EO, which is consistent with our findings. The methane production tended to be lower in sheep fed ANI compared to control indicating beneficial activity of ANI in mitigating methane production. However, the lack of the EUC effect on methane production in sheep study might be due to insufficient dose fed to sheep as we discussed above. In addition, the difference in microbial community and activity between in vitro and in vivo (Kamra et al., 2012) might contribute to the discrepant results as well. Thus, our study confirms the necessity to conduct in vivo study to confirm the EO effects on methane production.

CONCLUSIONS

Our study indicated that the supplementation of EUC and ANI inhibited in vitro methane emission in linear fashion, and the EUC showed no adverse impact on GP and VFA concentration. The ANI would inhibit the microbial protein synthesis potentially by mainly restricting the ammonia N utilization in the rumen. The ruminal methane production had a tendency to be less in sheep fed ANI at daily dose of 0.5 g without adversely affecting rumen fermentation, nutrient intake, and digestibility, indicating the potential activity of ANI in inhibiting ruminal methane emission of sheep. Further study using large number of animals with incremental EO doses during long experimental period is warranted to confirm its methane-inhibition activity.

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