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Evaluation of origanum oil, hydrolysable tannins and tea saponin in mitigating ruminant methane: In vitro and in vivo methods

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Abstract

The objective of this study was to investigate the effects of origanum oil (ORO), hydrolysable tannins (HYT) and tea saponin (TES) on methane (CH_4) emission, rumen fermentation, productive performance and gas exchange in sheep by using in vitro and in vivo methods. The ORO, HYT and TES additive levels were normalized per kg dry matter (DM) in both in vitro and in vivo experiments: ORO-0, 10, 20 and 40 ml/kg; HYT-0, 15, 30 and 60 g/kg; and TES-0, 15, 30 and 60 g/kg, respectively. During in vitro incubation, 40 ml/kg ORO linearly decreased CH_4 emission ($p < 0.05$); 20 and 40 ml/kg ORO cubically decreased carbon dioxide (CO_2) production ($p < 0.05$), and rumen pH was cubically raised with the increasing ORO additive level ($p < 0.01$). The 60 g/kg HYT cubically decreased CH_4 production ($p < 0.05$). The pH of 60 g/kg HYT was higher than that of 15 and 30 g/kg ($p < 0.01$); the pH of 20 g/kg TES was higher than that of 5 g/kg ($p < 0.05$). In the in vivo experiments, 40 ml/kg ORO inhibited dry matter intake ($p < 0.01$) cubically and reduced average daily gain (ADG) and feed conversion ratio (FCR) cubically ($p < 0.05$), and 20 or 40 ml/kg ORO linearly decreased CH_4 production based on per day or metabolic weight ($W^{0.75}$) ($p < 0.05$). Both 30 and 60 g/kg HYT linearly inhibited CH_4 emission on the bases of per day and $W^{0.75}$ ($p < 0.05$). The 20 g/kg TES improved the apparent digestibility of crude protein ($p < 0.05$), 10 and 20 g/kg of TES decreased CH_4 emission ($p < 0.05$), and 5 g/kg of TES reduced O_2 consumption and CO_2 production ($p < 0.05$). In conclusion, these three plant extracts all showed the abilities on mitigating CH_4 emission of sheep with appropriate additive ranges.

KEYWORDS
in vitro and in vivo, methane mitigation, plant extracts, productive performance, rumen fermentation, sheep

1 | INTRODUCTION

Rumen microbial fermentation is essential for yielding of metabolizable energy for ruminants; however, methane (CH_4) is also produced during this process, being not only an energy loss, but a greenhouse gas (GHG) pollutant. Approximately 2% to 12% of ruminant feed ingested energy is lost in the form of CH_4 (Johnson & Johnson, 1995), and these enteric CH_4 emissions from livestock contribute about 18% of the global GHG emission (Dangal et al., 2013). Reducing livestock CH_4 emission is necessary to support global carbon mitigation goals but must be achieved while maintaining or increasing meat supply. Therefore, finding efficient and safe strategies for modulating the rumen microecological environments to reduce CH_4 emissions has attracted significant attention.

Diet manipulations are important strategies for modulating CH_4 emission by controlling rumen fermentation pattern, repartitioning hydrogen use by microorganisms, and either directly or indirectly suppressing methanogen activity. Plant extracts and their secondary metabolites present a strong potential in mitigation with the advantages of efficiency, low risk of host toxicity or residues for the host, in contrast to antibiotics, ionophores or other chemical compounds. Plant essential oils (Paraskevakis, 2018), tannins (Hassanat & Benchaar, 2013; Salami et al., 2018) and saponins (Guyader et al., 2017; Liu et al., 2019) are the main plant extracts presently studied in ruminant CH_4 mitigation. However, the results of these experiments are diverse even across studies with the same plant extracts, which might be attributed to the plant varieties, additive levels or content of active ingredient. Therefore, intensive studies are necessary to clarify the exact effects of plant extracts on ruminant CH_4 mitigation, especially by using in vivo method.

In this study, origanum oil (ORO), hydrolysable tannins (HYT) and tea saponin (TES) were chosen for evaluating their effects on CH_4 and CO_2 production, volatile fatty acids (VFA) concentration by using in vitro batch fermentation system. The influences of ORO, HYT and TES on productive performance, nutrients digestibility and respiratory gases in vivo using sheep were simultaneously observed. We hoped that these results of the presented study would provide beneficial reference for establishing feasible CH_4 mitigation strategies.

2 | MATERIALS AND METHODS

2.1 | Plant extracts

The ORO, HYT and TES used in this study were sourced from Beijing Biolink Biotechnology. The ORO was analysed using gas chromatography-mass spectrometer (Banu et al., 2017), the main components of which were 3-methyl-4-isopropylphenol (64.37%), carvacrol (10.07%), thymol (4.67%), 2-methylphenol (4.04%) and 4-isopropyl-phenol (1.01%). The HYT was extracted from Chinese nutgall, and the purity of HYT monomer was 81.30% analysed with spectrophotometric method (Fecka, 2009). The TES was extracted from tea seeds (*Camellia sinensis*), and the purity of TES monomer was 65.50% analysed by vanillin-sulphuric acid assay (Wu et al., 2019).

2.2 | In vitro batch fermentation

An in vitro batch fermentation system was used to assess effects on fermentation. The incubations were as described by Sun et al. (2017), with 5 × 450 ml stainless steel incubation vessels, data acquisition controller, data analyzer (all data acquisition controller and data analyzer were Qtfxy-6, Haerbin Wuge Electronic) and thermostatic oscillating water bath (SHB-82, Changzhou City Jintan Huate Experimental Instrument) maintained at 39°C. The headspace in each incubation vessel was purged with nitrogen (N_2) in every 6 min and composition of the outflowing gas of fermentation gases monitored for CH_4 and CO_2 (CH_4 and CO_2 sensors were integration, AGM 10, Sensors Europe GmbH) in data acquisition controller successively collected instantaneous gases production of each cylinder every 6 minutes controlled by solenoid-based multiple switching system. The data analyzer recorded and transferred all information to a computer.

Rumen fluid was collected from 5-year-old Small Tailed Han × Dorper rumen fistulated ram (n = 4; 50.32 ± 2.63 kg) 2 h after feeding, and basal diet was formulated according to the nutrient requirements for meat-producing sheep (NY/T 816-2004, 2004; Table 1). Dry matter intake (DMI) was limited to 1.3 kg/day and calculated daily gain was 100 g/day, with drinking water available ad libitum. Rumen fluid (100 ml) collected from each of the four sheep was then

TABLE 1 Basal diet composition and nutrient levels (% DM basis)

Ingredients	Ratio	Nutrients	Level
Corn	3.26	Dry matter	86.80
Soybean meal	3.80	Digestive energy (MJ/kg)[c]	12.50
Wheat bran	10.39	Metabolic energy (MJ/kg)	10.25
Rapeseed meal	3.28	Crude protein	10.39
Rice bran meal	6.56	Ether extract	2.80
Cottonseed meal	2.47	Ash	7.72
DDGS	1.51	Neutral detergent fibre	53.36
Alfalfa hay	22.95	Acid detergent fibre	31.13
Chinese wildrye	44.26	Calcium	0.74
Limestone	0.72	Phosphorus	0.22
NaCl	0.50		
Additive premix	0.30		
Total	100.00		

Note: Premix provided the following per kg of the diet: Fe 38 mg, Zn 44 mg, Cu 15 mg, I 0.5 mg, Mn 50 mg, Se 0.3 mg, Co 0.05 mg, VA 354 IU, VD 94.4 IU and VE 1.06 mg. Digestive energy was a measured value.

Abbreviation: DDGS, distillers dried grain with solubles.

filtered into one beaker through four-layer medical gauze and stirred to homogeneity under a N_2 blanket. Mixed fluid was dispensed into five 39°C preheating vessels (50 ml each) that had been purged with N_2 accompanied with phosphate-bicarbonate buffer solution (100 ml each) (Menke et al., 1979) and 2 g of basal diet (Table 1) and plant extract additives. The incubation vessels were sealed, placed into oscillating water bath, gas lines connected and left to incubate for 12 h at 39°C.

A Latin square test design was adopted for the in vitro incubation experiment basing to different cylinders, with treatments replicated over days and each daily replicate considered as a block. Each plant extract trial was continuously proceeded for five days as five replications, and five cylinders were used each day. Each level of one plant extract was added in every cylinder in turn. The ORO, HYT and TES additive levels (DM basis) were: 0, 10, 20, 40 ml/kg; 0, 15, 30, 60 g/kg; 0, 5, 10, 20 g/kg, respectively. The last cylinder was set as blank control without diet or any additive. Rumen fluid sample of each vessel was collected immediately after twelve h of incubation for pH measurement and then stored at −20°C for VFA analysis.

2.3 | Experiments in vivo

Three consecutive in vivo experiments were carried out to assess the individual effects of ORO, HYT or TES on sheep productive performance, apparent nutrient digestibility and metabolic gas exchange by using a completely randomized design. These experiments were conducted at the Jilin Academy of Agricultural Sciences, Gongzhuling, Jilin, China. Animal feeding and management were according to the China government principles of Administration of Affairs Concerning Experimental Animals (Revised Edition, 2017).

In each of the experiments, twenty-four 12-month-old Small Tailed Han ×Dorper male castrated sheep were allocated to four treatments by using stratified randomization based on body weight with six sheep allocated into each treatment and housed in individually feeding pens. The initial body weight of sheep in ORO, HYT and TES experiments were 48.81 ± 2.15 kg, 47.86 ± 2.44 kg and 48.37 ± 1.97 kg, respectively. The basal diet formulation and feeding amount, plant extract sources and inclusion levels were same as for the in vitro experiment, and the additive levels of ORO, HYT and TES also were as follows: 0, 10, 20 and 40 ml/kg DM; 0, 15, 30 and 60 g/kg DM; and 0, 5, 10 and 20 g/kg DM, respectively. Plant extracts replaced equivalent wheat bran in the ration, and which were progressively adequately blended with corn and mixed with other feed ingredients in preparing the total mixed ration diet. The feed ingredients were all sourced from Wellhope Agri-tech Joint Stock.

Each experiment lasted twenty-four days including fourteen days of diet introduction, which allowed sheep to adapt to the trial diet with plant extracts. The sheep were fed twice daily with equal amounts at 07:00 and 19:00 h and water was freely available all times. Feed refusals were weighed before each feeding, samples taken for DM content and DMI subsequently was calculated. Each sheep was weighted on the morning of the 15th and 24th day with liveweight used for average daily gain (ADG) and feed conversion ratio (FCR) calculations.

At the 18:00 h of the 14th day in each experiment, eight sheep, with two sheep from each dietary treatment, were randomly selected from their individual pens and placed in metabolism cages equipped with faeces and urine separator, and then, the cages were placed individually inside each of 8 open-circuit respiratory calorimetry chambers for gas exchange and nutrient digestibility measurements. Emissions of O_2, CO_2 and CH_4 were measured starting at 07:00 h after morning feeding on the 15th day and lasted three days. The chamber doors were opened at 07:00 h and 19:00 h daily for feeding and faeces collections. This measurement program was applied other two times in succession with the rest of the sheep avoiding same sheep reused. The experimental processes of these additives were same with different sets of 24 new sheep each.

The volume of each respiratory chamber was 3.2 m^3, the internal temperature and humidity were controlled at 22°C and 70%, all the welds and joints were tightly sealed with structural adhesive and the door sealed with adhesive tape in case of leakage. The environmental and chamber outflowing concentrations of O_2, CO_2 and CH_4 were detected by the sensors of O_2 (Zirconium oxide sensor, Model 65–4–20, AMI), CO_2 and CH_4 (AGM 10, Sensors Europe GmbH). Before the starting of each respiratory experiment, all the gas sensors were individually calibrated by standard gas consisting of 21.2% O_2, 3440 ppm of CO_2, and 927 ppm of CH_4 (Northern Gas). Average chamber gas recover rate was 92.00% ± 2.16%, which was determined with 5 L 99.99% CO_2 (Northern Gas), cycle sampling period of eight chambers was 24 min including gas purge (2.5 min) and monitor (0.5 min) for each chamber, and ambient air composition was also measured at the beginning of each sampling cycle. The sampling cycle was 3 min to sample and measure from each of the 8 chambers. A custom-built multiplexer system controlled the sequential switching of sample streams, and computer software (Haerbin Wuge Electronic) collected and managed gas production rates automatically.

The O_2 consumption, CO_2 and CH_4 emission of in vivo or in vitro experiments were computed using the following equations. The in vivo gases exchanges basing on sheep metabolic weight ($W^{0.75}$) were then calculated. Respiratory quotient (RQ) was the ratio of CO_2 to O_2.

$$V_{O_2}(L/min) = V(L/min) \times \frac{[C_{O_{2,1}}(\%) - C_{O_{2,2}}(\%)]}{100}$$

$$V_{CO_2(CH_4)}(L/min) = V(L/min) \times \frac{[C_{CO_{2,2}(CH_{4,2})}(\%) - C_{CO_{2,1}(CH_{4,1})}(\%)]}{100}$$

Where, V_{O_2} was the O_2 consumption (L/min); V was the chamber or incubation vessel gas flow volume; $C_{O_{2,1}}$ was the environmental O_2 concentration; $C_{O_{2,2}}$ was the chamber or cylinder interior O_2 concentration; $V_{CO_2(CH_4)}$ was the CO_2 or CH_4 emission; $C_{CO_{2,2}(CH_{4,2})}$ was the chamber or incubation vessel interior CO_2 or CH_4 concentration; $C_{CO_{2,1}(CH_{4,1})}$ was the environmental CO_2 or CH_4 concentration.

2.4 | Fluid and faecal sampling and analysis

The pH of the rumen fluid samples incubated in vitro was measured with a general pH meter (pHs-3c, Rex, INESA). The VFA concentrations, including acetic acid (AA), propionic acid (PA) and butyric acid (BA), were analysed following the procedure of Zhang et al. (2008), using a gas chromatograph (6890 N, Agilent Technologies) fitted with a flame ionization detector and a capillary column (HP-INNOWax, 19091 N-133, Agilent Technologies).

The sheep faeces were completely collected for three days in the respiration chambers, and the faeces of each day were weighted and a 10% sub-sample immediately dried (80°C) and ground through a 1 mm sieve and were analysed according to the methods of AOAC (2000) with crude protein (CP) measured by a Kjeltec 2400 autoanalyzer (Foss, Analytical A/S). The neutral detergent fibre (NDF) and acid detergent fibre (ADF) concentrations were determined by using filter bag technology with automatic fibre analyzer (200, Ankom Technology). The results of the above nutrient contents and faecal output were utilized to calculate apparent digestibilities of CP, NDF and ADF.

2.5 | Statistical analysis

IBM SPSS version 20.0 (IBM SPSS Statistics, 2011) was used for all statistical analyses. The in vitro and in vivo experimental data were initially assessed with two-factor ANOVA (block and treatment), but the block effects of incubation day and gases exchange measurement day were not significant ($p > 0.05$), so one-way ANOVA was adopted for data re-assessment, and Tukey's multiple range tests were used to identify different treatments additives. The orthogonal polynomial contrasts were used to test the linear, quadratic and cubic effects of treatments. Differences between means were considered as significant at $p < 0.05$ or highly significant at $p < 0.01$.

3 | RESULTS

3.1 | Plant extracts induced changes of rumen in vitro incubation

The effects of the three plant extracts on rumen in vitro incubations of buffered digesta are presented in Tables 2–4. With the additive levels increasing, the CH_4 emission in ORO treatment decreased linearly ($p < 0.05$), and the CH_4 emission in HYT treatment decreased cubically ($p < 0.05$), and the CH_4 emissions of 40 ml/kg ORO and 60 g/kg HYT were both lower than their controls, respectively ($p < 0.05$). The CO_2 emission in ORO treatment decreased linearly ($p < 0.01$), quadratically and cubically ($p < 0.05$), which in HYT treatment was only linearly decreased ($p < 0.05$), ORO at 20 and 40 ml/kg significantly inhibited CO_2 generation compared with control ($p < 0.05$). The pH values of both ORO and HYT treatments demonstrated linear, quadratic and cubic increases ($p < 0.01$). The pH of 40 ml/kg ORO treatment was significantly higher than 20 ml/kg ($p < 0.01$) while the pH of 20 ml/kg ORO was significantly higher than 0 and 10 ml/kg treatment ($p < 0.01$). The pH of 60 g/kg HYT was significantly higher than 15 and 30 g/kg treatments, 15 and 30 g/kg HYT were higher than 0 g/kg ($p < 0.01$). The pH of 20 g/kg TES treatment was higher than that in 5 g/kg treatments ($p < 0.05$). The concentrations of VFA or VFA ingredients were not affected by any plant extracts.

3.2 | Plant extracts induced changes in productive performance, apparent nutrients digestibility and gas exchanges in vivo

The results of our in vivo experiments are showed in Tables 5–7. When the ORO additives increased, the DMI decreased linearly ($p < 0.05$), quadratically ($p < 0.01$) and cubically ($p < 0.01$), the ADG and FCR demonstrated quadratic and cubic responses ($p < 0.05$), and

TABLE 2 The ORO induced changes of rumen in vitro incubation

Item	ORO (ml/kg)				SEM	p value			
	0	10	20	40		Treat	L	Q	C
CH_4 (ml/g DM)	20.26[a]	16.79[ab]	13.60[ab]	12.97[b]	1.272	0.014	0.033	0.088	0.089
CO_2 (ml/g DM)	141.42[a]	86.23[ab]	76.14[b]	62.44[b]	11.770	0.045	0.004	0.017	0.017
pH	7.17[c]	7.37[c]	7.72[b]	7.98[a]	0.099	0.001	0.003	<0.001	<0.001
Total VFA (mmol/L)	38.82	38.89	38.47	33.22	1.699	0.641	0.530	0.468	0.457
Acetate	25.88	25.71	25.96	22.77	1.107	0.752	0.605	0.618	0.605
Propionate	8.17	8.69	7.85	6.78	0.472	0.598	0.597	0.368	0.368
Butyrate	4.77	4.48	4.67	3.67	0.741	0.267	0.247	0.242	0.228
Acetate to propionate ratio	3.18	3.01	3.31	3.45	0.120	0.670	0.659	0.461	0.466

Note: [a,b,c]Means within a row differ significant at $p < 0.05$.

Abbreviations: C, cubic; CH_4, methane; CO_2, carbon dioxide; L, liner; ORO, origanum oil; Q, quadratic; SEM, standard error of the mean; VFA, volatile fatty acid.

the CH_4 emission based on per day and $W^{0.75}$ both demonstrated linear decrease ($p < 0.05$). The 40 ml/kg ORO significantly decreased DMI compared to other treatments ($p < 0.01$), and 40 ml/kg ORO also reduced ADG and FCR compared to 10 and 20 ml/kg ORO ($p < 0.05$). The 20 ml/kg ORO significantly decreased CH_4 emission per day, and 20 and 40 ml/kg reduced CH_4 emission based on $W^{0.75}$ compared to 0 ml/kg ($p < 0.05$).

The CH_4 emissions both based on per day and $W^{0.75}$ were all demonstrated linear decreases when HYT additive levels was increasing; the 30 and 60 g/kg HYT significantly inhibited CH_4 production, on the basis of per day or $W^{0.75}$, compared to 0 g/kg ($p < 0.05$). The 20 g/kg TES improved the apparent digestibility of CP compared with other treatments ($p < 0.05$); the CH_4 and CO_2 productions and O_2 consumption based on per day or per day per $W^{0.75}$ in TES treatment all demonstrated linear, quadratic and cubic decreases ($p < 0.05$); the 10 and 20 g/kg TES significantly decreased CH_4 emission ($p < 0.05$); and 5 g/kg TES reduced O_2 consumption and CO_2 production compared with 0 g/kg treatment ($p < 0.05$).

4 | DISCUSSION

4.1 | Plant extracts induced changes of rumen in vitro incubation

As the main constituents of ORO, carvacrol and thymol have similar chemical structures and are verified to have valid antimicrobial activities (Patra & Yu, 2015). The linear reduction of in vitro CH_4 and CO_2 emissions may be directly related to the specific inhibition by carvacrol and thymol on *Prevotellaceae*, *Lachnospiraceae* and *Ruminococcaceae* (Paraskevakis, 2018; Patra & Yu, 2015). In addition, the 40 mg/kg ORO significantly reduced CH_4 by 35.98%, and 20 and 40 mg/kg ORO significantly inhibited CO_2 by 46.16% and 55.85% compared to 0 mg/kg ORO, respectively. The results of rumen fluid pH value corresponded to the reduction of methanogen abundance influenced by ORO were coincided with the study of Zhou et al. (2020), who observed that the ORO linearly decreased rumen in vitro CH_4 emission and pH value.

TABLE 3 The HYT induced changes of rumen in vitro incubation

Item	HYT (g/kg)				SEM	p value			
	0	15	30	60		Treat	L	Q	C
CH_4 (ml/g DM)	36.11[a]	30.29[ab]	27.52[ab]	25.26[b]	1.706	0.047	0.018	0.039	0.041
CO_2 (ml/g DM)	125.90	110.63	102.54	99.79	4.627	0.181	0.029	0.078	0.083
pH	7.48[c]	7.64[b]	7.69[b]	7.82[a]	0.040	0.001	0.003	<0.001	<0.001
Total VFA (mmol/L)	40.56	59.32	53.28	56.80	5.301	0.668	0.214	0.467	0.467
Acetate	26.07	37.49	33.54	36.13	3.197	0.657	0.211	0.464	0.479
Propionate	9.00	13.35	12.74	13.47	1.322	0.652	0.181	0.429	0.391
Butyrate	5.49	8.49	6.99	7.20	0.846	0.727	0.335	0.544	0.576
Acetate to propionate ratio	2.90	2.81	2.67	2.74	0.041	0.261	0.090	0.229	0.258

Note: [a,b,c]Means within a row differ significant at $p < 0.05$.

Abbreviations: C, cubic; CH_4, methane; CO_2, carbon dioxide; HYT, hydrolysable tannins treatment; L, liner; Q, quadratic; SEM, standard error of the mean; VFA, volatile fatty acid.

TABLE 4 The TES induced changes of rumen in vitro incubation

Item	TES (g/kg)				SEM	p value			
	0	5	10	20		Treat	L	Q	C
CH_4 (ml/g DM)	33.38	33.10	32.97	32.07	1.886	0.997	0.880	0.975	0.906
CO_2 (ml/g DM)	111.82	103.83	107.95	108.28	1.761	0.519	0.242	0.335	0.374
pH	7.84[ab]	7.75[a]	7.90[ab]	7.94[b]	0.029	0.040	0.666	0.054	0.056
Total VFA (mmol/L)	33.92	48.31	54.28	57.25	5.350	0.474	0.113	0.266	0.280
Acetate	23.53	33.18	37.12	38.96	3.596	0.487	0.117	0.277	0.301
Propionate	7.53	10.89	12.46	13.40	1.288	0.441	0.107	0.240	0.244
Butyrate	2.86	4.25	4.69	4.89	0.469	0.465	0.101	0.258	0.237
Acetate to propionate ratio	3.13	3.07	2.99	2.93	0.035	0.184	0.087	0.078	0.081

Note: [a,b,c]Means within a row differ significant at $p < 0.05$.

Abbreviations: C, cubic; CH_4, methane; CO_2, carbon dioxide; L, liner; Q, quadratic; SEM, standard error of the mean; TES, tea saponin; VFA, volatile fatty acid.

TABLE 5 The ORO induced in vivo changes in productive performance, apparent nutrient digestibility and gas exchange

| Item | ORO (ml/kg) | | | | SEM | p value | | | |
	0	10	20	40		Treat	L	Q	C
Productive performance									
DMI (g/day)	1275[a]	1274[a]	1258[a]	1222[b]	5.660	0.001	0.020	<0.001	<0.001
ADG (g/day)	102[ab]	108[a]	107[a]	94[b]	1.963	0.019	0.826	0.018	0.014
FCR (DMI/ADG)	12.47[ab]	11.82[b]	11.84[b]	13.06[a]	0.194	0.046	0.864	0.045	0.038
Apparent nutrients' digestibility									
CP (% CP)	59.03	62.45	59.88	57.94	1.683	0.839	0.902	0.650	0.627
NDF (% NDF)	21.23	27.24	21.12	19.66	1.814	0.503	0.906	0.355	0.362
ADF (% ADF)	30.30	41.89	39.20	36.03	2.755	0.526	0.223	0.313	0.327
Gases' exchange									
CH_4 (L/day)	20.86[a]	17.72[ab]	13.38[b]	14.21[ab]	1.245	0.042	0.028	0.075	0.088
O_2 (L/day)	219.16	221.45	192.29	239.52	15.355	0.790	0.983	0.908	0.890
CO_2 (L/day)	200.65	212.11	186.75	222.71	13.177	0.828	0.809	0.935	0.925
CH_4 (L/day/$W^{0.75}$)	1.07[a]	0.91[ab]	0.69[b]	0.74[b]	0.062	0.029	0.025	0.068	0.071
O_2 (L/day/$W^{0.75}$)	11.30	11.41	9.92	12.46	0.805	0.779	0.970	0.906	0.888
CO_2 (L/day/$W^{0.75}$)	10.34	10.93	9.63	11.58	0.690	0.814	0.794	0.929	0.918
RQ (CO_2/O_2)	0.92	0.97	0.98	0.94	0.014	0.343	0.224	0.243	0.238

Note: [a,b,c]Means within a row differ significant at $p < 0.05$.

Abbreviations: ADF, acid detergent fibre; ADG, average daily gain; C, cubic; CH_4, methane; CO_2, carbon dioxide; CP, crude protein; DMI, dry matter intake; FCR, feed conversion ratio; L, liner; NDF, neutral detergent fibre; O_2, oxygen; ORO, origanum oil; Q, quadratic; RQ, respiratory quotient; SEM, standard error of the mean; $W^{0.75}$, metabolic weight.

TABLE 6 The HYT induced in vivo changes in productive performance, apparent nutrient digestibility and gas exchange

| Item | HYT (g/kg) | | | | SEM | p value | | | |
	0	15	30	60		Treat	L	Q	C
Productive performance									
DMI (g/day)	1274	1273	1273	1272	0.320	0.545	0.439	0.461	0.452
ADG (g/day)	109	112	110	113	1.550	0.777	0.452	0.750	0.742
FCR (DMI/ADG)	11.71	11.39	11.63	11.25	0.161	0.774	0.451	0.738	0.723
Apparent nutrients' digestibility									
CP (% CP)	65.93	63.70	63.95	62.84	2.025	0.967	0.613	0.879	0.901
NDF (% NDF)	28.52	24.29	19.36	30.46	2.268	0.340	0.533	0.495	0.486
ADF (% ADF)	43.85	42.26	38.58	52.64	2.984	0.427	0.857	0.480	0.463
Gases' exchange									
CH_4 (L/day)	26.54[a]	18.86[ab]	16.54[b]	16.87[b]	1.560	0.040	0.015	0.053	0.053
O_2 (L/day)	240.59	204.74	209.18	223.69	9.146	0.546	0.216	0.337	0.324
CO_2 (L/day)	223.68	195.12	195.05	215.94	8.376	0.560	0.311	0.385	0.362
CH_4 (L/day/$W^{0.75}$)	1.36[a]	0.97[ab]	0.85[b]	0.86[b]	0.082	0.038	0.017	0.061	0.063
O_2 (L/day/$W^{0.75}$)	12.30	10.47	10.72	11.45	0.469	0.554	0.235	0.357	0.349
CO_2 (L/day/$W^{0.75}$)	11.44	9.97	10.00	11.06	0.433	0.575	0.342	0.410	0.425
RQ (CO_2/O_2)	0.93	0.96	0.93	0.96	0.005	0.167	0.214	0.466	0.449

Note: [a,b,c]Means within a row differ significant at $p < 0.05$.

Abbreviations: ADF, acid detergent fibre; ADG, average daily gain; C, cubic; CH_4, methane; CO_2, carbon dioxide; CP, crude protein; DMI, dry matter intake; FCR, feed conversion ratio; HYT, hydrolysable tannins; L, liner; NDF, neutral detergent fibre; O_2, oxygen; Q, quadratic; RQ, respiratory quotient; SEM, standard error of the mean; $W^{0.75}$, metabolic weight.

Tannins are polyphenol compounds widely found in the leaves, roots, barks and flesh of natural plants (Makkar, 2003) and are mainly divided into condensed tannins and HYT. The linear decreases of CH_4 productions without affecting VFA with increasing levels of HYT additive especially to 60 g/kg HYT reducing CH_4 emission by 30% in our study were consistent with Hassanat and

TABLE 7 The TES induced in vivo changes in productive performance, apparent nutrient digestibility and gas exchange[a]

Item	TES (g/kg)				SEM	p value			
	0	5	10	20		Treat	L	Q	C
Productive performance									
DMI (g/day)	1271	1271	1273	1269	0.357	0.328	0.882	0.745	0.717
ADG (g/day)	110	109	104	114	1.882	0.185	0.869	0.541	0.525
FCR (DMI/ADG)	11.77	11.71	12.24	11.11	0.203	0.293	0.832	0.591	0.574
Apparent nutrients' digestibility									
CP (% CP)	60.63[b]	58.65[b]	57.44[b]	65.07[a]	1.092	0.048	0.990	0.102	0.095
NDF (% NDF)	39.36	29.50	36.93	29.74	2.166	0.273	0.151	0.370	0.382
ADF (% ADF)	53.76	45.69	48.78	38.91	2.952	0.373	0.167	0.299	0.291
Gases' exchange									
CH_4 (L/day)	27.92[a]	20.08[ab]	17.92[b]	14.54[b]	1.717	0.019	0.003	0.006	0.006
O_2 (L/day)	227.52[a]	163.10[b]	183.81[ab]	181.88[ab]	9.342	0.034	0.013	0.033	0.036
CO_2 (L/day)	212.60[a]	152.61[b]	168.20[ab]	166.93[ab]	8.839	0.042	0.010	0.029	0.031
CH_4 (L/day/$W^{0.75}$)	1.42[a]	1.03[ab]	0.93[b]	0.75[b]	0.087	0.022	0.003	0.006	0.006
O_2 (L/day/$W^{0.75}$)	11.63[a]	8.33[b]	9.44[ab]	9.33[ab]	0.485	0.026	0.017	0.039	0.041
CO_2 (L/day/$W^{0.75}$)	10.86[a]	7.79[b]	8.64[ab]	8.58[ab]	0.462	0.028	0.014	0.037	0.040
RQ (CO_2/O_2)	0.94	0.93	0.92	0.92	0.009	0.865	0.551	0.771	0.774

Note: [a,b,c]Means within a row differ significant at $p < 0.05$.

Abbreviations: ADF, acid detergent fibre; ADG, average daily gain; C, cubic; CH_4, methane; CO_2, carbon dioxide; CP, crude protein; DMI, dry matter intake; FCR, feed conversion ratio; L, liner; NDF, neutral detergent fibre; O_2, oxygen; Q, quadratic; RQ, respiratory quotient; SEM, standard error of the mean; TES, tea saponin; $W^{0.75}$, metabolic weight.

Benchaar (2013), who observed that 50, 100, 150 and 200 g/kg chestnut and valonea (HYT) decreased CH_4 production, but not VFA concentration in vitro. The pH value and CO_2 concentration also presented cubic increasing and linear reducing tendency, indicated that HYT modulating CH_4 production and ruminal fermentation has specific dosage range. Salami et al. (2018) verified that the HYT of 4% chestnut displayed specific methanogens inhibition and altered the microbiome composition without compromising ruminal fermentation.

The TES is a compound extracted from tea seeds, leaves and roots, which is pentacyclic triterpene and composed of ligands, sugars and organic acids (Liu et al., 2019). In our study, except for the pH value, other in vitro indexes showed no discrepancy. Previous studies found that 0.4 mg/ml (Guo et al., 2008; Hu et al., 2006), 0.8 mg/ml (Hu et al., 2006) and 0.5 mg/ml (Guyader et al., 2017) TES inhibited CH_4 emission with decreasing protozoa and fungi abundance, but VFA and pH were only marginally affected. The CH_4 value divergences between our study and previous works were mainly ascribed to dose-dependent effects of TES. The maximum additive level (20 g/kg) of TES in our study, equalled to 0.27 mg/ml according to rumen fluid volume and substrate, was lower than the effective doses of the above studies. The difference of pH between low and high levels of TES treatments in our study might correlate with effects of TES on rumen nitrogen metabolism, ammonia-N concentration and microbial protein (Hu et al., 2005).

4.2 | Plant extracts induced changes in productive performance, apparent nutrients digestibility and gas exchanges in vivo

The in vitro rumen incubation has the limitations of short-term fermentation, buffered medium, diversity and viability of the microbiome (Benchaar, 2020), so that the in vivo experiment is necessary to verify the in vitro results with live animal. The decrease in DMI and ADG with increasing level of ORO in our study could be directly attributed to the irritating odour, in which compositions, thymol has the most intense odour (Díaz-Maroto et al., 2005) that might suppress the sheep's appetite. The decrease in DMI likely contributed to the poorer FCR at 40 ml/kg, which indicated that a high level of ORO might have negative effects on productive performance. The results of ORO linearly reducing in vivo CH_4 emission supported our in vitro observations, especially to 20 and 40 ml/kg additive levels. This result was consistent with the research of Paraskevakis (2018), who found that 1 ml/day ORO suppressed specific rumen microorganisms in goats by inhibiting methanogens activity. Benchaar (2020) reported that 50 mg/kg DM of ORO had no effect on the total-tract apparent nutrients digestibility and enteric CH_4 emission of dairy cow. In contrast, Olijhoek et al. (2019) found that low and high levels of oregano plant (0.12% and 4.12% essential oil per kg DM) linearly decreased the apparent digestibility of DM and NDF without any effect on CH_4 production in dairy cows. The different results of these studies might be attributed to variations in the ORO

additive formulations (dried oregano plant or oregano oil), additive levels, or valid dose ranges for different ruminants.

In our in vivo experiments, 30 and 60 g/kg HYT decreased CH_4 by 37.6% and 36.4% respectively, compared to 0 g/kg. This linear CH_4 reduction might be related to the induced change of some rumen methanogen genera. Salami et al. (2018) found that HYT (chestnut and tara) inhibited the protozoal abundance in lamb rumen and suppressed the fibrolytic bacteria *Fibrobacter* and *Methanosphaera*. The contradictory DMI results between our study and the research of Aderao et al. (2020) might be attributed to the models of limit feeding and ad libitum feeding, in addition to the additive formulations of tannins monomer and *Acacia nilotica* leaves (rich in HYT). Tannins extracted from chestnut and tara could induce different DMI in lamb, even they all belonged to the HYT (Valenti et al., 2018).

In our additive range, the HYT did not show any obvious effects on sheep productive performance and apparent digestibility of nutrients. These results supported the conclusions of Mueller-Harvey (2006) and Patra and Saxena (2011), who considered that 25 to 50 g/kg tannins in the basal diet was the general dosage threshold for maintaining normal nutrient intake and productive performance. In our study, 60 g/kg HYT was actual 48.8 g/kg according to the analysed concentration of the tannin monomer (81.3%).

The result of 20 g/kg TES improved CP digestibility of sheep was coincident with the findings of Liu et al. (2019), who observed that TES enhanced both the apparent digestibility of nitrogen and nitrogen retention in the Dorper crossbred ewe. The increased CP digestibility might be due to TES inhibiting the protozoa population in the rumen, which contributed 10% to 40% to rumen nitrogen, resulting in less predation and lysis of bacteria, and the reduced release of fewer protein breakdown products (Guo et al., 2008; Liu et al., 2019). Inconsistent with the in vitro incubation, the TES presented potential of CH_4 inhibition within the in vivo experiment. The reduction of rumen CH_4 induced by TES might relate to a decreased protozoan population and hydrogen availability for methanogenic archaea without affecting methanogens activity (Guo et al., 2008).

It was interesting that CH_4 and CO_2 productions and O_2 consumption all presented linear, quadratic and cubic reducing tendency when TES additive level was increased, especially to 5 g/kg, which resulted in significant reduction of both O_2 consumption and CO_2 production. Although there was no significant difference compared to 0, 10 and 20 g/kg of TES also showed the similar effects. Plant saponins have a wide range of physiological and pharmacological activities, which mainly manifested in their immune regulation and sedative-hypnotic activities (Shi et al., 2016). Whether the reduced metabolic rate of sheep was closely related with the physiological function of TES or not, which was needed to be explored deeply.

5 | CONCLUSION

In conclusion, the plant extracts of ORO, HYT and TES all had the ability to modulate ruminant CH_4 metabolism. The in vitro techniques could provide reference to the variation tendencies of ORO and HYT

affecting on rumen fermentation of live ruminant rather than TES with its limitations. However, differences in results between previous studies and our research indicate plant complex effects or additive doses effects. In the future, clarifying suitable ranges for additive levels and more deeply exploring the modulation of rumen microorganism by these plant extracts according to pure monomer concentrations will be necessary for practical application and reasonable utilization.

CONFLICT OF INTEREST

We verify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ANIMAL WELFARE STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been revised. The authors confirm that they have followed the standards of China government principles of Regulations on Administration of Experimental Animal for protecting animals used in this experiment for scientific purposes.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at https://doi.org/10.3168/jds.2019-16611, Zhou et al. (2020).

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