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, partitioning 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 & Benchar, 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-isopropylphenol (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 thermostat 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   | 12.50 |
| Wheat bran      | 10.39 | Metabolic energy   | 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 N2 blanket. Mixed fluid was dispensed into five 39°C preheating vessels (50 ml each) that had been purged with N2 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 replicates, 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 O2, CO2 and CH4 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 m3, 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 O2, CO2 and CH4 were detected by the sensors of O2 (Zirconium oxide sensor, Model 65−4−20, AMI), CO2 and CH4 (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% O2, 3440 ppm of CO2, and 927 ppm of CH4 (Northern Gas). Average chamber gas recovery rate was 92.00% ± 2.16%, which was determined with 5 L 99.99% CO2 (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 O2 consumption, CO2 and CH4 emission of in vivo or in vitro experiments were computed using the following equations. The in vivo gases exchanges basing on sheep metabolic weight (W0.75) were then calculated. Respiratory quotient (RQ) was the ratio of CO2 to O2.

\[
V_{O2}(L/min) = V(L/min) \times \frac{[C_{O2}] - [CO2] \times 100}{[C_{O2}] - [CO2]}
\]

\[
V_{CO2(CH4)}(L/min) = V(L/min) \times \frac{[C_{CO2(CH4)}] - [CO2(CH4)] \times 100}{[C_{CO2(CH4)}] - [CO2(CH4)]}
\]

Where, \( V_{O2} \) was the O2 consumption (L/min); \( V \) was the chamber or incubation vessel gas flow volume; \( C_{O2} \) was the environmental O2 concentration; \( C_{O2(CH4)} \) was the chamber or cylinder interior O2 concentration; \( V_{CO2(CH4)} \) was the CO2 or CH4 emission; \( C_{CO2(CH4)} \) was the chamber or incubation vessel interior CO2 or CH4 concentration; \( C_{CO2(CH4)} \) was the environmental CO2 or CH4 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

| Item | ORO (ml/kg) | SEM | 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$^a$ | 7.37$^a$ | 7.72$^b$ | 7.98$^b$ | 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 CH₄ emission based on per day and W⁰.₇₅ 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 CH₄ emission per day, and 20 and 40 ml/kg reduced CH₄ emission based on W⁰.₇₅ compared to 0 ml/kg (p < 0.05).

The CH₄ emissions both based on per day and W⁰.₇₅ were all demonstrated linear decreases when HYT additive levels was increasing; the 30 and 60 g/kg HYT significantly inhibited CH₄ production, on the basis of per day or W⁰.₇₅, 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 CH₂ and CO₂ productions and O₂ consumption based on per day or per day per W₀.₇₅ in TES treatment all demonstrated linear, quadratic and cubic decreases (p < 0.05); the 10 and 20 g/kg TES significantly decreased CH₄ emission (p < 0.05); and 5 g/kg TES reduced O₂ consumption and CO₂ 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 CH₄ and CO₂ 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 CH₄ by 35.98%, and 20 and 40 mg/kg ORO significantly inhibited CO₂ 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 CH₄ emission and pH value.

### TABLE 3 The HYT induced changes of rumen in vitro incubation

| Item                  | HYT (g/kg) | p value               | SEM | Treat | L | Q | C |
|-----------------------|------------|-----------------------|-----|-------|---|---|---|
|                       | 0          | 15                    | 30  | 60    |   |   |   |
| CH₄ (ml/g DM)         | 36.11<sup>a</sup> | 30.29<sup>ab</sup>   | 27.52<sup>ab</sup> | 25.26<sup>b</sup> | 1.706 | 0.047 | 0.018 | 0.039 | 0.041 |
| CO₂ (ml/g DM)         | 125.90     | 110.63                | 102.54 | 99.79 | 4.627 | 0.181 | 0.029 | 0.078 | 0.083 |
| pH                    | 7.48<sup>b</sup> | 7.64<sup>b</sup>   | 7.69<sup>b</sup>  | 7.82<sup>a</sup>  | 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: <sup>a,b</sup>Means within a row differ significant at p < 0.05.
Abbreviations: C, cubic; CH₄, methane; CO₂, carbon dioxide; HYT, hydrolysable tannins treatment; L, linear; 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) | p value               | SEM | Treat | L | Q | C |
|-----------------------|------------|-----------------------|-----|-------|---|---|---|
|                       | 0          | 5                     | 10  | 20    |   |   |   |
| CH₄ (ml/g DM)         | 33.38      | 33.10                 | 32.97 | 32.07  | 1.886 | 0.997 | 0.880 | 0.975 | 0.906 |
| CO₂ (ml/g DM)         | 111.82     | 103.83                | 107.95 | 108.28 | 1.761 | 0.519 | 0.242 | 0.335 | 0.374 |
| pH                    | 7.84<sup>ab</sup> | 7.75<sup>a</sup>   | 7.90<sup>ab</sup> | 7.94<sup>b</sup> | 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: <sup>a,b</sup>Means within a row differ significant at p < 0.05.
Abbreviations: C, cubic; CH₄, methane; CO₂, carbon dioxide; L, linear; Q, quadratic; SEM, standard error of the mean; TES, tea saponin; VFA, volatile fatty acid.
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 \( \text{CH}_4 \) productions without affecting VFA with increasing levels of HYT additive especially to 60 g/kg HYT reducing \( \text{CH}_4 \) emission by 30% in our study were consistent with Hassanat and

| Item                  | ORO (ml/kg) | SEM | p value | Treat | L | Q | C |
|-----------------------|-------------|-----|---------|-------|---|---|---|
|                       | 0  | 10  | 20   | 40   |    |   |   |
| 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\(^b\) | 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 |

| Item                  | HYT (g/kg) | SEM | p value | Treat | L | Q | C |
|-----------------------|------------|-----|---------|-------|---|---|---|
|                       | 0  | 15  | 30  | 60   |    |   |   |
| 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 | 0.451 | 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 |

| Item                  | HYT (g/kg) | SEM | p value | Treat | L | Q | C |
|-----------------------|------------|-----|---------|-------|---|---|---|
|                       | 0  | 10  | 20  | 40   |    |   |   |
| 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 | 0.451 | 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 |

Note: a,b,cMeans within a row differ significant at \( p < 0.05 \).

Abbreviations: ADF, acid detergent fibre; ADG, average daily gain; C, cubic; \( \text{CH}_4 \), methane; \( \text{CO}_2 \), carbon dioxide; CP, crude protein; DMI, dry matter intake; FCR, feed conversion ratio; L, linear; NDF, neutral detergent fibre; \( \text{O}_2 \), oxygen; ORO, origanum oil; Q, quadratic; RQ, respiratory quotient; SEM, standard error of the mean; \( W^{0.75} \), metabolic weight.

Note: a,b,cMeans within a row differ significant at \( p < 0.05 \).

Abbreviations: ADF, acid detergent fibre; ADG, average daily gain; C, cubic; \( \text{CH}_4 \), methane; \( \text{CO}_2 \), carbon dioxide; CP, crude protein; DMI, dry matter intake; FCR, feed conversion ratio; HYT, hydrolysable tannins; L, linear; NDF, neutral detergent fibre; \( \text{O}_2 \), oxygen; ORO, origanum oil; Q, quadratic; RQ, respiratory quotient; SEM, standard error of the mean; \( W^{0.75} \), metabolic weight.
The TES induced in vivo changes in productive performance, apparent nutrient digestibility and gas exchange

| Item                     | TES (g/kg) | SEM | p value |
|--------------------------|------------|-----|---------|
|                         | 0          | 5   | 10      | 20      | Treat | L   | Q   | C   |
| 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)                | 60.63b     | 58.65b| 57.44b  | 65.07a  | 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**

| CH₄ (L/day)              | 27.92a     | 20.08ab| 17.92b  | 14.54b  | 1.717 | 0.019| 0.003| 0.006| 0.006|
| O₂ (L/day)               | 227.52a    | 163.10b| 183.81b | 181.88b | 9.342 | 0.034| 0.013| 0.033| 0.036|
| CO₂ (L/day)              | 212.60a    | 152.61b| 168.20ab | 166.93ab | 8.839 | 0.042| 0.010| 0.029| 0.031|
| CH₄ (L/day/W₀.75)        | 1.42a      | 1.03ab | 0.93b   | 0.75b   | 0.087 | 0.022| 0.003| 0.006| 0.006|
| O₂ (L/day/W₀.75)         | 11.63a     | 8.33ab | 9.44ab  | 9.33ab  | 0.485 | 0.026| 0.017| 0.039| 0.041|
| CO₂ (L/day/W₀.75)        | 10.86a     | 7.79ab | 8.64ab  | 8.58ab  | 0.462 | 0.028| 0.014| 0.037| 0.040|
| RQ (CO₂/O₂)              | 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; CH₄, methane; CO₂, carbon dioxide; CP, crude protein; DMI, dry matter intake; FCR, feed conversion ratio; L, linear; NDF, neutral detergent fibre; O₂, oxygen; Q, quadratic; RQ, respiratory quotient; SEM, standard error of the mean; TES, tea saponin; W₀.75, metabolic weight.

Benchaa (2013), who observed that 50, 100, 150 and 200 g/kg chestnut and valonea (HYT) decreased CH₄ production, but not VFA concentration in vitro. The pH value and CO₂ concentration also presented cubic increasing and linear reducing tendency, indicated that HYT modulating CH₄ 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 CH₄ emission with decreasing protozoa and fungi abundance, but VFA and pH were only marginally affected. The CH₄ 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 (Benchaa, 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 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 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 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 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 results of ORO linearly reducing in vivo CH₄ 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. Benchaa (2020) reported that 50 mg/kg DM of ORO had no effect on the total-tract apparent nutrients digestibility and enteric CH₄ 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 CH₄ 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 CH₄ by 37.6% and 36.4% respectively, compared to 0 g/kg. This linear CH₄ 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 analyzed 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 CH₄ inhibition within the in vivo experiment. The reduction of rumen CH₄ induced by TES might relate to a decreased protozoa population and hydrogen availability for methanogenic archaea without affecting methanogens activity (Guo et al., 2008).

It was interesting that CH₄ and CO₂ productions and O₂ 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 O₂ consumption and CO₂ 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 CH₄ 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.

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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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