Effects of Oregano Oil on Rumen Fermentation Characteristics and Methane Production in Sheep Using In Vitro Gas Production Method: Postprint

Authors: Zhang Ran, Zheng Chen, Yan Xiaogang, Liang Hao, Yang Huaming

Date: 2018-12-24T00:00:00+00:00

Abstract

This experiment used the in vitro gas production method to investigate the effects of adding oregano oil to the incubation medium on rumen fermentation characteristics and methane production in sheep. A single-factor completely randomized experimental design was adopted, with 0 (control), 100, 200, 300, and 400 mg/L of oregano oil added to the medium in each group, with 5 replicates per group. After 12 h of fermentation, total gas production, methane production, medium pH, and concentrations of total nitrogen, ammonia nitrogen, urea nitrogen, protein nitrogen, and volatile fatty acid concentrations and proportions were measured. The results showed: 1) The addition of 100, 200, 300, and 400 mg/L oregano oil to the medium significantly increased the medium pH ($P<0.05$); high addition levels (300, 400 mg/L) of oregano oil significantly decreased the total volatile fatty acid concentration and propionate proportion in the medium ($P<0.05$), significantly increased the acetate proportion and acetate/propionate ratio in the medium ($P<0.05$), and inhibited rumen fermentation. 2) Compared with the control group, ammonia nitrogen concentration in the medium was significantly decreased in all treatment groups except the 100 mg/L oregano oil group ($P<0.05$). 3) High addition levels (300, 400 mg/L) of oregano oil significantly decreased methane production while also significantly decreasing total gas production ($P<0.05$). It can be concluded that oregano oil can regulate rumen fermentation and reduce methane production, but excessively high addition levels have an inhibitory effect on rumen fermentation, with the optimal addition level under in vitro conditions being 200 mg/L.

Full Text

Effects of Oregano Oil on Ruminal Fermentation Characteristics and Methane Production in Sheep Using In Vitro Gas Production Technique

ZHANG Ran[1],[2], ZHENG Chen[2], YAN Xiaogang[1], LIANG Hao[1], YANG Huaming[1]*

[1]Branch Academy of Animal Science, Jilin Academy of Agricultural Sciences, Gongzhuling 136100, China
[2]College of Animal Science and Technology, Gansu Agricultural University, Lanzhou 730070, China

Abstract

This study investigated the effects of oregano oil on ruminal fermentation characteristics and methane production in sheep using in vitro gas production technique. A single-factor completely randomized design was employed, with oregano oil added to the culture medium at concentrations of 0 (control), 100, 200, 300, and 400 mg/L, each group having five replicates. After 12 hours of fermentation, total gas production, methane production, pH, concentrations of total nitrogen, ammonia nitrogen, urea nitrogen, and protein nitrogen, as well as volatile fatty acid concentrations and proportions were measured. The results showed: (1) Addition of oregano oil at 100, 200, 300, and 400 mg/L all significantly increased culture medium pH ($P<0.05$). High supplementation levels (300 and 400 mg/L) significantly decreased total volatile fatty acid concentration and propionate proportion ($P<0.05$), while significantly increasing acetate proportion and acetate/propionate ratio ($P<0.05$), indicating inhibited ruminal fermentation. (2) Compared with the control group, ammonia nitrogen concentration was significantly reduced in all treatment groups except the 100 mg/L group ($P<0.05$). (3) High supplementation levels (300 and 400 mg/L) significantly decreased both methane production and total gas production ($P<0.05$). In conclusion, oregano oil can regulate ruminal fermentation and reduce methane production, but excessive supplementation levels inhibit ruminal fermentation. The optimal supplementation level under in vitro conditions is 200 mg/L.

Keywords: oregano oil; in vitro gas production technique; fermentation characteristics; methane yield

Introduction

Oregano essential oil (OEO) is a volatile plant oil extracted from the leaves and flowers of oregano (*Origanum vulgare*), containing over 30 antimicrobial compounds. Its active components are primarily carvacrol (2-methyl-5-isopropylphenol) and thymol (5-methyl-2-isopropylphenol) [1], which are

approved as antimicrobial growth promoters by China' s Ministry of Agriculture. Previous studies have demonstrated that dietary supplementation with oregano oil in ruminants can regulate ruminal fermentation, reduce ruminal methane emissions [2] and ammonia nitrogen concentration [3-4], and improve animal performance [5]. As global warming intensifies, greenhouse gas (CO , CH , and N O) emission reduction has garnered widespread attention. Methane, with its long atmospheric lifetime, has a global warming potential approximately 25 times that of carbon dioxide [6]. In the rumen, 82% of methane is produced by *Methanobrevibacter* spp. utilizing metabolic hydrogen to reduce CO [7]; however, methane is not utilized by the animal and is expelled through eructation, representing a significant energy loss. As a feed additive, oregano oil can control greenhouse gas emissions, reduce energy loss, and improve feed utilization efficiency. Therefore, in-depth research on oregano oil application in animal production is of great significance. This study employed in vitro gas production technique to investigate the effects of different oregano oil supplementation levels on ruminal fermentation characteristics and methane production, aiming to determine the optimal supplementation level and provide scientific basis for oregano oil application in ruminant production.

Materials and Methods

1.1 Materials

Oregano oil was purchased from Jinan Shangda Bioengineering Co., Ltd., with a purity of 90%.

1.2 Experimental Animals and Management

Four crossbred male sheep (Xinjiang fine-wool × Dorper), aged 4 years and weighing 40.83 ± 4.11 kg, fitted with permanent rumen fistulas, served as rumen fluid donors. The basal diet was formulated according to nutrient requirements recommended in *Feeding Standard of Meat Sheep* (NY/T816-2004), with a concentrate-to-forage ratio of 30:70. Diet composition and nutrient levels are shown in . Animals were fed equal amounts twice daily at 07:00 and 17:00 (forage first, then concentrate) with free access to water. A 10-day pre-feeding period preceded rumen fluid collection for in vitro fermentation trials.

1.3 Experimental Design

A single-factor completely randomized design was adopted. The in vitro fermentation system consisted of six fermentation vessels (effective volume: 200 mL each). One vessel served as a blank control (no substrate or oregano oil, for gas production correction), while the remaining five vessels received culture medium + fermentation substrate + oregano oil. Oregano oil concentrations in culture medium were set at 0 (control), 100, 200, 300, and 400 mg/L, with fermentation lasting 12 hours. The experiment ran for five consecutive days, providing five replicates per supplementation level.

1.4 Experimental Procedures

1.4.1 Apparatus The in vitro gas production system was the "Six-channel Instantaneous Fermentation Micro-gas Automatic Recording Device and Software System" (Model Qtfxy-6) independently developed by the Branch Academy of Animal Science, Jilin Academy of Agricultural Sciences. The apparatus comprises a constant-temperature anaerobic fermentation system and a data acquisition system. Each of the six stainless steel fermentation vessels is connected to two latex tubes: one linked to a nitrogen tank providing continuous nitrogen flow to maintain anaerobic conditions, and another connecting to the data acquisition system to deliver fermentation gases. Methane (Semsorseurope, Germany), carbon dioxide (Semsorseurope, Germany), oxygen (AMI, USA), and hydrogen (CITY, UK) concentration sensors detect gas composition. The data acquisition instrument collects gas data from each vessel sequentially (1-6) at 1-minute intervals, completing a cycle every 6 minutes.

1.4.2 Buffer Preparation Anaerobic artificial rumen buffer was prepared according to Menke et al. [8].

1.4.3 Rumen Fluid Collection On the experimental day, before morning feeding, sufficient rumen fluid was collected from different sites in the rumen of the four fistulated sheep using negative pressure. The fluid was mixed under anaerobic and sterile conditions, then filtered through four layers of cheesecloth to separate solid and liquid fractions.

1.4.4 Experimental Process The culture medium (150 mL) consisted of rumen fluid and buffer at a 1:2 ratio. Fermentation substrate (2 g) was composed of diet with a concentrate-to-forage ratio of 30:70. Oregano oil was prepared by dissolving 0, 150, 300, 450, and 600 mg in 10 mL anhydrous ethanol to create solutions of 0, 15, 30, 45, and 60 mg/mL, respectively. One milliliter of each solution was added to the corresponding culture medium to achieve final concentrations of 0, 100, 200, 300, and 400 mg/L. The blank control also received 1 mL anhydrous ethanol. The water bath shaker was maintained at 39°C with a rotation speed of 80 r/min.

1.4.5 Sample Collection After 12 hours of fermentation, pH was measured immediately, and culture medium samples were collected and stored at -20°C for subsequent determination of total nitrogen, ammonia nitrogen, urea nitrogen, and volatile fatty acid concentrations.

1.5 Measurement Indicators and Methods

pH: Measured using a pH meter (pHS-3C, Shanghai Leici Instrument Factory).

Volatile fatty acid concentration: Determined by gas chromatography (6890N, Agilent, USA) using an HP19091N-213 capillary column (Agilent,

USA). Chromatographic conditions: injector temperature 220°C, nitrogen flow rate 2.0 mL/min, split ratio 40:1, injection volume 0.6 L, temperature program (120°C for 3 min, then increased to 180°C at 10°C/min, held for 1 min), flame ionization detector (FID) temperature 250°C, FID air, hydrogen, and nitrogen flow rates of 45, 40, and 45 mL/min, respectively.

Total nitrogen concentration: Determined by Kjeldahl method [9].

Ammonia nitrogen concentration: Measured by modified colorimetric method according to Feng et al. [10].

Urea nitrogen concentration: Determined by diacetyl monoxime method using assay kits purchased from Nanjing Jiancheng Bioengineering Institute.

Protein nitrogen concentration was calculated as: Protein nitrogen (mg/dL) = Total nitrogen (mg/dL) - Ammonia nitrogen (mg/dL) - Urea nitrogen (mg/dL).

Total gas production and methane production: Collected and recorded in real-time by the data analyzer.

1.6 Statistical Analysis

Data were analyzed using one-way ANOVA in SPSS 19.0 software. When significant differences were detected, multiple comparisons were performed using Turkey's test (for homogeneous variances) or Tamhane's test (for heterogeneous variances). Significance was declared at $P<0.05$.

Results

2.1 pH and Volatile Fatty Acid Concentration and Proportion in Culture Medium

As shown in , compared with the control group, pH values in all treatment groups increased by 2.6%, 5.6%, 7.2%, and 6.8%, respectively, and were all significantly higher ($P<0.05$). The 300 and 400 mg/L groups showed significantly lower total volatile fatty acid concentration and propionate proportion compared with other groups ($P<0.05$), while acetate proportion and acetate/propionate ratio were significantly increased ($P<0.05$). Butyrate and other acid proportions showed consistent changes: the 200 mg/L group was significantly higher than the control ($P<0.05$), while the 400 mg/L group was significantly lower ($P<0.05$).

2.2 Nitrogen Concentration in Culture Medium

As shown in , ammonia nitrogen concentration was significantly reduced in all treatment groups except the 100 mg/L group compared with the control ($P<0.05$). Total nitrogen concentration in the 300 mg/L group was significantly higher than in the 100 and 400 mg/L groups ($P<0.05$) but did not differ significantly from other groups ($P>0.05$). Urea nitrogen concentration showed no

significant difference compared with the control (P>0.05), though the 400 mg/L group was significantly higher than the 200 mg/L group (P<0.05). Protein nitrogen concentration in the 300 mg/L group was significantly higher than in the 400 mg/L group (P<0.05), with no significant differences among other groups (P>0.05).

2.3 Total Gas Production and Methane Production

As shown in , total gas production and methane production showed consistent patterns: both were significantly reduced in the 300 and 400 mg/L groups compared with the control (P<0.05), with no significant differences among other groups (P>0.05). The dynamic changes in methane production at different time points are illustrated in [Figure 1: see original paper]. At all time points, the control group showed the highest methane production, while the 300 and 400 mg/L groups were substantially lower.

Discussion

3.1 Effects of Oregano Oil on pH and Volatile Fatty Acid Concentration and Proportion in Culture Medium

Ruminal pH is a primary indicator for diagnosing ruminal acidosis in ruminants, with volatile fatty acids and lactic acid being the main factors causing pH changes [11]. In this study, pH values in all treatment groups ranged from 6.63 to 7.17, significantly higher than the control and within the optimal range (5.5-7.5) for rumen microbial growth, development, and fermentation [12], indicating that oregano oil can increase in vitro culture medium pH. Xu [13] investigated the effects of different oregano oil levels (0, 27, 53, 80 mg/dL) on ruminal fermentation characteristics of yellow cattle using in vitro batch culture with a 60:40 concentrate-to-forage diet, reporting that oregano oil increased culture medium pH in a dose-dependent manner. Evans et al. [14] demonstrated that thymol inhibited the growth of *Streptococcus bovis* and *Selenomonas ruminantium*, which are associated with ruminal energy metabolism, thereby reducing lactic acid concentration. Lactic acid is a strong acid with a dissociation constant (pKa 3.9) lower than that of volatile fatty acids (4.8), contributing nearly ten times more to ruminal pH reduction than volatile fatty acids [15-16], thus reducing organic acid accumulation and increasing ruminal pH.

Volatile fatty acids are the end products of rumen microbial fermentation and the primary form of energy supply for ruminants [17]; therefore, any oregano oil level that reduces total volatile fatty acid concentration is considered detrimental. In this study, high supplementation levels (300 and 400 mg/L) significantly decreased total volatile fatty acid concentration (by 21.6% and 35.5%, respectively) and propionate proportion (by 21.3% and 24.9%, respectively), while significantly increasing acetate proportion (by 3.8% and 7.3%, respectively) and acetate/propionate ratio (by 32.3% and 43.2%, respectively), indicating that oregano oil concentrations above 300 mg/L inhibited ruminal fermentation and

microbial activity. Lin et al. [18] studied the effects of oregano oil and its main component carvacrol on in vitro ruminal fermentation, adding 0, 50, 200, 500, and 750 mg/L oregano oil to culture medium, and found that total volatile fatty acid concentration decreased with increasing oregano oil levels, with high levels (500, 750 mg/L) inhibiting ruminal fermentation. This effect is attributed to loss of cell membrane integrity and reduced glucose uptake [14]. Castillejos et al. [19] investigated the effects of various plant oils at different concentrations (5, 50, 500, 5,000 mg/L) on in vitro fermentation products, finding that 500 mg/L thymol significantly reduced total volatile fatty acid concentration and propionate proportion while significantly increasing pH, acetate proportion, and acetate/propionate ratio, whereas low levels (5, 50 mg/L) had no significant effects. Patra et al. [20-21] reported that oregano oil supplementation in vitro reduced populations of rumen protozoa, bacteria, and major cellulolytic bacteria (*Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus*) in a dose-dependent manner. In this study, total volatile fatty acid concentration decreased with increasing oregano oil levels, showing a dose-response effect likely because high oregano oil levels inhibited fiber degradation by cellulolytic bacteria and protozoa, thereby reducing total volatile fatty acid concentration. However, some studies reported that plant oil supplementation had no effect [22-23] or even increased total volatile fatty acid concentration [24-25], possibly because the supplementation level and chemical structure of the main components determine the effect. Plant essential oils have differential effects on individual volatile fatty acids. Some studies reported that plant essential oils selectively inhibit rumen microbial populations, with high oregano oil levels promoting Gram-positive bacteria (acetate producers) while inhibiting Gram-negative bacteria (propionate producers), thereby increasing acetate proportion, decreasing propionate proportion, and elevating acetate/propionate ratio [14], consistent with our findings.

3.2 Effects of Oregano Oil on Nitrogen Concentration in Culture Medium

In this study, total nitrogen, urea nitrogen, and protein nitrogen concentrations showed no significant differences compared with the control, while ammonia nitrogen concentration ranged from 8.81 to 12.64 mg/dL, within the normal ruminal ammonia nitrogen range (85-300 mg/L) [26]. Compared with the control, oregano oil at 100, 200, 300, and 400 mg/L reduced ammonia nitrogen concentration by 9.8%, 21.0%, 13.1%, and 20.3%, respectively, with significant reduction only at levels above 100 mg/L. These results align with Bai [27], who added different concentrations (0, 45, 450, and 4,500 mg/L) of oregano oil and thymol to in vitro culture medium and found no significant difference at 45 mg/L oregano oil after 24 hours, while other treatment groups were significantly lower than the control; all thymol groups were significantly lower than the control. Our results are similar to those of Castillejos et al. [19] and Cobellis et al. [2]. The mechanism for reduced ammonia nitrogen concentration involves: (1) inhibition of hyper-ammonia-producing bacteria (e.g., *Clostridium sticklandii*, *Peptostrepto-*

coccus anaerobius) and reduced deaminase activity, leading to decreased amino acid deamination [28-29]; and (2) reduced protozoal numbers, as protozoa are net ammonia nitrogen producers in the rumen [30].

3.3 Effects of Oregano Oil on Total Gas Production and Methane Production

Total gas production is an important indicator reflecting feed fermentability and rumen microbial activity [31]. The primary pathway for methane synthesis in the rumen involves hydrogen reduction of carbon dioxide, with secondary pathways from volatile fatty acids such as acetate, formate, and butyrate [12]; thus, any factor affecting methane formation pathways alters methane production. This study showed that total gas production decreased with increasing oregano oil concentration, with significant reduction at high levels (300 and 400 mg/L), indicating inhibited ruminal fermentation. Similarly, only high oregano oil levels (300 and 400 mg/L) significantly reduced methane production. Evans et al. [14] reported that thymol at 50, 100, and 200 mg/L had no effect on methane production in vitro, while 400 mg/L significantly reduced methane production. Chaudhary et al. [32] reported that 450 mg/L thyme oil and oregano oil significantly reduced in vitro methane production (by 68.8% and 82.5%, respectively), while 50 and 150 mg/L had no significant effects. Studies by Günal et al. [33] and Macheboeuf et al. [34] are consistent with our results, possibly because rumen microorganisms develop tolerance to low plant essential oil levels, rendering them ineffective. Wang et al. [35] demonstrated in vivo that 0.25 g/d oregano oil mixture reduced methane production in sheep. Some scholars propose that plant essential oils reduce methane production by directly inhibiting methanogen activity or indirectly reducing protozoal numbers that symbiotically associate with methanogens [20-21,36]. Another theory suggests an inverse relationship between propionate proportion and methane production, as propionate acts as a hydrogen sink, utilizing hydrogen required for methane synthesis [12]. However, this relationship was not observed in our study, warranting further investigation.

Conclusion

(1) High supplementation levels (300 and 400 mg/L) of oregano oil significantly reduced methane production but also significantly decreased total gas production and total volatile fatty acid concentration, inhibiting ruminal fermentation and proving detrimental to ruminant nutrition. In contrast, 200 mg/L oregano oil significantly reduced ammonia nitrogen concentration without significantly affecting total gas production or total volatile fatty acid concentration. (2) Based on comprehensive evaluation of all indicators, the optimal supplementation level under in vitro conditions is 200 mg/L.

References

[1] ZHANG Yong, WANG Meng, LI Fangfang, et al. Effects of tributyrin and oregano oil on growth performance, serum biochemical indices and nutrient apparent digestibility in weaned piglets[J]. Chinese Journal of Animal Nutrition, 2016, 28(9): 2786-2794.

[2] COBELLIS G, PETROZZI A, FORTE C, et al. Evaluation of the effects of mitigation on methane and ammonia production by using *Origanum vulgare* L. and *Rosmarinus officinalis* L. essential oils on in vitro rumen fermentation systems[J]. Sustainability, 2015, 7(9): 12856-12869.

[3] BUSQUET M, CALSAMIGLIA S, FERRET A, et al. Plant extracts affect in vitro rumen microbial fermentation[J]. Journal of Dairy Science, 2006, 89(2): 761-771.

[4] XIE Zhongquan, NIU Shuqi. Production technology and quality standards of natural plant feed additives[M]. Beijing: China Agricultural Science and Technology Press, 2004: 3-4.

[5] ZHANG Shanzhi. Effects of dietary oregano oil supplementation on growth performance of beef cattle in summer[J]. Animal Husbandry and Veterinary Medicine, 2011, 43(7): 39-41.

[6] ZHOU Y Y, MAO H L, JIANG F, et al. Inhibition of rumen methanogenesis by tea saponins with reference to fermentation pattern and microbial communities in Hu sheep[J]. Animal Feed Science and Technology, 2011, 166-167: 93-100.

[7] ZHOU Yi, DIAO Qiyu. Regulation of ruminal methane gas production in ruminants[J]. Grass-Feeding Livestock, 2008(4): 21-24.

[8] MENKE K H, STEINGASS H. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid[J]. Animal Research and Development, 1988, 28: 7-55.

[9] YANG Sheng. Feed analysis and feed quality detection technology[M]. Beijing: Beijing Agricultural University Press, 1993.

[10] FENG Zongci, GAO Min. Improvement of colorimetric method for determining rumen fluid ammonia nitrogen content[J]. Inner Mongolia Animal Science, 2010(4): 40-41.

[11] OWENS F N, SECRIST D S, HILL W J, et al. Acidosis in cattle: a review[J]. Journal of Animal Science, 1998, 76(1): 275-286.

[12] HAO Zhengli, LIU Shimin, MENG Xiancheng. Ruminant nutrition[M]. Lanzhou: Gansu Nationalities Publishing House, 2000: 3-4.

[13] XU Fanghua. Study on effects of oregano oil and its main components on ruminal fermentation characteristics and methane production[D]. Master' s thesis. Yanji: Yanbian University, 2014: 15-17.

[14] EVANS J D, MARTIN S A. Effects of thymol on ruminal microorganisms[J]. Current Microbiology, 2000, 41(5): 336-340.

[15] ENEMARK J M D, JØRGENSEN R J, ST ENEMARK P. Rumen acidosis with special emphasis on diagnostic aspects of subclinical rumen acidosis: a review[J]. Veterinarija Ir Zootechnika, 2002, 20(42): 16-29.

[16] WU Yuhang, LIU Dacheng, HU Honglian, et al. Research progress on effects of subacute ruminal acidosis on rumen fermentation and microbiota[J]. Chinese Journal of Animal Science, 2013, 49(19): 86-90.

[17] VAN SOEST P J. Nutritional ecology of the ruminant[M]. Ithaca: Cornell University Press, 1994.

[18] LIN Bo, JI Miaomiao, LIANG Quan, et al. Effects of cinnamon oil and oregano oil and their main components on in vitro ruminal fermentation and methane production[J]. Chinese Journal of Veterinary Science, 2011, 31(2): 279-282, 287.

[19] CASTILLEJOS L, CALSAMIGLIA S, FERRET A. Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in in vitro systems[J]. Journal of Dairy Science, 2006, 89(7): 2649-2658.

[20] PATRA A K, YU Z T. Effects of essential oils on methane production and fermentation by, and abundance and diversity of, rumen microbial populations[J]. Applied and Environmental Microbiology, 2012, 78(12): 4271-4280.

[21] PATRA A K, YU Z T. Essential oils affect populations of some rumen bacteria in vitro as revealed by microarray (RumenBactArray) analysis[J]. Frontiers in Microbiology, 2015, 6: 297.

[22] BEAUCHEMIN K A, MCGINN S M. Methane emissions from beef cattle: effects of fumaric acid, essential oil, and canola oil[J]. Journal of Animal Science, 2006, 84(6): 1489-1496.

[23] CHAVES A V, HE M L, YANG W Z, et al. Effects of essential oils on proteolytic, deaminative and methanogenic activities of mixed ruminal bacteria[J]. Canadian Journal of Animal Science, 2008, 88(1): 117-122.

[24] CASTILLEJOS L, CALSAMIGLIA S, FERRET A, et al. Effects of a specific blend of essential oil compounds and the type of diet on rumen microbial fermentation and nutrient flow in continuous culture system[J]. Animal Science Technology, 2005, 119(1/2): 29-41.

[25] CHAVES A V, STANFORD K, GIBSON L L, et al. Effects of carvacrol and cinnamaldehyde on intake, rumen fermentation, growth performance, and carcass characteristics of growing lambs[J]. Animal Feed Science and Technology, 2008, 145(1/2/3/4): 396-408.

[26] ZHOU Anguo, CHEN Daiwen. Animal nutrition[M]. 3rd ed. Beijing: China Agriculture Press, 2010: 40.

[27] BAI Wurihan. Study on effects of plant essential oils and other active components on ruminal fermentation function in dairy cows[D]. Master' s thesis. Hohhot: Inner Mongolia Agricultural University, 2009: 36-37.

[28] FLYTHE M D. The antimicrobial effects of hops (*Humulus lupulus* L.) on ruminal hyper ammonia-producing bacteria[J]. Letters in Applied Microbiology, 2009, 48(6): 712-717.

[29] BENCHAAR C, CALSAMIGLIA S, CHAVES A V, et al. A review of plant-derived essential oils in ruminant nutrition and production[J]. Animal Science Technology, 2008, 145(1/2/3/4): 209-228.

[30] MCINTOSH F M, WILLIAMS P, LOSA R, et al. Effects of essential oils on ruminal microorganisms and their protein metabolism[J]. Applied and Environmental Microbiology, 2003, 69(8): 5011-5014.

[31] CLARK J H, KLUSMEYER T H, CAMERON M R. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows[J]. Journal of Dairy Science, 1992, 75(8): 2304-2323.

[32] CHAUDHARY P P, GOEL N, BAKER G, et al. Influence of essential oils supplementation on rumen fermentation profile and ruminal microbial population in vitro[J]. Journal of Science, 2016, 1(4): 25-34.

[33] GÜNAL M, PINSKI B, ABUGHAZALEH A A. Evaluating the effects of essential oils on methane production and fermentation under in vitro conditions[J]. Italian Journal of Animal Science, 2017, 16(3): 500-506.

[34] MACHEBOEUF D, MORGAVI D P, PAPON Y, et al. Dose-response effects of essential oils on in vitro fermentation activity of the rumen microbial population[J]. Animal Feed Science and Technology, 2008, 145(1/2/3/4): 335-350.

[35] WANG C J, WANG S P, ZHOU H. Influences of flavomycin, ropadiar, and saponin on nutrient digestibility, rumen fermentation, and methane emission from sheep[J]. Animal Feed Science and Technology, 2009, 148(2/3/4): 157-166.

[36] CIESLAK A, SZUMACHER-STRABEL M, STOCHMAL A, et al. Plant components with specific activities against rumen methanogens[J]. Animal, 2013, 7(Suppl. 2): 253-265.

Note: Figure translations are in progress. See original paper for figures.