American Journal of Animal and Veterinary Sciences

Original Research Paper

# Effect of Rumen Culture Adaptation to *Origanum vulgare* L. Essential Oil on Rumen Methane and Fermentation

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*Article history*
Received: 14-07-2019
Revised: 21-07-2019
Accepted: 31-08-2019

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**Abstract:** The main objective of this study was to investigate the impact of adding oregano essential oil (OEO) to adapted and unadapted rumen cultures on methane gas ($CH_4$) production and rumen fermentation under *in vitro* condition. Rumen fluid for culture fermenters was collected from a Holstein cow located in Southern Illinois University farms. The adapted and unadapted rumen cultures were obtained from continuous culture fermenters fed a control diet, or control diet plus OEO at 250 mg $day^{-1}$ for 10 days. After 10 days of adaptation, the fermenter's contents were incubated in a 24 h gas production experiment. Treatments were unadapted culture, unadapted culture plus OEO, adapted culture and adapted culture plus OEO. The OEO was added to rumen cultures at the rate of 500 mg $L^{-1}$. After 24-h of incubation, total gas production decreased ($p<0.03$) with the addition of OEO in both cultures and total gas production tended to be lower when added to the adapted cultures. As a percentage of total gas, $CH_4$ production decreased ($p<0.05$) with OEO addition only in the adapted cultures. Total VFA and acetate concentrations were lower ($p<0.05$) in the adapted than unadapted cultures and their concentrations decreased ($p<0.05$) with the addition of OEO, particularly when added to the adapted cultures. Propionate concentrations were also lower ($p<0.05$) in the adapted than the unadapted cultures and concentrations decreased ($p<0.05$) with the addition of OEO. The greater effect of OEO on $CH_4$ production in the adapted cultures might have resulted from lower microbial fermentation activity which also might have caused a shift in microbial fermentation favoring less $CH_4$ formation in these cultures.

**Keywords:** Oregano Essential Oil, Methane, Adapted Rumen Inoculum

## Introduction

It has been estimated that agricultural activities are responsible for about 10-12% of the total worldwide greenhouse gas emissions and about 50% of methane ($CH_4$) emissions (Smith *et al*., 2014). Methane is considered the second most responsible gas for global warming and ruminant animals contribute for about 33% of the global $CH_4$ emission because of the fermentation process in their digestive systems (Gerber *et al*., 2010). In addition to methane gas impact on climate change, it's also considered as an energy loss. It has been estimated that between 2-12% of gross energy can be lost during methanogenesis (Johnson and Johnson, 1995).

Plant extracts and secondary metabolites such as essential oil (EO) were widely evaluated as feed additives to reduce rumen $CH_4$ production due to their antimicrobial properties and negative impact on ruminal microorganisms such as methanogenic *Archaea* (Benchaar and Greathead, 2011). Oregano essential oil (OEO) contains phenolic monoterpens as thymol and carvacrol known for their strong and broad antimicrobial activity due to the presence of an oxygenated cyclic hydrocarbon in its structure (Helander *et al*., 1998; Dorman and Deans, 2000; Benchaar and Greathead, 2011).

Although EO or their active components were effective in reducing rumen $CH_4$ production (Evans and Martin, 2000; Macheboeuf *et al*., 2008; Patra and Yu, 2012), most studies were short term where effects measured after 24-48 h of EO addition (Hariadi and Santoso, 2010; Cobellis *et al*., 2015; Pinski *et al*., 2016; Günal *et al*., 2017). However, rumen microbial ecosystem can acclimatize or develop resistance to feed

additives causing effects to diminish overtime. Cardozo *et al.* (2004) reported the acclimatization of rumen microorganism to the addition of plant extracts after 6 days of supplementation. Some studies in food preservation have also shown that pathogenic bacteria inhibited by carvacrol or thymol can adapt to non-lethal concentrations of these phenolic compounds (Ultee *et al*., 2000; Nazzaro *et al*., 2013). Hariadi and Santoso (2010) reported shifts in the effect of a variety of plant species on $CH_4$ production after incubation for 6, 24 or 48 h. More recently, Yáñez-Ruiz *et al.* (2016) reviewed the use of *in vitro* batch culture technique to assess enteric $CH_4$ production and recommended the use of inoculum from animals that have been adapted to the treatment. There is a limited literature investigating the shifts in microbial populations or the ability of individual microorganisms to acclimatize to the addition of EO and their active components is limited. Therefore, the objective of this study was to evaluate the effects of adding OEO to adapted and unadapted rumen cultures on $CH_4$ production and fermentation characteristics.

## Materials and Methods

The study was approved from the Ethical Committee of the Institutional Animal Care and Use Committee-Southern Illinois University Carbondale, with the tracking number of 18-25.

### *Experimental Design, Incubation Procedure and Laboratory Analysis*

Rumen fluid for continuous culture fermenters was collected from a lactating cannulated Holstein cow (60:40 forage to concentrate diet; dry matter basis). Ruminal fluid inoculum for the batch experiment was collected from four continuous culture fermenters (Teather and Sauer, 1988) fed either control diet (two fermenters) or control diet plus OEO (*Origanum vulgare* L.) at 250 mg $day^{-1}$ (two fermenters). The diet (50:50 forage to concentrate) was fed at the rate of 45 g $day^{-1}$ (dry matter basis) in three equal portions at 08.00, 15.00 and 24.00 h. The forage portion of the diet consisted of alfalfa pellets, while the concentrate mix contained ground corn (240 g $kg^{-1}$), soybean meal (94 g $kg^{-1}$), soy hulls (60 g $kg^{-1}$), limestone (3 g $kg^{-1}$) and minerals mix (3 g $kg^{-1}$). Anaerobic conditions inside the fermenters were maintained by continuous flushing of $CO_2$ at 45 mL $min^{-1}$ and cultures were stirred continuously at 45 rpm. Fermenters' temperature was maintained at 39°C using a circulating water bath. The buffer (Goering and Van Soest, 1970) was delivered continuously to each fermenter at a flow rate of 1.16 mL $min^{-1}$, using a precision pump.

On day 10, ruminal cultures from the continuous fermenters were collected and strained through two layers of cheesecloth and then used within approximately 15 min after collection. Seventy milliliters of the fluid inoculum from the continuous fermenters fed the control diet was incubated with 130 mL of buffer solution (Goering and Van Soest, 1970) in 250 mL ANKOM gas with or without OEO. Similarly, seventy mililiters of the fluid inoculum from the continuous fermenters fed the control diet plus OEO were incubated with 130 mL of buffer solution in 250 mL ANKOM gas with or without OEO. The OEO was added to each jar at 500 mg $L^{-1}$. Each jar contained 3 g diet that composed of (on a dry matter basis) alfalfa hay (500 g $kg^{-1}$), ground corn (300 g $kg^{-1}$), soybean meal (100 g $kg^{-1}$), soy hulls (80 g $kg^{-1}$) and mineral-vitamin mix (20 g $kg^{-1}$). Each treatment was run in triplicate.

Each jar was flushed with $CO_2$ to maintain the anaerobic conditions. Gas samples were collected using Tedlar gas collection bag connected to each jar (CEL Scientific Corp., Santa Fe Springs, CA, USA). Jars were placed into a water bath at 39°C for 24 h. Gasses from jars were programmed to be released into connected bags when the psi exceeded 1.0. Every two hours, the jars were shaken by hand for approximately 30 sec. After 24 h, gas bags were detached from jars and analyzed immediately for gas composition.

Three replicates were drawn from each gas bag, using a 1 mL gas tight needle syringe (27G 1 $14^{-1}$; Fisher Scientific, Chicago, IL, USA) and analyzed for gas composition using gas chromatography (SRI 8610C, Torrance, CA, USA) equipped with TCD detector (6"×1/8" S.S. Shin Carbon) and ST 80/800 column (2 m × 2 mm ID). The oven temperature was set at 38°C for five min, then increased at 5°C $min^{-1}$ to 270°C and held for five min. The carrier gas was argon. The samples gas peaks ($CO_2$ and $CH_4$) were recognized by comparing the retention times with those of the corresponding standard (Scotty Analyzed Gases 14, Sigma-Aldrich, St. Louis, MO, USA). Total gas production of the head-space sample was converted from pressure readings to mL according to Avogadro's Law equation:

$$N = P\left(VRT^{-1}\right)$$

where, $N$ represent the gas produced in moles, while $P$ is the pressure inside jars measured in kilopascal, $V$ is the head–space volume in the jars measured in liters, $T$ is the temperature in Kelvin scale and $R$ is the gas constant. Each gas was measured through measuring the relative proportion of each peak to the total gas production. After 24 h of incubation, samples were collected from each culture jar under cooling conditions to measure the volatile fatty acids (VFA) and ammonia N ($NH_3$-N) determination. The pH inside the jars was measured using a portable pH meter (Oakton Vernon Hills, IL 60061, USA). Samples for VFA's analysis were added to

1 mL of freshly prepared 25% meta-phosphoric acid, then centrifuged (IEC Centra GP8R, Needham Heights, MA, USA) at 20,000g at 4°C for 20 min. The supernatant was taken and added to 100 µL of 2-ethylbutyric acid as an internal standard (Jenkins, 1987). A Shimadzu GC-2010 gas chromatograph (Shimadzu Scientific Instruments Inc., Columbia, MD, USA) containing a flame-ionization detector and 30-m SP-2560 fused silica capillary column (Restek Stabil WAX DA column, Bellefonte, PA, USA) was used to measure the concentration of the VFAs. The carrier gas was helium, which was maintained at a linear velocity of 23 cm $s^{-1}$. GC temperature protocol was set to 65°C for 3 min, then get raised to a final temperature of 225°C with an increasing rate of 12°C/min. The column temperature was maintained at 65°C and flameionization detector temperature at 225°C. For ammonia-N, the 5 mL collected sample was centrifuged at 20,000 g (IEC CentraGP8R, Needham Heights, MA, USA) at 4°C for 10 min. The supernatant was then acidified with 0.5 mL of 0.1 N HCL and analyzed for ammonia N, as outlined by Cotta and Russell (1982).

### *Statistical analysis*

Data were analyzed using the general linear model procedure of SAS (SAS Inst., Inc., Gary, NC). The statistical model was:

$$y_{ij} = \mu + C_i + O_j + C_iO_j + e_{ij}$$

where, $y_{ij}$ is the observation; $\mu$ the overall mean; $c_i$ the culture ($i$ = adapted, unadapted); $O_j$ the essential oil ($j$ = with, without); $C_iO_j$ the interaction between culture and essential oil and $e_{ij}$ the residual error. Differences among means were tested using Duncan's multiple range tests. A significant level of 0.05 was used.

## Results

The effect of treatments on gas production and fermentation parameters is presented in Table 1. Compared with the control (no essential oil), adding OEO decreased ($p<0.05$) total gas production in both adapted and unadapted cultures and there was a tendency ($p<0.13$) for lower gas production when OEO was added to the adapted cultures. Compared with the control, the addition of OEO reduced ($p<0.05$) $CH_4$ production, as a percentage of total gas, however a significant essential oil by culture interaction showed that the OEO effect was significant ($p<0.05$) only when added to the adapted cultures. Total VFA and acetate concentrations were lower ($p<0.05$) in the adapted than the unadapted cultures and the addition of OEO reduced concentrations in both cultures. However, a significant interaction showed that the reductions in the total VFA and acetate concentrations with OEO addition were greater in the adapted than the unadapted cultures. Similar results were also observed with valerate and *iso*-valerate. Propionate concentrations were also lower ($p<0.05$) in the adapted than the unadapted cultures and concentrations decreased ($p<0.05$) with the addition of OEO. Butyrate concentrations, however, were greater ($p<0.05$) in the adapted than the unadapted cultures and concentrations increased ($p<0.05$) with the addition of OEO. Similarly, the acetate to propionate ratio was higher ($p<0.05$) in the adapted than the unadapted cultures and the ratio increased ($p<0.05$) with the addition of OEO. Ammonia-N concentration and cultures pH were not affected ($p>0.05$) by OEO or culture source.

**Table 1:** Effect of oregano essential oil on methane gas production and fermentation

| | Treatments | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Unadapted culture | | Adapted culture | | | p-value | | |
| | Control | OEO | Control | OEO | MSE | OEO | Culture | OEO × Culture |
| Total gas, ml | 179.06 | 140.25 | 174.51 | 117.64 | 11.234 | 0.01 | 0.70 | 0.41 |
| $CO_2$, % of total gas | 61.61 | 65.54 | 67.61 | 61.69 | 2.926 | 0.39 | 0.28 | 0.07 |
| $CH_4$, % of total gas | 21.44 | 21.38 | 23.13 | 20.42 | 0.541 | 0.03 | 0.23 | 0.04 |
| pH | 6.16 | 6.21 | 6.18 | 6.16 | 0.102 | 0.88 | 0.81 | 0.78 |
| NH3-N, mg $dL^{-1}$ | 11.16 | 10.79 | 10.46 | 9.45 | 0.836 | 0.44 | 0.26 | 0.71 |
| Total VFA, *m*M | 59.85 | 52.51 | 56.76 | 44.17 | 1.010 | 0.01 | 0.01 | 0.03 |
| Acetate (C2) | 23.39 | 19.52 | 22.39 | 15.23 | 0.418 | 0.01 | 0.01 | 0.01 |
| Propionate (C3) | 16.86 | 10.35 | 14.77 | 6.88 | 0.515 | 0.01 | 0.01 | 0.22 |
| Butyrate | 14.48 | 17.34 | 14.94 | 18.80 | 0.391 | 0.01 | 0.04 | 0.24 |
| *Iso*-butyrate | 0.61 | 0.64 | 0.52 | 0.55 | 0.086 | 0.74 | 0.33 | 0.99 |
| Valerate | 2.25 | 2.15 | 2.01 | 1.56 | 0.060 | 0.01 | 0.01 | 0.02 |
| *Iso*-valerate | 2.25 | 2.50 | 2.11 | 1.14 | 0.136 | 0.03 | 0.01 | 0.01 |
| C2:C3 ratio | 1.38 | 1.89 | 1.51 | 2.25 | 0.102 | 0.01 | 0.04 | 0.28 |

$CH_4$ is methane gas, $CO_2$ is carbon dioxide gas, $NH_3$-N is ammonia N, MSE is mean square of error, OEO is oregano essential oil, VFA is volatile fatty acids

## Discussion

Oregano essential oil's chemical composition has been investigated previously and reported to consist mainly of thymol and carvacrol (D'Antuono *et al*., 2000; De Falco *et al*., 2013). These phenolic compounds were thought to have an effect on rumen fermentation, methanogenic activity and $CH_4$ production in ruminants (Evans and Martin, 2000; Busquet *et al*., 2006; Günal *et al*., 2017). The addition of OEO to cultures in the present study resulted in reductions in VFA concentration and total gas production suggesting that the fermentation and microbial activity were negatively impacted at the dose utilized in this study. These effects are consistent with the findings of others (Castillejos *et al*., 2006; Patra and Yu, 2012; Cobellis *et al*., 2015). In general, at high doses, OEO or its components decrease the fermentation and microbial activity. Busquet *et al.* (2006) reported reductions in VFA concentration and $CH_4$ production with high doses of OEO (300 and 3000 mg $L^{-1}$) but not when included at 30 mg $L^{-1}$. Cobellis *et al.* (2015) also observed similar reductions in VFA concentration when OEO was added to rumen cultures at high doses.

The present study also showed that rumen culture source had no effect on total gas production but significantly affected VFA concentration. Total VFA concentration was greater in the unadapted than the adapted cultures possibly suggesting that fermentation activity was lower in the adapted cultures. Adding OEO or its components has been reported to negatively influence on rumen microbial population, especially protozoa, and fermentation (Patra and Yu,2012; Mbiriri *et al*., 2016). However, this explanation should be taken with caution, as the total gas production was not affected by culture source, which is usually affected by lower microbial fermentation activity. It is possible though that OEO effect on rumen microbes was selective, inhibiting some strains more than others (Tekippe *et al*., 2011; Patra and Yu, 2012). For example, in the current study, OEO may have affected more the microbial strains involved in grains than fiber digestion as suggested by the greater acetate to propionate ratio seen in the unadapted than adapted cultures (Table 1). Evans and Martin (2000) reported that adding thymol at 180 mg $L^{-1}$, completely inhibited the growth of *Streptococcus bovis*, one of amylolytic bacteria species in the rumen. Additionally, the bacterial populations colonizing soluble carbohydrate-rich substrates are affected to a greater extent by supplementation of the diet with a commercial blend of EO compounds (Duval *et al*., 2007), possibly due to the greater ability of starch granules to absorb some components of the EO (Misharina, 2004).

The reduction in $CH_4$ with the addition of OEO was consistent with the findings of others (Tekippe *et al*., 2011; Patra and Yu, 2012; Cobellis *et al*., 2015; Kolling *et al*., 2018) and could be attributed to the antimicrobial effects of the OEO on protozoa and methanogenic *Archaea* as reported by Patra and Yu (2012) and Paraskevakis (2018). Interestingly however, the reduction in $CH_4$ was significant only when OEO was added to the adapted culture suggesting that microbial adaptation and/or modification played a role in OEO effect. Compared to rumen, continuous culture fermenters were reported to have limited number of protozoa (Fenchel *et al*., 1977; Williams and Coleman, 1997) and in previous studies (Lin *et al*., 2012; Patra and Yu, 2012; Mbiriri *et al*., 2016), the effects of OEO and its components or other EO on $CH_4$ production were attributed in part to their effects on protozoa. Therefore, it is possible that the lack of OEO effect in the unadapted culture was due to protozoa absence in collected inoculum from continuous culture fermenters while the effect of OEO in the adapted culture could be due to lower methanogenic bacterial activity in these cultures. Another possible explanation for the lack of effect in the unadapted culture was the low dose of OEO used. Although the same concentration of OEO was added to both batch cultures, it is possible that the adapted cultures had traces of OEO and therefore more OEO was present in the adapted than the unadapted batch cultures. In general, EO effect on $CH_4$ production was reported to be proportional to dose level (Cobellis *et al*.,2015; Pinski *et al*., 2016; Günal *et al*., 2017). Finally, the reduction in $CH_4$ production in the adapted culture may also be explained by the greater suppression in the overall fermentation seen in these cultures. In the current study, the reduction in acetate formation was greater in the adapted than unadapted cultures (16.5 vs. 32%) suggesting also lower cellulolytic activity. Low acetate production in the rumen usually results in less $CH_4$ production because of lower hydrogen formation (Kreuzer *et al*., 1986). The ruminal methanogen population is associated with cellulolytic bacteria that produces hydrogen in the rumen (Szumacher-Strabel and Cieślak, 2010). Patra and Yu (2012) reported that OEO reduced cellulolytic bacteria abundance such as *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens*.

The reductions in the concentrations of acetate, propionate, valerate and *iso*-valerate and the increase in the concentrations of butyrate and *iso*-butyrate with OEO addition were consistent with the findings of others (Busquet *et al*.,2006; Benchaar *et al*., 2007; Patra and Yu,2012; Cobellis *et al*., 2015) and suggest inhibition for both Gram positive and negative bacteria. The main chemical components of OEO are carvacrol (24-53%) thymol (2-24%), p-cymene (4 -8%) and linalool (0.1-

14%) (Tuncer *et al*., 2009; De Falco *et al*., 2013) and previous studies reported that carvacrol and thymol have a negative effect on both Gram-negative and positive bacteria (Helander *et al*., 1998; Dorman and Deans, 2000). Carvacrol and thymol were reported to diffuse the cell membrane lipid layer, causing pores between the fatty acids and changes in the permeability of cell membranes for cations H+ and K+ (Helander *et al*., 1998; Ultee *et al*., 1999). The OEO by culture interaction in this study however, suggest that OEO adverse effects on fermentation were more pronounced in the adapted than unadapted cultures. For example, relative to control, the addition of OEO to the unadapted cultures reduced acetate and propionate concentrations by 16.5 and 38.2%, respectively, compared to 32 and 53.4% in the adapted cultures. Comparing the concentrations of propionate and acetate to propionate ratio between the adapted and unadapted cultures suggest that OEO effect was greater on the Gram-negative bacteria. In the rumen, Gram-positive bacteria generally produce acetate and butyrate, while Gram-negative bacteria generally produce propionate (Stewart, 1992). Gram-positive bacteria are usually more susceptible to EO due to lacking outer membrane compared to Gram-negative bacteria's complex cell wall (Cox *et al*., 2001). However, in the present study, the increase in the acetate to propionate ratio and butyrate concentration with OEO addition suggested that Gram negative bacteria were more affected than Gram positive bacteria.

The decrease in $CH_4$ production with EO supplementation to adapted rumen cultures may vary depending on dose-level. Rumen microorganisms can adapt to and/or degrade the active compounds of EO, particularly at low dosage rates (Chizzola *et al*., 2004; Klop *et al*., 2017). Mbiriri *et al.* (2016) reported a transient mitigating effect on the suppression of $CH_4$ with carvacrol and thymol supplementation at 300 mg $L^{-1}$. However, Fraser *et al.* (2007) observed a persistent effect with 500 mg EO $L^{-1}$ on $CH_4$ production in a continuous culture fermenter. In general, low doses of EO have a transient mitigating effect on $CH_4$ production while higher doses have a persistent effect. In the present study, total VFA, $CH_4$, acetate, valerate and *iso*-valerate concentrations were more affected with OEO addition in the adapted than unadapted culture suggesting more persistent effects for OEO on rumen microbes.

## Conclusion

The addition of OEO negatively affected rumen fermentation especially in the adapted cultures. Methane production was reduced with the addition of OEO only in the adapted cultures that also had lower VFA concentration suggesting that the adapted cultures had lower overall microbial efficiency and a shift in microbial fermentation favoring less $CH_4$ formation than the unadapted cultures. Future studies examining the effects of different dose levels of OEO and longer adaptation period on methanogenic microbes are needed.

## Authors Contributions

**Mohammed G. Embaby:** Conduction of study, a laboratory analysis and interpretation of data, drafting of manuscript

**Mevlüt Günal:** Drafting of manuscript.

**Amer A. AbuGhazaleh:** Experimental design, interpretation of data and drafting of manuscript.

## Conflict of Interest

All authors confirm that this article is original and there is no conflict of interest in this article to declare.

## References

Benchaar, C., A.V. Chaves, G.R. Fraser, Y. Wang and K.A. Beauchemin *et al*., 2007. Effects of essential oils and their components on *in vitro* rumen microbial fermentation. Can. J. Anim. Sci., 87: 413-419. DOI: 10.4141/CJAS07012.

Benchaar, C. and H. Greathead, 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Anim. Feed Sci. Technol., 166-167: 338-355. DOI: 10.1016/j.anifeedsci.2011.04.024

Busquet, M., S. Calsamiglia, A. Ferret and C. Kamel, 2006. Plant extracts affect *in vitro* rumen microbial fermentation. J. Dairy Sci., 89: 761-771. DOI: 10.3168/jds.S0022-0302(06)72137-3

Cardozo, P.W., S. Calsamiglia, A. Ferret and C. Kamel, 2004. Effects of natural plant extracts on ruminal protein degradation and fermentation profiles in continuous culture. J. Anim. Sci., 82: 3230-3236. DOI: 10.2527/2004.82113230x

Castillejos, L., S. Calsamiglia and A. Ferret, 2006. Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in *in vitro* systems. J. Dairy Sci., 89: 2649-2658. DOI: 10.3168/jds.S0022-0302(06)72341-4

Chizzola, R., W. Hochsteiner and S. Hajek, 2004. GC analysis of essential oils in the rumen fluid after incubation of *Thuja orientalis* twigs in the Rusitec system. Res. Vet. Sci., 76: 77-82. DOI: 10.1016/j.rvsc.2003.07.001

Cobellis, G., A. Petrozzi, C. Forte, G. Acuti and M. Orrù *et al*., 2015. 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. Sustainability, 7: 12856-12869. DOI: 10.3390/su70912856

Cotta, M.A. and J.B. Russell, 1982. Effects of peptides and amino acids on efficiency of rumen bacterial protein synthesis in continuous culture. J. Dairy Sci., 65: 226-234. DOI: 10.3168/jds.S0022-0302(82)82181-4

Cox, S.D., C.M. Mann, J.L. Markham, J.E. Gustafson and J.R. Warmington *et al*., 2001. Determining the antimicrobial actions of tea tree oil. Molecules, 6: 87-91: DOI: 10.3390/60100087

D'Antuono, L.F., G.C. Galletti and P. Bocchini, 2000. Variability of essential oil content and composition of *Origanum vulgare* L. populations from a North Mediterranean area (Liguria Region, Northern Italy). Ann. Bot., 86: 471-478. DOI: 10.1006/anbo.2000.1205

De Falco, E., E. Mancini, G. Roscigno, E. Mignola and O. Taglialatela-Scafati *et al*., 2013. Chemical composition and biological activity of essential oils of *Origanum vulgare* L. *subsp. vulgare* L. under different growth conditions. Molecules, 18: 14948-14960. DOI: 10.3390/molecules181214948

Dorman, H.J. and S.G. Deans, 2000. Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. J. Appl. Microbiol., 88: 308-316. DOI: 10.1046/j.1365-2672.2000.00969.x

Duval, S.M., N.R. Mcewan, R.C. Graham, R.J. Wallace and C.J. Newbold, 2007. Effect of a blend of essential oil compounds on the colonization of starch-rich substrates by bacteria in the rumen. J. Applied Microbiol., 103: 2132-2141. DOI: 10.1111/j.1365-2672.2007.03455.x

Evans, J.D. and S.A. Martin, 2000. Effects of thymol on ruminal microorganisms. Curr. Microbiol., 41: 336-340. DOI: 10.1007/s002840010145

Fenchel, T., T. Perry and A. Thane, 1977. Anaerobiosis and symbiosis with bacteria in free-living ciliates. J. Protozool., 24: 154-163. DOI: 10.1111/j.1550-7408.1977.tb05294.x

Fraser, G.R., A.V. Chaves, Y. Wang, T.A. Mcallister and K.A. Beauchemin *et al*., 2007. Assessment of the effects of cinnamon leaf oil on rumen microbial fermentation using two continuous culture systems. J. Dairy Sci., 90: 2315-2328. DOI: 10.3168/jds.2006-688

Gerber, P., T. Vellinga, C. Opio, B. Henderson and H. Steinfeld, 2010. Greenhouse gas emissions from the dairy sector: A life cycle assessment. Food and Agriculture Organization of the United Nations (FAO), Animal Production and Health Division, Rome, Italy.

Goering, H.K. and P.J. Van Soest, 1970. Forage fiber analysis (Apparatus, reagents, procedures and some applications). Agriculture Handbook No. 379. ARS-USDA, Washington, USA.

Günal, M., B. Pinski and A.A. AbuGhazaleh, 2017. Evaluating the effects of essential oils on methane production and fermentation under *in vitro* conditions. Ital. J. Anim. Sci., 16: 500-506. DOI: 10.1080/1828051X.2017.1291283

Hariadi, B.T. and B. Santoso, 2010. Evaluation of tropical plants containing tannin on *in vitro* methanogenesis and fermentation parameters using rumen fluid. J. Sci. Food Agric., 90: 456-461. DOI: 10.1002/jsfa.3839

Helander, I.M., H. Alakomi, T. Mattila-Sandholm, I. Pol and E.J. Smid *et al*., 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. J. Agric. Food Chem., 46: 3590-3595. DOI: 10.1021/jf980154m

Jenkins, T.C., 1987. Effect of fats and fatty acid combinations on ruminal fermentation in semi-continuous *in vitro* cultures. J. Anim. Sci., 64: 1526-1532. DOI: 10.2527/jas1987.6451526x

Johnson, K.A. and D.A. Johnson, 1995. Methane emissions from cattle. J. Anim. Sci., 73: 2483-2492. DOI: 10.2527/1995.7382483x.

Klop, G., S. Van Laar-van Schuppen, W.F. Pellikaan, W.H. Hendriks and A. Bannink *et al*., 2017. Changes in *in vitro* gas and methane production from rumen fluid from dairy cows during adaptation to feed additives *in vivo*. Animal, 11: 591-599. DOI: 10.1017/S1751731116002019

Kolling, G.J., S.C.B. Stivanin, A.M. Gabbi, F.S. Machado and A.L. Ferreira *et al*., 2018. Performance and methane emissions in dairy cows fed oregano and green tea extracts as feed additives. J. Dairy Sci., 101: 4221-4234. DOI: 10.3168/jds.2017-13841

Kreuzer, M., M. Kirchgeßner and H.L. Müller, 1986. Effect of defaunation on the loss of energy in wethers fed diferent quantities of cellulose and normal or steam flaked maize starch. Anim. Feed Sci. Technol., 16: 233-241. DOI: 10.1016/0377-8401(86)90114-8

Lin, B., Y. Lu, J.H. Wang, Q. Liang and J.X. Liu, 2012. The effects of combined essential oils along with fumarate on rumen fermentation and methane. J. Anim. Feed Sci., 21: 198-210. DOI: 10.22358/jafs/66064/2012

Macheboeuf, D., D.P. Morgavi and Y. Papon, 2008. Dose-response effects of essential oils on *in vitro* fermentation activity of the rumen microbial population. Anim. Feed Sci. Technol., 145: 335-350. DOI: 10.1016/j.anifeedsci.2007.05.044

Mbiriri, D.T., S. Cho, C.I. Mamvura and N.J. Choi, 2015. Assessment of rumen microbial adaptation to garlic oil, carvacrol and thymol using the consecutive batch culture system. J. Vet. Sci. Anim. Husb., 3: 401-401.

Misharina, T.A., 2004. Retention of essential oil components sorbed by native cornstarch during storage. Applied Biochem. Microbiol., 40: 104-107.

Nazzaro, F., F. Fratianni, L. De Martino, R. Coppola and V. De Feo, 2013. Effect of essential oils on pathogenic bacteria. Pharmaceuticals, 6: 1451-1474. DOI: 10.3390/ph6121451

Paraskevakis, N., 2018. Effects of dietary Greek oregano (*Origanum vulgare ssp. hirtum*) supplementation on rumen fermentation, enzyme profile and microbial communities in goats. J. Anim. Physiol. Anim. Nutr., 102: 701-705. DOI: 10.1111/jpn.12812

Patra, A.K. and Z. Yu, 2012. Effects of essential oils on methane production and fermentation by and abundance and diversity of rumen microbial populations. Applied Environ. Microbiol., 78: 4271-4280. DOI: 10.1128/AEM.00309-12

Pinski, B., M. Günal and A.A. AbuGhazaleh, 2016. The effects of essential oil and condensed tannin on fermentation and methane production under *in vitro* conditions. Anim. Prod. Sci., 56: 1707-1713. DOI: 10.1071/AN15069

Smith, P., M. Bustamante, H. Ahammad, H. Clark and H. Dong *et al*., 2014. Agriculture, Forestry and Other Land Use (AFOLU). In: Climate Change 2014: Mitigation of Climate Change. Cambridge University Press, UK, pp: 73-139.

Stewart, C.S., 1992. Lactic Acid Bacteria in the Rumen. In: The Lactic Acid Bacteria in Health and Disease, Wood, B.J.B. (Ed.), Springer, Boston, MA US, ISBN-13: 978-1-4615-3522-5, pp: 49-68.

Szumacher-Strabel, M. and A. Cieślak, 2010. Potential of phyto factors to mitigate rumen ammonia and methane production. J. Anim. Feed Sci., 19: 319-337. DOI: 10.22358/jafs/66296/2010

Teather, R.M. and F.D. Sauer, 1988. A naturally compartmented rumen simulation system for the continuous culture of rumen bacteria and protozoa. J. Dairy Sci., 71: 666-673. DOI: 10.3168/jds.S0022-0302(88)79605-8

Tekippe, J.A., A.N. Hristov, K.S. Heyler, T.W. Cassidy and V.D. Zheljazkov *et al*., 2011. Rumen fermentation and production effects of *Origanum vulgare* L. in lactating dairy cows. J. Dairy Sci., 94: 5065-5079. DOI: 10.3168/jds.S0022-0302(88)79605-8

Tuncer, O., S. Karaman, S. Kizil and E. Diraz, 2009. Changes in essential oil composition of oregano (*Origanum onites* L.) due to diurnal variations at different development stages. Not. Bot. Horti Agrobo., 37: 177-181. DOI: 10.15835/nbha3723188

Ultee, A., E.P. Kets, M. Alberda, F.A. Hoekstra and E.J. Smid, 2000. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. Arch. Microbiol., 174: 233-238. DOI: 10.1007/s002030000199

Williams, A.G. and G.S. Coleman, 1997. The Rumen Protozoa. In: The Rumen Microbial Ecosystem, Hobson, P.N. and C.S. Stewart (Eds.), Springer, Dordrecht, NL, ISBN-13: 978-94-009-1453-7, pp: 73-139.

Yáñez-Ruiz, D.R., A. Bannink, J. Dijkstra, E. Kebreab and D.P. Morgavi *et al*., 2016. Design, implementation and interpretation of *in vitro* batch culture experiments to assess enteric methane mitigation in ruminants-a review. Anim. Feed Sci. Technol., 216: 1-18. DOI: 10.1016/j.anifeedsci.2016.03.016