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

1Mohamed G. Embaby, 2Mevlüt Günal and 1Amer AbuGhazaleh

1Department of Animal Science, Food and Nutrition, Southern Illinois University, 62901- Carbondale, IL- USA
2Department of Animal Science, Isparta University of Applied Sciences, 32260-Isparta -Turkey

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 monoterpenes 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; Güral *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 from animals that have been adapted to the treatment.

There is a limited literature investigating the shifts in the proportion of each peak to the total gas production. After 24 h in incubation, samples were collected from 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 stratified 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 milliliters 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 comprised 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 jars were 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 1/4"; 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:

\[
N = P(VRT^{-1})
\]

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., Cary, NC). The statistical model was:

\[ y_{ij} = \mu + c_i + O_j + c_i O_j + e_{ij} \]

where, \( y_{ij} \) is the observation; \( \mu \) the overall mean; \( c_i \) the culture \((i = \text{adapted, unadapted}); O_j \) the essential oil \((j = \text{with, without}); C_{ij} \) 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 \( (p<0.05) \) CH₄ 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

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Unadapted culture</th>
<th>Adapted culture</th>
<th>MSE</th>
<th>OEO</th>
<th>OEO × Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>179.06</td>
<td>140.25</td>
<td>147.51</td>
<td>117.64</td>
<td>11.234</td>
</tr>
<tr>
<td>OEO</td>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>0.70</td>
</tr>
<tr>
<td>CH₄, % of total gas</td>
<td>61.61</td>
<td>65.54</td>
<td>67.61</td>
<td>61.69</td>
<td>2.926</td>
</tr>
<tr>
<td>CH₃N, mg dL⁻¹</td>
<td>21.44</td>
<td>21.38</td>
<td>23.13</td>
<td>20.42</td>
<td>0.541</td>
</tr>
<tr>
<td>pH</td>
<td>6.16</td>
<td>6.21</td>
<td>6.18</td>
<td>6.16</td>
<td>0.102</td>
</tr>
<tr>
<td>NH₃-N, mg dL⁻¹</td>
<td>11.16</td>
<td>10.79</td>
<td>10.46</td>
<td>9.45</td>
<td>0.836</td>
</tr>
<tr>
<td>Total VFA, mM</td>
<td>59.85</td>
<td>52.51</td>
<td>56.76</td>
<td>44.17</td>
<td>1.010</td>
</tr>
<tr>
<td>Acetate (C2)</td>
<td>23.39</td>
<td>19.52</td>
<td>22.39</td>
<td>15.23</td>
<td>0.418</td>
</tr>
<tr>
<td>Propionate (C3)</td>
<td>16.86</td>
<td>10.35</td>
<td>14.77</td>
<td>6.88</td>
<td>0.515</td>
</tr>
<tr>
<td>Butyrate</td>
<td>14.48</td>
<td>17.34</td>
<td>14.94</td>
<td>18.80</td>
<td>0.391</td>
</tr>
<tr>
<td>iso-butyrate</td>
<td>0.61</td>
<td>0.64</td>
<td>0.52</td>
<td>0.55</td>
<td>0.086</td>
</tr>
<tr>
<td>Valerate</td>
<td>2.25</td>
<td>2.15</td>
<td>2.01</td>
<td>1.56</td>
<td>0.060</td>
</tr>
<tr>
<td>iso-valerate</td>
<td>2.25</td>
<td>2.50</td>
<td>2.11</td>
<td>1.14</td>
<td>0.136</td>
</tr>
<tr>
<td>C2/C3 ratio</td>
<td>1.38</td>
<td>1.89</td>
<td>1.51</td>
<td>2.25</td>
<td>0.102</td>
</tr>
</tbody>
</table>

CH₄ is methane gas, CO₂ is carbon dioxide gas, NH₃-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 CH₄ 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 CH₄ 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 CH₄ 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 CH₄ 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 CH₄ 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 CH₄ 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 CH₄ 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 CH₄ 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 Cieslak, 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-
produce propionate (Stewart, 1992). Gram-positive bacteria generally produce acetate and propionate and acetate to propionate ratio between the adapted cultures. Comparing the concentrations of 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. 

The decrease in CH₄ 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 CH₄ 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 CH₄ production in a continuous culture fermenter. In general, low doses of EO have a transient mitigating effect on CH₄ production while higher doses have a persistent effect. In the present study, total VFA, CH₄, 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 CH₄ 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 Günsel**: 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**


