Potential effect of some essential oils on rumen methane reduction and digestibility by In Vitro incubation technique

M N Rofiq¹*, W Negara¹, S Martono¹, R A Gopar¹ and M Boga²

¹Center of Agricultural Production Technology- Agency of National Research and Innovation (BRIN), LABTIAP 610 bld. Kawasan Puspiptek Serpong, Tangerang Selatan 15314, Indonesia
²Niğde Ömer Halisdemir University, Food Processing Departmen, Nigde 51240, Turkey

Corresponding author: nasir.rofiq@bppt.go.id

Abstract. Since excessive use of in-feed antibiotics and chemical feed supplements can potentially affect health risks and environmental problems, organic feed supplement became alternative save material. Organic material such as essential oils (EO) is potential for feed additive in the livestock industry because of their main activity as rumen manipulator. Their potential as rumen manipulators has not enough for type and doses of essential oils experiment. Therefore, the objective of this study was to evaluate 6 essential oils/EO’s (garlic/, thyme, clove, orange peel, mint, and cinnamon) with different doses (100, 200 and 300 ppm) on in vitro rumen methane reduction and in vitro true digestibility using in vitro gas production technique. The experiment resulted highest level in in-vitro methane reduction potential (MRP, %) 72.83% at orange peel oils 300 ppm after 6 h incubation, while using clove oils 300 ppm had highest in vitro true digestibility of dry matter (IVTDDM) 81.41% after 30-hour incubation. Using Cinnamon oils had high value both of MRP level and IVTDDM (48.91 and 79.12 %). In Conclusion, all essential oils reduced in vitro rumen methane production at 6 h incubation compared to control at 300 ppm and there was no negative effect on In Vitro Digestibility.

1. Introduction
Livestock contribute to greenhouse gases (GHG) through CH₄ and N₂O gas from rumen fermentation, nitrogenous fertilizers, waste or manure management and deposition of animal manures on pastures. Enteric methane emissions are estimated increasing by over 30% from 2000 to 2020 because of increasing food demand [1]. Methane gas (CH₄) production from livestock mainly produced by rumen fermentation and storage of manure which influences global warming 28 times higher than carbon dioxide. Nitrous oxide (N₂O) is obtained from storage of manure has a global warming potential 265 times higher than carbon dioxide. The carbon dioxide equivalent is a standard unit used to account for the global warming potential [2]. Reducing or mitigation CH₄ from rumen fermentation had been assessed by some researchers since the CH₄ emission from ruminants livestock significantly related to feed conversion efficiency [3] and GHGs emission. Resulted from the experiment gave some recommendation of mitigation strategies which could be applied in current strategies and future [4]. Nutrient improvement and rumen modification strategies are the current strategies from some experiments results. It could be developed to some level in experimental objects such as animal level, microbial level, nutrient level and plant level assessment strategies. However, nutrient level strategies
are generally based around one of all rumen CH4 mitigation strategies. It is important that any mitigation strategy would be only adopted when it passes regulatory requirements and there is an economic profit [5].

Nutrient strategies including the use of feed additive to reduce rumen methane production is a future strategy which has positive effect on animal productivity and environment friendly. Livestock industry started to be assessed using feed supplement. However, some chemical feed supplements could affect health risk and environmental problems. Livestock industry is seeking feed supplement materials which it shall not harm animal health, human health, or the environment. Potential feed supplement from plant secondary metabolites is used as organic feed additive in livestock industry. Essential oils (EO’s) from plant is one of plant secondary metabolites which is obtained from volatile fraction of plants distillation process [6].

Essential oils (EO’s) was reported has bioactive compounds which was could be selected inhibit rumen methanogenesis by their antimicrobial activity [7]. The bioactive compound was derived from some plant thyme, oregano, cinnamon, garlic, rhubarb and frangula decreased in vitro rumen gas production in a dose dependent manner. However, this essential oil potential should be developed with any type of plants and doses. The object of this study was to assay the effect of some essential oils with different bioactive compounds and different doses on in vitro rumen methane production, gas production and digestibility value using in vitro incubation technique.

2. Material and Methods

2.1. Essential oils
Six essential oils (EO’s) were used for this experiment which were selected because of their major bioactive compounds. Garlic (Gar), Clove (Clo), Thyme (Thy), Cinnamon (Cin), Orange peel (Ora) and Mint (Min) EOs have different major bioactive compounds as in Table 1. The oils were evaluated with three doses (100, 200 and 300 ppm) in in vitro test used total mixed ration (TMR) as a substrate.

| Essential Oil | Bioactive compounds | % Compound/EO* |
|---------------|---------------------|----------------|
| Garlic (Gar)  | Diallyl disulfide   | 51.17          |
|               | Allyl trisulfide    | 30.57          |
| Clove (Clo)   | Eugenol             | 97.26          |
| Thyme (Thy)   | Thymol              | 79.81          |
| Cinnamon (Cin)| Cinnamaldehyde      | 17.79          |
|               | Benzyl alcohol      | 34.59          |
|               | Pyrrolidinenedione  | 21.08          |
|               | Propeoic acid       | 21.89          |
| Mint (Min)    | α, -Pinene          | 38.99          |
|               | Pulegone            | 39.98          |
| Orange Peel (Ora) | D-Limonene    | 98.08          |

2.2. In Vitro incubation technique
There are two incubation technique were used in this experiment for measure in vitro rumen gas total, methane, and digestibility. First incubation, in vitro rumen gas production technique Hohenheim Gas Test (HGT) system is described in detail by Menke and Steingass [8]. The operation of in Vitro HGT used 200 mg total mixed ration as a substrate and was incubated with essential oils and rumen fluid for 96 hours. Total mixed ration as a substrate was analyzed for proximate fraction [9] and fiber fraction
This incubation technique resulted in gas production data, methane gas production and organic matter digestibility (OMD) estimation. Second incubation, in vitro batch culture using in vitro Daisy II incubator was used measuring in vitro true digestibility of Dry matter (IVTDDM). In vitro digestibility with Daisy II incubator is conducted as Daisy II incubator Ankom Technology method [11]. This incubation technique used 0.25 g substrate which was filled into filter bag F57. The filter bag was incubated with rumen fluid as an inoculums and buffer solution for 30 hours. After incubation, the NDF value was analyzed from substrate to calculate its digestibility (dNDF).

2.3. In Vitro gas and methane measurement

Gas production data was collected from in vitro gas production technic at 3, 6, 9, 12, 24, 48, 72, 96 hours. After 6 h of incubation, methane gas was measured using a katharometer methane sensor OLC20. Cumulative gas production data were fitted to the model of Ørskov [12]:

\[
y = a + b(1 - \exp^{-ct})
\]

Where: \(a\) = gas production from soluble fraction (ml), \(b\) = gas production from the insoluble fraction (ml), \([a+b]\) = potential gas production (ml), \(c\) = gas production rate constant for the insoluble fraction (ml/h), \(t\) = incubation time (h), \(y\) = gas produce at time \(t\) (ml).

In vitro rumen methane gas reduction was calculated as a different between control without essential oils and with essential oils incubation. The methane reduction potential (MRP) is percentage value of increasing methane gas production from control (without using EO’s).

2.4. In Vitro rumen digestibility measurement

In Vitro digestibility value which was resulted from In Vitro ANKOM DAISY II incubator is calculated using NDF digestibility values (IVNDFD). Using the NDF Value for in vitro digestibility obtained In Vitro true dry matter digestibility for ruminant (IVTDDM). They were calculated with below equation (2 and 3).

- \[IVNDFD \ (\% \ DM) = 100 \times \frac{(W_2 \times \%NDF_{Feed}) - (W_3 - (W_1 \times C_1))}{(W_2 \times \%DM_{Feed})}\] (2)
- \[IVTDDM \ (\%DM) = 100 - \left[\frac{(W_3 - (W_1 \times C_1)) \times 100}{(W_2 \times \%DM_{Feed})}\right]\] (3)

Where: \(W_1\) is weight of filter bag, \(W_2\) is weight of sample, \(W_3\) is final weight (Filter bag + sample). NDF_{Feed} is % of NDF contain in Feed (%DM). DM_{Feed} is % of dry matter contain in feed and \(C_1\) is correction of factor (blank filter bag NDF value).

Organic matter digestibility (OMD) also estimated from gas production 24 hours by equation below for forage and concentrate [8].

- \[OMD_{forage} \ (\%) : 14.88 + 0.8893 \times GP_{24}(ml/200mgDM) + 0.448CP(\%DM) + 0.65Ash\] (4)
- \[OMD_{concentrate} \ (\%) : 22.53 + 0.76 \times GP_{24}(ml/200mgDM) + 0.6365CP(\%DM)\] (5)

Where GP_{24} is gas production at 24 hours incubation, CP is crude protein and Ash is as in proximate fractionation of TMR as a substrate.

2.5. Statistical analysis

The effect of six EO’s (Gar, Clo. Cin, Thy, Min, and Ora) and four doses (0, 100, 200, and 300 ppm) on in vitro rumen gas production, methane reduction, and digestibility values was assigned in a factorial complete randomize design. Resulted data was analyzed using GLM procedure of SAS 9.1.3 for windows statistical packaged analyzed [13].

3. Results and discussion

3.1. In vitro rumen gas production (GP) and methane reduction potential (MRP)

In vitro incubation technique using gas production system resulted gas production curve with different characteristics between EO’s and doses (Figure 1). The Curve of gas production (GP) characteristics
indicated significantly different with different EO’s and doses (P < 0.01), except for rate of GP (c; %h). The value for “a” intercept (ml) for all treatments ranged from -6.47 to 1.73. The “a” intercept value ideally reflects the fermentation of the soluble fraction or quickly soluble fraction [12].

A high in vitro GP was positively associated with digestibility and reflects greater fermentation to support rapid rumen microbial growth. At the end of fermentation (96 hours), the GP of incubation using 300 ppm clove EO’s (Clo-100) and 100 ppm garlic EO’s (Gar-100) were higher than others. Thyme (Thy) EO’s resulted lower gas production than control (without EO’s) at the end of incubation. Gas produced at 6 hours incubation was a good estimate of the develop fermentation of non-structural carbohydrate (NSC) (i.e., estimated primarily as sugars, pectin, and starches); therefore, gas methane production also better was measured at this time. After 6-hour incubation, range of GP value was ranged from 8.35 ml to 20.91 ml. Thy-300 had lowest gas production (8.35 ml) after 6 hours incubation, while Clo-300 had highest (20.91 ml). Cin-300, Min all doses and Clo all doses had higher gas production after 6 hours incubation than control (13.71 ml). All essential oils at high doses (300 ppm) had gas production higher than low doses (100 ppm). For that reason, some essential oils which were used in this experiment could be used at high doses (300 ppm).

Methane gas production was decrease at 6 h incubation compared by addition of all essential oils, except Clo-100 (Table 2). Decreasing value of methane gas production was calculated as a percentage value of increasing methane gas production from control (without using EO’s), as a methane reduction potential (MRP). All essential oils had highest MRP doses at 300 ppm, while the lowest value of MRP is using Gar-100 (3.70%) and the highest is using Ora-300 (72.83%). It was also indicated that garlic EO’s as 300 ppm reduced in vitro methane production 42.96 %, lower than other study with same doses in batch culture fermentation (17 h) which reduced methane as 74% [14, 15]. Similar with other
experiment result that Gar EO’s 100 and 200 ppm reduced methane concentration after 6 hours incubation [16,17]. The Garlic oils can inhibit Archaea microorganisms directly in the rumen because the Garlic EO’s has four main components (dially sulfide, diallyly disulfide, allyl mercaptan and allycin) [16]. Thus, the garlic oils 250 ppm could reduce methane production 16.32%, but not impact on asetat/propionate ratio and in vitro organic matter digestibility [18].

Table 2. In Vitro rumen methane production and methane reduction potent (MRP) at 6 hours Incubation of EO’s as doses 100, 200 and 300 ppm in vitro rumen incubation technique.

| Essential oils (EO) | Doses (ppm) | Gas 6 h (ml) | Methane production | MRP (%) |
|---------------------|------------|--------------|--------------------|---------|
| **Control**         | 0          | 13.71c       | 31.37f             | 21.54   |
| **Clove (Clo)**     | 100        | 18.85e       | 23.52d             | 22.21   | -3.09  |
|                     | 200        | 19.20c       | 16.51c             | 15.85   | 26.42  |
|                     | 300        | 20.91f       | 13.61b             | 14.22   | 33.99  |
| **Garlics (Gar)**   | 100        | 12.78b       | 32.27f             | 20.74   | 3.70   |
|                     | 200        | 12.06b       | 30.04f             | 18.11   | 15.92  |
|                     | 300        | 10.54abc     | 23.09de            | 12.29   | 42.96  |
| **Thyme (Thy)**     | 100        | 11.54b       | 29.97f             | 17.12   | 20.52  |
|                     | 200        | 13.67c       | 25.65e             | 17.49   | 18.80  |
|                     | 300        | 8.35a        | 26.61e             | 11.10   | 48.44  |
| **Cinnamon (Cin)**  | 100        | 13.13c       | 18.88cde           | 12.40   | 42.41  |
|                     | 200        | 13.38c       | 16.43c             | 10.98   | 49.04  |
|                     | 300        | 14.63cd      | 15.36bc            | 11.20   | 48.01  |
| **Mint (Min)**      | 100        | 14.61d       | 22.17de            | 16.21   | 24.75  |
|                     | 200        | 17.27c       | 19.56ed            | 16.85   | 21.78  |
|                     | 300        | 20.30f       | 15.16bce           | 15.40   | 28.49  |
| **Orange peels (Ora)** | 100   | 9.41ab       | 15.56bce           | 7.30    | 66.10  |
|                     | 200        | 11.93b       | 12.52b             | 7.48    | 65.28  |
|                     | 300        | 12.73b       | 9.16a              | 5.85    | 72.83  |

P < 0.05

| EO      | Doses | EO*Doses |
|---------|-------|----------|
| ****    | **    | **       |

Where: ns = no significant, ** = very significant, MRP= methane reduction potent, same superscript letter in one column refer to not difference significantly

This experiment resulted that the cinnamon (Cin) EO’s at all doses could reduce methane production approximately similarly with 100 ppm, 200 ppm and 300 ppm (42.41; 49.04; 48.01) respectively, while other experiment was reported at 250 ppm dose [17]. The bioactive component of Cin EO’s which was used in this experiment has main component benzyl alcohol, cinnamaldehyde and propenoic acid. The component is non-phenolic phenylpropene, does exhibit antimicrobial activity by binding and inactive microbial enzymes [19]. Other phenolic components such as eugenol in clove (Clo) EO’s and thymol in thyme (Thym) EO’s had a greatest antimicrobial effect the hydroxyl group (-OH) in addition to being involved in the transport of ion across the plasma membrane [20], is also thought to be involved in the inactivation of microbial enzymes [19]. It was approved from this experiment using Clo EO’s 300 ppm and Thym EO’s 300 ppm reduced methane 33.99% and 48.44%. Limonene in orange peel (Ora) EO’s and alpha-pinene in mint oils are monoterpenes nonphenolic which appear to have limited antimicrobial activity compared to the phenolic monoterpenes (thymol). In contrast to the theory about...
limonene above, this experiment resulted that Ora EO’s had high methane reduction potential at doses 100, 200 and 300 ppm (66.10; 65.28; and 72.83) respectively. It could be explained by recovery of limonene exposure to rumen fluid after 24 hours was lower than 10% [21, 22]. It might be due to the interaction with the rumen microbe as well as acidity and could have an impact on methane production, but its effect lost after 24 hours.

3.2. In Vitro rumen digestibility values
Effect of EO’s at some doses is significantly different (P<0.05) in resulted in Vitro rumen digestibility values (Table 3). The highest value of in vitro rumen true digestibility of dry matter (IVTDDM) was observed for using 300 ppm of clove EO’s (81.41%), while the lowest values of IVTDDM was using 100 ppm doses of thyme EO’s (68.13%). Some EO’s (Thyme 100, 200, 300 ppm and orange peel oils 200, 300 ppm) had lower values of IVTDDM than control.

Table 3. The Value of in vitro rumen true digestibility of Dry Matter (IVTDDM) and in vitro rumen NDF Digestibility of EO’s as doses 100, 200 and 300 ppm after 30 hours of incubation (dNDF30).

| Essential Oils (EO) | IVTDDM | dNDF30 |
|--------------------|--------|--------|
|                     | %DM    | SE     | %NDF  | SE     |
| Control             | 76.19b | 37.71bc|        |        |
| Garlic              |        |        |        |        |
| 100                 | 76.34b | 1.33   | 38.10bc| 1.33   |
| 200                 | 75.31b | 4.74   | 35.40bc| 4.74   |
| 300                 | 77.17b | 2.35   | 40.27c | 2.35   |
| Thyme               |        |        |        |        |
| 100                 | 68.13a | 7.89   | 16.62a | 7.89   |
| 200                 | 68.29a | 2.21   | 17.05a | 2.21   |
| 300                 | 68.56a | 3.70   | 17.74a | 3.70   |
| Clove               |        |        |        |        |
| 100                 | 75.02b | 9.62   | 34.64bc| 9.62   |
| 200                 | 78.83c | 2.56   | 44.62cd| 2.56   |
| 300                 | 81.41c | 3.70   | 51.37d | 0.88   |
| Orange Peel         |        |        |        |        |
| 100                 | 72.87ab| 7.19   | 29.02b | 7.19   |
| 200                 | 66.57a | 2.25   | 12.54a | 2.25   |
| 300                 | 67.36a | 4.10   | 14.61a | 4.10   |
| Mint                |        |        |        |        |
| 100                 | 78.26c | 6.42   | 43.11cd| 6.42   |
| 200                 | 73.26ab| 4.06   | 30.06b | 4.06   |
| 300                 | 73.15ab| 9.49   | 29.76b | 9.49   |
| Cinnamon            |        |        |        |        |
| 100                 | 75.88b | 6.38   | 36.91bc| 6.38   |
| 200                 | 78.86c | 8.91   | 44.69cd| 8.91   |
| 300                 | 79.12c | 4.99   | 45.37cd| 4.99   |

Where: EO = essential oils, ** = significantly different (P <0.05), Same letter in same column indicated for not significant

Decreasing values of IVTDDM of control due to decreasing of gas production which had also low in vitro digestibility values from incubation gas technique test. Carvacrol and thymol founded in thyme (Thymus ssp) were reported had a strong antimicrobial activity against a wide range of gram-positive
and negative bacteria [23]. It might have negative effect of thymol in thyme and limonene in orange peel oils caused to unbalanced rumen microorganism including the microorganism for nutrient digestibility in rumen (protozoa, cellulose bacterial or others).

Some doses of EO’s (garlic 100, 200, 300 ppm; clove 100 ppm; orange peel 100 pm; mint 200, 300 ppm and cinnamon 100 ppm) had no effect both on IVTDDM and NDF digestibility (dNDF). Orange peel oils (200, 300 ppm) which had high effect for reducing methane production (> 50%) due to low IVTDDM and dNDF as negative effect of unbalance rumen microbial in rumen.

3.3. General result effect of EO on In Vitro rumen methane production and digestibility

General result of this experiment showed that all doses 300 ppm of EO had positive effect on in vitro rumen gas production, rumen methane reduction and digestibility except thyme oil (Table 4). The best using garlic EO’s which had high effect to reduce in vitro rumen production and increased organic matter digestibility. Some researcher explained that active compound of garlic such as Allycin diallilsulfide may increase dry matter digestibility in vitro [24,25] and NDF digestibility [18], decreasing methane in vitro [14,26], inhibit deamination in rumen [27] and decreasing total VFA production [28]. Garlic oil also decreased in vitro methane gas production due to reducing effect of protozoa [18]. Other doses of EO also had positive effect on in vitro gas production, digestibility and reducing methane gas production except thyme which it could not keep in vitro digestibility at least similar with control. Orange peel EO’s was determined has strong effects for highest methane reduction potential but had negative effect on digestibility at doses 300 ppm.

| Essential Oils    | Doses | GP₄₈ | CH₄ | OMD | IVTDDM | dNDF |
|------------------|-------|------|-----|-----|--------|------|
| Garlic           | 100   | +    | =   | +   | =      | =    |
|                  | 200   | +    | -   | +   | =      | =    |
|                  | 300   | +    | -   | =   | =      | =    |
| Thyme            | 100   | =    | -   | =   | =      | -    |
|                  | 200   | =    | -   | =   | =      | -    |
|                  | 300   | -    | -   | =   | =      | -    |
| Clove            | 100   | +    | =   | +   | =      | =    |
|                  | 200   | +    | -   | +   | =      | =    |
|                  | 300   | +    | -   | +   | =      | =    |
| Orange Peel      | 100   | =    | -   | =   | =      | =    |
|                  | 200   | =    | -   | =   | =      | -    |
|                  | 300   | +    | -   | =   | =      | -    |
| Mint             | 100   | +    | -   | =   | =      | =    |
|                  | 200   | =    | -   | +   | =      | =    |
|                  | 300   | -    | -   | +   | =      | =    |
| Cinnamon         | 100   | +    | -   | =   | =      | =    |
|                  | 200   | +    | -   | =   | =      | =    |
|                  | 300   | +    | -   | =   | =      | =    |

Where = “=” same with control, “+”, increase from control, “−”, decrease from control, (“−−” more than 50% decrease).
4. Conclusion
All the essential oils in this study able to reduce in vitro rumen methane production at 6 h incubation compared to control at 300 ppm, and there was no negative effect on In Vitro Digestibility. This result would be important to decide using EO’s as feed additive which are optimum for environment also increasing productivity. There are some recommendations for future strategy using EO’s as feed additive assessed by in vitro and in vivo test with different doses and combination. The EO for feed additive could be marketable because of environment friendly and profitable.

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