

Rumen modulatory effect of thyme, clove and peppermint oils *in vitro* using buffalo rumen liquor

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Abstract

Aim: The present study was conducted to examine the rumen modulatory effect of thyme, clove and peppermint oils on rumen fermentation pattern *in vitro* using roughage based diet.

Materials and Methods: Thyme, clove and peppermint oils were tested at concentration of 0, 30, 300 and 600 mg/l (ppm) of total culture fluid using *in vitro* gas production technique in wheat straw based diet (concentrate: Wheat straw 50:50). Different *in vitro* parameters e.g., total gas production, methane production, nutrient degradability, volatile fatty acid (VFA) production and ammonia nitrogen concentration were studied using buffalo rumen liquor.

Results: Thyme oil at higher dose level (600 ppm) reduced ($p < 0.05$) total gas production, feed degradability and ammonia nitrogen ($\text{NH}_3\text{-N}$) concentration whereas total VFA concentration was significantly lower ($p > 0.05$) in 300 and 600 ppm dose levels. 600 ppm dose level of clove oil reduced ($p < 0.05$) total gas production, feed degradability, total VFA and acetate to propionate ratio. Methane production was significantly reduced ($p < 0.05$) in 300 and 600 ppm dose levels of clove and peppermint oil.

Conclusion: Right combination of these essential oils may prove to enhance performance of animals by reducing methane production and inhibiting protein degradation in rumen.

Keywords: ammonia nitrogen, essential oil, rumen fermentation, methane, wheat straw.

Introduction

Appearance of residue and resistant strain of bacteria, acceptance of antibiotics as growth promoter and rumen fermentation modulator is gradually reducing or totally banned in some countries. Essential oils (EOs) are emerging as a potent alternative of feed additive due to its natural availability. They are plant secondary metabolites and present in some spice, condiments and different parts of plants. The word "EOs" has come from "essence," which means sweet fragrance. Thymol, carvacol, eugenol, limonene, allicin, diallyldisulfide are the active compounds present in the Eos, which are responsible for the odour.

Some EOs has antimicrobial activities [1] and is currently considered safe for human and animal consumption, and termed as generally recognized as safe or GRAS [2]. Several workers reported promising effect of different EOs in modulating rumen function *in vitro* [3-5]. Potentiality of different EOs and their combination have also been studied in different ruminant species [6-9] where most of the workers reported positive results due to supplementation. A total of 6-8% of Gross energy of the feed is reported to be lost

by methane. In addition, ruminant livestock contributes about 80 million metric tons of methane annually, accounting for about 28% of global methane emissions from human-related activities [10].

However, most of the studies were conducted using some patented EOs or their combination. Due to paucity of studies of effect of different EOs on rumen fermentation parameters *in vitro* in Indian condition, present study was conducted to observe the effect of thyme, clove and peppermint oil on rumen fermentation pattern *in vitro* using buffalo rumen liquor in straw based diet.

Materials and Methods

Ethical approval

Ethical approval for fistulation of buffalo bull and collection of rumen liquor from those bulls was taken from Institutional Animal Ethics Committee constituted as per the article no. 13 of the CPCSEA rules laid down by Government of India.

Animal feeding and sample analysis

Rumen liquor was collected from two donors fistulated buffalo bull maintained by the herd of National Dairy Research Institute, Karnal, Haryana, India. Fistulation of buffalo bull was performed by surgeon as per regulation of Institutional Animal Ethics Committee. Rumen liquor was collected before morning feeding into a pre-warmed thermo-flask and brought to the laboratory. Donor animals were fed on

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wheat straw and concentrate based diet (3.0 kg concentrate mixture and wheat straw ad libitum).

Source of EOs

Thyme, clove and peppermint oils standards were supplied by Sigma-Aldrich chemicals Pvt. Limited (USA). Each EO was diluted to prepare three dilutions, i.e., 30, 300 and 600 ppm in 30 ml of incubation medium from standard supplied by Sigma-Aldrich chemicals Pvt. Limited.

Proximate analyses of substrate

Diet samples were collected and analyzed for dry matter (DM) (ID number 930.15), organic matter (OM) and ash (ID number 942.05) and crude protein ($N \times 6.25$, ID number 954.01), ether extract (ID number 920.39) contents, in accordance with the AOAC [11]. The neutral detergent fiber and acid detergent fiber analysis were based on the procedures described by Van Soest and Robertson [12].

In vitro gas and methane production

In vitro gas production was analyzed following the methodology adapted by Menke and Steingass [13]. 0.2 g of wheat straw (*Triticum aestivum*) and concentrate mixture (50:50 ratio) were used as substrates and mulberry leaves (*Morus alba*) as an internal standard. Concentrate mixture was consist of maize 33%, groundnut cake (oiled) 21%, mustard oil cake (oiled) 12%, wheat bran 20%, deoiled rice bran 11%, mineral mixture 2% and common salt 1%. Chemical composition of concentrate mixture and wheat straw used in the experiment is presented in Table-1. Gas production in blank (containing inoculum) and test syringe (containing inoculum and substrate) were measured after 24 h. The gas produced in standard syringe (containing mulberry leaves) was used to check the day to day variation of inoculum. Methane was estimated by using Nucon-5700 gas chromatograph (GC) equipped with flame ionization detector and stainless steel column packed with Porapak-Q. The standard gas used for methane estimation (Spantech Calibration gas, Surrey, England) composed of 50% methane and 50% CO₂. The methane produced from the substrate during 24 h incubation was corrected for the blank values. The volume of CH₄ (ml) produced was calculated as follows;

Methane production (ml) = Total gas produced (ml) × % methane in the sample.

In vitro degradability of feed, ammonia- N (NH₃-N) and volatile fatty acid (VFA) estimation

True DM and OM degradability was determined by transferring the content of each syringe quantitatively into a centrifuge tube and centrifuged

at 5000 rpm for 15 min. True degradability was estimated after 24 h incubation as per the method outlined by Goering and Soest [14]. Truly degradable OM in rumen (TDOMR) was estimated by subtracting incubated OM with substrate recovered as residue after ND solution treatment. However, partitioning factor was calculated as the ratio of TDOMR (mg) to gas volume (ml) produced during 24 h incubation. For NH₃-N estimation, 5 ml of supernatant was mixed with 2 ml of 1 N NaOH and steam distilled using KEL PLUS - N analyzer (Pelican, India). Released NH₃-N was trapped in boric acid solution having mixed indicator and titrated against 0.01N H₂SO₄ [15]. Total VFA concentration was estimated as per method described by Barnett and Reid [16]. For the estimation of individual VFAs, 5 ml of supernatant was treated with 1 ml meta-phosphoric acid (25%) and kept overnight at 4°C. Different VFA's of the samples were identified using GC (Nucon 5700, India) on the basis of their retention time and their concentration (mmol) was calculated by comparing the retention time as well as the peak area of standards after deducting the corresponding blank values [17].

Statistical analysis

The generated data were statistically analyzed by analysis of variance considering dose of EOs as factor using the general linear model procedure (univariate) according to $Y_{ij} = \mu + D_i + e_{ij}$ with Y_{ij} as the studied parameter (j^{th} observation on the i^{th} treatment), μ as population mean, D_i as effect of the EOs (effect of the i^{th} group) and e_{ij} as residual error associated with the ij^{th} observation. Means were compared using Tukey's HSD test [18]. Significant differences were accepted if $p > 0.05$. All statistical analyses were performed using SPSS 19 [19].

Results and Discussion

Proximate principle and fibre fraction of concentrate mixture and wheat straw used in the experiment is presented in Table-1. The values of nutrient composition are comparable with the findings of Ranjhan [20] and Ayyappan *et al.* [21]. 600 ppm dose level of thyme and clove oil resulted in a decrease ($p < 0.05$) in gas production, OM and DM degradability (Tables-2 and -3). Thyme oil at 300 and 600 ppm reduced total VFA concentration by 20.2 and 40.4%, respectively (Table-2), compared with control. Whereas, relative to control, the concentration of total VFA was reduced by 14.7% at 600 ppm dose rate of clove oil (Table-3). The main active compounds of thyme and clove oil are thymol and eugenol, respectively. Overall decrease in rumen fermentation in high dose level of thyme and clove

Table-1: Chemical compositions (%DM basis) of substrate (concentrate mixture and wheat straw).

| | DM | OM | Crude protein | Ether extract | Crude fiber | NFE | Total Ash | NDF | ADF |
|---------------------|------|------|---------------|---------------|-------------|------|-----------|------|------|
| Concentrate mixture | 90.1 | 92.8 | 21.5 | 3.1 | 6.6 | 61.5 | 7.7 | 30.7 | 12.1 |
| Wheat straw | 90.4 | 89.4 | 3.6 | 0.9 | 38.1 | 47.8 | 11.3 | 83.6 | 50.1 |

NFE=Nitrogen free extract, NDF=Neutral detergent fibre, ADF=Acid detergent fibre, OM=Organic matter, DM=Dry matter

Table-2: Effect of different levels of thyme oil on *in vitro* fermentation parameters (mean±SD) in wheat straw based (concentrate: Roughage=50:50) diet.

| Attributes | Control | 30 ppm | 300 ppm | 600 ppm | p value |
|--------------------------------------|--------------------------|--------------------------|---------------------------|--------------------------|---------|
| Total gas (ml/24 h/200 mg substrate) | 36.00 ^a ±1.65 | 35.50 ^a ±1.54 | 34.83 ^a ±1.75 | 23.16 ^b ±1.30 | <0.01 |
| True OM degradability (%) | 65.13 ^a ±2.17 | 62.08 ^a ±2.55 | 61.90 ^a ±3.65 | 51.30 ^b ±5.11 | <0.01 |
| True DM degradability (%) | 67.16 ^a ±2.84 | 65.33 ^a ±3.67 | 66.50 ^a ±1.73 | 55.16 ^b ±4.64 | <0.01 |
| Methane (ml/24 h) | 8.10±3.48 | 10.11±2.25 | 10.67±1.82 | 7.07±1.21 | >0.05 |
| Methane (ml/g OMD) | 67.08±28.86 | 87.73±19.57 | 93.00±15.99 | 74.33±12.75 | >0.05 |
| Methane (ml/g DMD) | 60.33±25.95 | 77.42±17.26 | 80.28±13.79 | 64.10±10.99 | >0.05 |
| Ammonia nitrogen (mg/100 ml) | 32.28 ^a ±1.80 | 31.42 ^a ±1.54 | 27.99 ^{ab} ±1.93 | 24.90 ^b ±3.17 | 0.01 |
| Total VFAs (mmol/100 ml) | 51.3 ^a ±1.87 | 48.27 ^a ±1.85 | 40.94 ^b ±1.38 | 30.59 ^c ±1.96 | 0.01 |
| VFA (molar proportion) | | | | | |
| Acetate | 56.28±2.92 | 57.91±3.21 | 58.23±10.68 | 56.24±3.47 | >0.05 |
| Propionate | 22.30±1.57 | 20.32±1.92 | 20.37±1.26 | 21.65±0.89 | >0.05 |
| Butyrate | 6.37±1.15 | 6.47±1.14 | 7.77±0.73 | 10.18±3.22 | >0.05 |
| Partitioning factor | 3.35 ^a ±0.14 | 3.24 ^a ±0.10 | 3.33 ^{ab} ±0.45 | 4.10 ^b ±0.33 | 0.02 |

Different superscript a, b, c in a row differ significantly (p<0.05). OM=Organic matter, DM=Dry matter, OMD=Organic matter digested, DMD=Dry matter digested, VFA=Volatile fatty acid

Table-3: Effect of different levels of clove oil on *in vitro* fermentation parameters (mean±SD) in wheat straw based (Concentrate: Roughage=50:50) diet.

| Attributes | Control | 30 ppm | 300 ppm | 600 ppm | p value |
|--------------------------------------|--------------------------|--------------------------|---------------------------|--------------------------|---------|
| Total gas (ml/24 h/200 mg substrate) | 41.33 ^a ±1.52 | 41.20 ^a ±3.86 | 37.33 ^{ab} ±2.36 | 29.33 ^b ±5.01 | <0.01 |
| True OM degradability (%) | 68.72 ^a ±3.27 | 69.98 ^a ±1.71 | 64.05 ^{ab} ±3.41 | 59.56 ^b ±2.84 | <0.01 |
| True DM degradability (%) | 70.16 ^a ±1.44 | 71.66 ^a ±0.76 | 63.83 ^{ab} ±5.33 | 59.16 ^b ±3.24 | <0.01 |
| Methane (ml/24 h) | 12.38 ^a ±0.48 | 12.80 ^a ±0.19 | 10.86 ^b ±0.92 | 9.09 ^c ±0.57 | <0.01 |
| Methane (ml/g OMD) | 99.22 ^a ±3.97 | 99.67 ^a ±1.42 | 95.17 ^{ab} ±8.14 | 86.99 ^b ±5.46 | 0.03 |
| Methane (ml/g DMD) | 88.23 ^a ±3.52 | 89.34 ^a ±1.28 | 85.09 ^{ab} ±7.27 | 76.81 ^b ±4.81 | 0.02 |
| Ammonia nitrogen (mg/100 ml) | 18.89±1.75 | 17.34±0.54 | 17.51±0.43 | 18.63±2.22 | >0.05 |
| Total VFAs (mmol) | 47.4 ^a ±1.45 | 47.62 ^a ±0.56 | 46.30 ^a ±0.72 | 40.44 ^b ±0.69 | 0.04 |
| VFA (molar proportion) | | | | | |
| Acetate | 52.13 ^a ±1.07 | 50.41 ^a ±0.65 | 49.80 ^a ±1.82 | 42.73 ^b ±1.24 | 0.04 |
| Propionate | 20.41 ^a ±0.48 | 21.85 ^a ±1.10 | 22.52 ^a ±1.50 | 25.26 ^a ±0.60 | 0.03 |
| Butyrate | 8.46±0.54 | 8.74±1.05 | 7.69±1.06 | 9.00±1.53 | >0.05 |
| Partitioning factor | 3.08±0.05 | 3.17±0.28 | 3.18±0.23 | 3.82±0.54 | >0.05 |

Different superscript a, b, c in a row differ significantly (p<0.05). OM=Organic matter, DM=Dry matter, OMD=Organic matter digested, DMD=Dry matter digested, VFA=Volatile fatty acid, SD=Standard deviation

oil may be due to their active principles (thymol and eugenol, respectively), which are more effective antimicrobials in comparison with other non-phenolic secondary plant metabolites because of the presence of a hydroxyl group in the phenolic structure and results into loss of integrity of bacterial cell membrane which ultimately resulted in reduction in glucose uptake by bacteria [22-25]. Reduction in total VFA concentration was also reported other workers using thyme and clove oil *in vitro* [26,27]. Peppermint oil did not adversely affect feed degradability in all the dose level, which is similar to the findings of Craig [28]. Methane concentration was reduced by 16.6 and 18.1% in 300 and 600 ppm of peppermint oil compared with control, whereas 12.3 and 26.6% reduction in methane concentration was found in clove oil in above dose levels, respectively (Tables-3 and-4). Present finding corroborate the observation of Agarwal *et al.* [29] who reported reduction in methane production by 19.9%, 46.0% and 75.6% at 0.33, 1.0 and 2.0 µl of peppermint oil. It may be postulated that the reduction of methane was due to the reduction of total protozoa [30] by menthol (active principle of peppermint oil). Similar to our

finding, Patra *et al.* [31] reported inhibition of methane production with reduced digestibility of the feed *in vitro* with clove oil. The concentration of NH₃-N was reduced (p<0.05) in high dose level (600 ppm) of thyme oil (Table-2). Reduction (p<0.05) in acetate to propionate ratio was observed in high (600 ppm) dose level of clove oil. Reduction in concentration of NH₃-N is suggesting the potentiality of thymol in inhibiting deamination and corroborating the study of past workers [26,32] who found a similar result when incubated rumen fluid with thymol *in vitro*. Evans and Martin [33] in their study in pure culture reported that thymol affected the energy metabolism of two major rumen deaminating bacteria, *Streptococcus bovis* and *Selenomonas ruminantium*. Corroborating the study of Busquet *et al.* [27], present study also reported reduction of acetate propionate ratio in high dose level of clove oil which could be a good indicator of simultaneous methane reduction in the rumen [34].

Conclusion

Higher levels of tested EOs were detrimental to rumen microbes reflecting reduction in total gas

Table-4: Effect of different levels of peppermint oil on *in vitro* fermentation parameters (mean±SD) in wheat straw based (Concentrate: Roughage=50:50) diet.

| Attributes | Control | 30 ppm | 300 ppm | 600 ppm | p value |
|--------------------------------------|---------------------------|----------------------------|---------------------------|---------------------------|---------|
| Total gas (ml/24 h/200 mg substrate) | 36.83±1.59 | 34.83±2.87 | 31.33±6.65 | 37.66±3.05 | >0.05 |
| True OM degradability (%) | 47.70±3.76 | 48.57±2.03 | 50.27±3.29 | 50.22±2.42 | >0.05 |
| True DM degradability (%) | 48.16±1.83 | 50.00±1.29 | 53.16±3.03 | 53.00±1.75 | >0.05 |
| Methane (ml/24 h) | 12.46 ^a ±1.40 | 11.21 ^{ab} ±0.19 | 10.35 ^b ±0.02 | 10.21 ^b ±0.87 | <0.01 |
| Methane (ml/g OMD) | 144.12 ^a ±0.72 | 130.22 ^{ab} ±0.33 | 115.38 ^b ±0.78 | 113.94 ^b ±0.62 | <0.01 |
| Methane (ml/g DMD) | 128.36 ^a ±1.30 | 113.10 ^{ab} ±1.54 | 96.34 ^b ±1.06 | 95.32 ^b ±1.00 | <0.01 |
| Ammonia nitrogen (mg/100 ml) | 24.06±1.37 | 24.16±2.18 | 25.18±1.13 | 22.76±1.56 | >0.05 |
| Total VFAs (mmol) | 38.57±3.85 | 39.96±1.40 | 44.3±5.22 | 44.82±5.01 | >0.05 |
| VFA (molar proportion) | | | | | |
| Acetate | 57.00±5.67 | 61.23±3.05 | 55.03±4.30 | 55.70±4.62 | >0.05 |
| Propionate | 21.82±3.92 | 20.25±2.08 | 22.05±3.21 | 25.58±3.59 | >0.05 |
| Butyrate | 8.54±1.07 | 9.43±2.31 | 11.51±3.32 | 11.55±1.66 | >0.05 |
| Partitioning factor | 2.33 ^a ±0.12 | 2.51 ^a ±0.26 | 2.89 ^b ±0.17 | 2.40 ^a ±0.21 | 0.02 |

Different superscript a, b, c in a row differ significantly ($p < 0.05$). OM=Organic matter, DM=Dry matter, OMD=Organic matter digested, DMD=Dry matter digested, VFA=Volatile fatty acid

production and degradability of feed. Clove and peppermint oil at higher dose rate reduce methane production. Concentration of $\text{NH}_3\text{-N}$ was decreased at 600 ppm dose rate of thyme oil due to its selective inhibition of certain deaminating bacteria that may enhance the bypass activity of feed protein. Judicial selection and a careful combination of these EOs allows manipulation of rumen fermentation in a favorable direction.

Authors' Contributions

DR planned and carried out research work to compare different EOs *in vitro* for his Ph.D. thesis programme in collaboration with advisory members and guide SKT. VK helped DR in setting overall *in vitro* experiment. All authors participated in draft and revision of the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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