Veterinary World, EISSN: 2231-0916
Available at www.veterinaryworld.org/Vol.7/April-2014/5.pdf

RESEARCH ARTICLE
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# Efficacy of different essential oils in modulating rumen fermentation *in vitro* using buffalo rumen liquor

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Received: 25-01-2014, Revised: 03-03-2014, Accepted: 10-03-2014, Published online: 07-04-2014

doi: 10.14202/vetworld.2014.213-218
How to cite this article: Roy D, Tomar SK, Sirohi SK, Kumar V and Kumar M (2014) Efficacy of different essential oils in modulating rumen fermentation *in vitro* using buffalo rumen liquor, *Veterinary World* 7(4): 213-218.

## Abstract

**Aim:** Present study was conducted to examine the modulatory effect of different essential oils on rumen fermentation pattern *in vitro* using wheat straw based diet (concentrate: wheat straw 50:50).

**Materials and Methods:** Four essential oils i.e. cinnamon, garlic, oregano and rosemary oils were tested at concentration of 0, 30, 300 and 600 mg/litre (ppm) of total culture fluid using *in vitro* gas production technique. Total gas production, methane production, nutrient degradability, volatile fatty acid (VFA) production and ammonia nitrogen concentration were studied *in vitro* using buffalo rumen liquor.

**Results:** Results indicated that all four essential oils decreased gas production significantly ($P<0.05$) at 600ppm concentration. However, in case of garlic oil, 300 ppm concentration was also found to be effective in decreasing total gas production. Reduction in methane production was found maximum ($P<0.05$) at higher doses in most of the oils. Maximum reduction in methane was noticed with garlic oil at 600ppm dose. Ammonia-N concentration was also decreased significantly ($P<0.05$) with essential oils and was found minimum with oregano oil at 600 ppm dose. Partition factor was found to be significantly ($P<0.05$) higher in 600 ppm concentration of garlic and oregano oil. The degradability of dry matter decreased significantly with higher concentration of essential oil in most of treatment combinations.

**Conclusion:** Supplementation with different essential oils on wheat straw based diet modulates rumen fermentation and reduced methane and ammonia- N production and improved utilization of nutrients.

**Keywords:** ammonia nitrogen, *in vitro*, essential oils, methane, rumen fermentation.

## Introduction

The use of antibiotics as animal feed additive is facing reduced social acceptance due to the appearance of residues and resistant strains of bacteria. As alternatives, plants and their extracts are attractive with consumer opinion that most things 'natural' are good. Essential oils (EO) are plant secondary metabolites and the word “Essential oils” has come from “essence” which means sweet fragrance. The odour of EOs is due to the presence of active compounds (thymol, carvacol, eugenol, limonene, allicin, diallyldisulphide etc.). Some EOs have antimicrobial activities and are currently considered safe for human and animal consumption, and are categorized as Generally Recognized as Safe (GRAS) [1]. Potential use in the diet of ruminants has been reviewed recently [2, 3]. A number of researchers have conducted studies using some patented EO combinations got promising result both *in vitro* [4-7] and *in vivo* [8-15]. Use of essential oil as antimicrobial substances has also been studied [16].

Due to paucity of studies on the effect of individual EO on rumen fermentation pattern in Indian condition, present study was conducted to see the effect of different EO on rumen fermentation *in vitro* in buffaloes on wheat straw based diet.



## Materials and Methods

Ethical approval: Rumen liquor was collected from two donor fistulated Buffalo bull maintained by the herd of National Dairy Research Institute (NDRI), Karnal, Haryana, India. Fistulation of Buffalo bull was performed by surgeon as per regulation of Institutional Animal Ethics committee constituted as per the article no. 13 of the CPCSEA rules laid down by Government of India.

Source of essential oils: Cinnamon, garlic, oregano, rosemary oils 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 30ml of incubation medium from standard supplied by Sigma-Aldrich chemicals Pvt. Limited.

Animal feeding and sample analysis: Rumen liquor was collected from donor animals fitted with permanent rumen fistula, before morning feeding into a pre-warmed thermo-flask and brought to the laboratory. Donor animals were fed on wheat straw and concentrate based diet (3.0 kg concentrate mixture and

wheat straw *ad libitum*).

Proximate analyses of substrate: Dry matter (DM) (ID number 930.15), Organic matter (OM) and ash (ID number 942.05) and Crude protein (CP) (N×6.25, ID number 954.01), Ether extract (EE) (ID number 920.39), Neutral detergent fibre (NDF) and Acid detergent fibre (ADF) of concentrate mixture and wheat straw were determined by standard procedures [17, 18].

*In vitro* gas production test: The incubations were carried out in 100 ml graduated glass syringes [19]. 200 mg of concentrate mixture and wheat straw (Triticum aestivum) in 50:50 ratio were used as substrates and mulberry leaves (Morus alba) as internal standard. Concentrate mixture consists 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%. The rumen liquor was bubbled with $CO_2$ for about 2 minutes and filtered through 4 layers of muslin cloth. After medium became colorless, the required amount of strained rumen liquor (SRL) was added. The ratio of medium to rumen liquor was 2:1. Corresponding dose of EO were injected in specified syringes before injecting inoculums. Each treatment was incubated in triplicate. 30 ml of incubation medium was injected to each syringe using auto dispenser. The syringes were shaken gently and residual air or air bubble, if any, was removed and the outlet was closed. The level of piston was recorded and the syringes were placed in an incubator (39 ± 0.5°C). The syringes were shaken every 30 minutes for first 2 h from the start of the incubation and thereafter every 2 h up to 24 h of incubation.

Estimation of total gas and methane production: Gas production in blank (containing inoculum) and test syringe (containing inoculum and substrate) was measured after 24 h. The gas produced in standard syringe (containing mulberry leaves) was used to examine day to day variation in the quality of inoculum.

Estimation of methane production: Methane ($CH_4$) was estimated using Nucon-5700 gas chromatograph equipped with flame ionization detector (FID) and stainless steel column packed with Porapak-Q. For this 3 ml gas was sampled from the headspace of syringe in an airtight syringe and injected into Nucon-5700 gas chromatograph. The standard gas used for methane estimation (Spantech Calibration gas, Surrey, England) composed of 50% methane and 50% $CO_2$. The peak of methane gas was identified on the basis of retention time of standard methane gas and the response factor obtained was used to calculate methane percentage in the gas sample. The methane produced from the substrate during 24 h incubation was corrected for the blank values. The volume of $CH_4$ (ml) produced was calculated as follows;

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

Degradability of feed: For the determination of true DM and OM degradability, the content of each syringe was transferred quantitatively into centrifuge tube and centrifuged at 5000 rpm for 15 min. True degradability was estimated after 24 h incubation as per the standard procedure [20]. Truly degradable organic matter in rumen (TDOMR) was calculated as the amount of substrate OM incubated minus the amount of substrate recovered as residue after ND solution treatment, and the partitioning factor (PF) was calculated as the ratio of TDOMR (mg) to gas volume (ml) produced from it during 24 h of incubation.

Ammonia-N concentration ($NH_3$-N): 5 ml of supernatant was mixed with 1 N NaOH (2 ml) and steam distilled using KEL PLUS - N analyzer (Pelican, India) and the $NH_3$-N evolved was collected in boric acid solution having mixed indicator and titrated against 0.01N $H_2SO_4$. $NH_3$-N concentration was calculated as per standard procedure [21].

Volatile fatty acid (VFA) estimation: For total VFA estimation [22], one ml of supernatant was taken into Markham's distillation apparatus and steam distilled with 1 ml of oxalate buffer containing 10% potassium oxalate and 5 percent oxalic acid. About 100 ml of distillate was collected and then titrated against standard 0.01N sodium hydroxide solution. Phenolphthalein was used as indicator, which gave light pink colour as an end point. For the estimation of individual VFAs, a 5 ml of supernatant was treated with 1 ml meta- phosphoric acid (25%) and kept overnight at 4°C [23]. Thereafter, it was centrifuged at 3000 rpm for 10 min and used for the VFA estimation using gas chromatograph (Nucon 5700, India) equipped with flame ionization detector (FID) and stainless steel column (length 4'; o.d ¼ "; i.d 3 mm) packed with Chromosorb – 101. Temperature of injection port, column and detector was set at 200, 180 and 210°C, respectively. The flow rate of carrier gas (nitrogen) through the column was 40 ml/min; and the flow rate of hydrogen and air through FID was 30 and 300ml/ min, respectively. Sample (2µl) was injected through the injection port using Hamilton syringe (10µl). Different VFA's of the samples were identified 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.

Statistical analysis: The generated data were statistically analyzed by one way ANOVA considering dose of essential oil as factor using the general linear model procedure (univariate) according to $Yij = \mu + Di + e_{ij}$ with *Yij* as the studied parameter ($j^{th}$ observation on the $i^{th}$ treatment), µ as population mean, *Di* as effect of the essential oil (effect of the $i^{th}$ group) and $e_{ij}$ as residual error associated with the $ij^{th}$ observation. Means were compared using Tukey's test [24]. Significant differences were accepted if $P < 0.05$. All statistical

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

| Substrate | Dry matter | Organic matter | 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

Table-2: Effect of different levels of cinnamon oil on in vitro fermentation in wheat straw based (Concentrate: Roughage= 50:50) diet

| Attributes | Control | 30ppm | 300ppm | 600ppm | SEM[1] | P Value |
|---|---|---|---|---|---|---|
| Total gas (ml/24h/200mg substrate) | 38.1 | 41.6 | 38.0 | 34.1 | 1.8 | 0.12 |
| True OM degradability (%) | $64.9^{ab}$ | $70.7^{a}$ | $61.3^{b}$ | $62.9^{ab}$ | 1.6 | 0.03 |
| True DM degradability (%) | $66.8^{abc}$ | $73.5^{a}$ | $63.5^{bc}$ | $63.0^{c}$ | 1.7 | 0.01 |
| $CH_4$ (ml/24h) | 13.3 | 13.8 | 13.5 | 12.6 | 0.3 | 0.22 |
| $CH_4$ (ml/g OMD) | 110.7 | 105.6 | 118.9 | 108.2 | 3.0 | 0.06 |
| $CH_4$ (ml/g DMD) | 99.7 | 94.2 | 106.5 | 100.4 | 2.7 | 0.07 |
| $NH_3$-N (mg/100 ml) | $23.5^{ac}$ | $25.2^{a}$ | $21.9^{bc}$ | $22.1^{c}$ | 0.5 | 0.02 |
| Total VFA (mM/100 ml) | $28.3^{a}$ | $41.5^{b}$ | $43.7^{b}$ | $45.5^{b}$ | 0.8 | <0.01 |
| VFA (molar proportion) | | | | | | |
| Acetate | $52.2^{a}$ | $59.3^{b}$ | $59.8^{b}$ | $59.5^{b}$ | 0.5 | 0.03 |
| Propionate | 26.3 | 23.5 | 22.4 | 24.0 | 0.3 | 0.14 |
| Butyrate | 9.6 | 9.0 | 9.8 | 8.7 | 0.7 | 0.07 |
| Partitioning factor | 3.2 | 3.1 | 3.1 | 3.4 | 0.1 | 0.54 |

Different superscript $^{a,b,c}$ in a row differ significantly ($P < 0.05$). [1]SEM = Standard error of the means, OMD= Organic matter degraded, DMD= Dry matter degraded, VFA= Volatile fatty acid.

Table-3: Effect of different levels of garlic oil on *in vitro* fermentation in wheat straw based (Concentrate: Roughage= 50:50) diet

| Attributes | Control | 30ppm | 300ppm | 600ppm | SEM[1] | P Value |
|---|---|---|---|---|---|---|
| Total gas (ml/24h/200mg substrate) | $38.6^{a}$ | $31.6^{a}$ | $19.3^{b}$ | $19.3^{b}$ | 2.2 | <0.01 |
| True OM degradability (%) | $65.8^{ab}$ | $68.2^{a}$ | $54.5^{b}$ | $57.7^{ab}$ | 2.5 | 0.02 |
| True DM degradability (%) | $65.8^{ab}$ | $67.0^{a}$ | $52.5^{b}$ | $58.6^{ab}$ | 2.9 | 0.02 |
| Ch4 (ml/24h) | $11.8^{a}$ | $7.5^{b}$ | $1.3^{c}$ | $0.1^{c}$ | 0.3 | <0.01 |
| Ch4 (ml/g OMD) | $97.0^{a}$ | $58.6^{b}$ | $13.3^{c}$ | $1.0^{d}$ | 2.6 | <0.01 |
| Ch4 (ml/g DMD) | $90.0^{a}$ | $56.2^{b}$ | $12.8^{c}$ | $0.9^{d}$ | 2.4 | <0.01 |
| NH3-N (mg/100 ml) | $22.0^{a}$ | $17.8^{ab}$ | $14.7^{b}$ | $20.2^{ab}$ | 1.2 | 0.04 |
| Total VFA (mM/100 ml) | $43.7^{a}$ | $37.5^{a}$ | $24.5^{b}$ | $23.1^{b}$ | 0.5 | 0.02 |
| VFA (molar proportion) | | | | | | |
| Acetate | $60.2^{a}$ | $59.5^{ab}$ | $56.3^{ab}$ | $55.9^{b}$ | 1.1 | 0.03 |
| Propionate | $20.3^{a}$ | $22.3^{ab}$ | $24.7^{b}$ | $25.8^{b}$ | 0.2 | 0.02 |
| Butyrate | 10.3 | 9.1 | 10.9 | 9.2 | 0.3 | 0.10 |
| Partitioning factor | $3.2^{a}$ | $4.0^{ab}$ | $5.3^{ab}$ | $5.8^{b}$ | 0.4 | 0.04 |

Different superscript $^{a,b,c}$ in a row differ significantly ($P < 0.05$). [1]SEM = Standard error of the means, OMD= Organic matter degraded, DMD= Dry matter degraded, VFA= Volatile fatty acid.

analyses were performed using standard software package [25].

## Results and Discussion

Chemical composition: Chemical compositions of concentrate mixture and wheat straw are given in Table-1. The DM, OM, CP, EE, CF, NFE and total ash contents are similar to the findings of earlier workers [26, 27]. The NDF and ADF contents of the substrates are close to the values reported by other worker [27].

Effect of essential oils on gas and methane production: Gas production remains unaffected in cinnamon oil supplemented groups (Table-2), whereas it reduced significantly ($P < 0.05$) in 600 ppm garlic and oregano oil treated groups (Table-3 and 4). In case of rosemary oil supplementation, gas production was found similar in control and treated groups (Table-2 and Table-5). Methane production per unit of DM and OM degradability was also significantly ($P<0.01$) reduced in all the concentration of garlic oil compared to control (Table-3).

With increase in dose rate, oregano oil decreased methane production significantly ($P<0.01$); whereas, rosemary oil reduced it significantly ($P<0.01$) at 30ppm dose rate, but no further decrease was found at higher doses (Table-5). The main compounds of oregano oil (*Origanum vulgare*) are carvacrol, thymol, α-and β-pinene, β-bisabolene, cineol, *p*-cimene, borenol, linalool, linalyl acetate, γ and α-terpinene, myrcene, diopenten, and β-caryophyllene whereas cinnamon contains cinnamaldehyde [7, 28, 29]. Among those, Carvacrolis is the major active compounds of oregano oil (average of 660 mg/g) [29]. Decrease in gas and methane production by higher dose level of oregano oil in the present study might be due to depressing effect on microbial fermentation [30]. Previous reports [4, 5]

Table-4: Effect of different levels of oregano oil on *in vitro* fermentation in wheat straw based (Concentrate: Roughage= 50:50) diet

| Attributes | Control | 30ppm | 300ppm | 600ppm | SEM[1] | P Value |
|---|---|---|---|---|---|---|
| Total gas (ml/24h/200mg substrate) | 36.0[a] | 34.0[a] | 31.8[a] | 11.6[b] | 2.1 | <0.01 |
| True OM degradability (%) | 64.9[a] | 66.0[a] | 60.9[a] | 53.9[b] | 1.0 | <0.01 |
| True DM degradability (%) | 64.6[a] | 65.2[a] | 52.2[b] | 56.0[b] | 0.9 | <0.01 |
| $Ch_4$ (ml/24h) | 11.5[a] | 10.4[a] | 10.6[bc] | 3.6[d] | 0.1 | <0.01 |
| $Ch_4$ (ml/g OMD) | 95.8[a] | 84.4[b] | 113.4[c] | 36.2[d] | 1.5 | <0.01 |
| $Ch_4$ (ml/g DMD) | 89.2[a] | 79.3[b] | 102.3[c] | 32.4[d] | 1.3 | <0.01 |
| $NH_3$-N (mg/100 ml) | 18.2[a] | 18.5[a] | 19.7[a] | 15.2[b] | 0.3 | <0.01 |
| Total VFA (mM/100 ml) | 51.4[a] | 47.3[a] | 55.3[a] | 17.7[b] | 0.9 | <0.01 |
| **VFA (molar proportion)** | | | | | | |
| Acetate | 57.2[a] | 56.9[a] | 55.9[a] | 52.4[b] | 1.7 | 0.03 |
| Propionate | 23.7 | 23.6 | 24.6 | 24.5 | 0.5 | 0.05 |
| Butyrate | 10.0 | 8.3 | 10.7 | 11.5 | 0.6 | 0.09 |
| Partitioning factor | 3.3[a] | 3.6[a] | 3.0[a] | 8.8[b] | 0.4 | <0.01 |

Different superscript [a,b,c] in a row differ significantly (P < 0.05). [1]SEM = Standard error of the means, OMD= Organic matter degraded, DMD= Dry matter degraded, VFA= Volatile fatty acid.

Table-5: Effect of different levels of rosemary oil on *in vitro* fermentation in wheat straw based (Concentrate: Roughage= 50:50) diet

| Attributes | Control | 30ppm | 300ppm | 600ppm | SEM[1] | P Value |
|---|---|---|---|---|---|---|
| Total gas (ml/24h/200mg substrate) | 46.0 | 36.4 | 37.5 | 34.2 | 3.4 | 0.23 |
| True OM degradability (%) | 64.4 | 66.7 | 68.0 | 62.8 | 2.0 | 0.48 |
| True DM degradability (%) | 64.5 | 67.8 | 67.8 | 62.0 | 3.0 | 0.59 |
| $Ch_4$ (ml/24h) | 15.4[a] | 12.9[b] | 12.6[b] | 11.7[b] | 0.4 | <0.01 |
| $Ch_4$ (ml/g OMD) | 128.9[a] | 104.3[b] | 100.2[b] | 100.6[b] | 3.4 | <0.01 |
| $Ch_4$ (ml/g DMD) | 119.3[a] | 95.2[b] | 93.2[b] | 94.5[b] | 3.1 | <0.01 |
| $NH_3$-N (mg/100 ml) | 23.2[a] | 20.6[a] | 21.7[a] | 18.7[b] | 0.5 | 0.01 |
| Total VFA (mM/100 ml) | 36.8 | 40.8 | 38.7 | 39.7 | 0.8 | 0.23 |
| VFA (molar proportion) | | | | | | |
| Acetate | 60.8 | 60.2 | 59.5 | 54.0 | 1.1 | 0.07 |
| Propionate | 22.2 | 23.1 | 22.5 | 23.7 | 0.2 | 0.06 |
| Butyrate | 9.9 | 8.5 | 8.8 | 8.2 | 0.2 | 0.46 |
| Partitioning factor | 2.6 | 3.4 | 3.6 | 3.5 | 0.3 | 0.46 |

Different superscript [a,b,c] in a row differ significantly (P < 0.05). [1]SEM = Standard error of the means, OMD= Organic matter degraded, DMD= Dry matter degraded, VFA= Volatile fatty acid.

suggested that the antimethanogenic effect of garlic and its active components was the result of direct inhibition of *Archaea* microorganisms in the rumen. *Archaea* have unique membrane lipids that contain glycerol linked to long chain isoprenoid alcohols essential for the stability of the cell membrane. The synthesis of the isoprenoid units in methanogenic *Archaea* is catalyzed by hydroxymethylglutaryl coenzyme A (HMGCoA) reductase, an enzyme that has also been described in the liver and that participates in the synthesis of cholesterol. Garlic oil and some derived organosulfur compounds are strong inhibitors of HMG -CoA reductase, and as a result, the synthesis of the isoprenoid unit is inhibited, the membrane of *Archaea* becomes unstable, and the cells die. *In vitro* studies demonstrated that garlic reduced $CH_4$ (µmol): VFA (µmol) ratio from 0.20 to 0.05 [5]. In the present study, we also found significant (P<0.05) decrease in methane production at all the dose level of garlic oil supplemented group.

Effect of essential oils on feed degradability, partitioning factor and VFA concentration: *In vitro* DM and OM degradability percent was reduced significantly (P<0.05) at higher doses of garlic and oregano oil (Table-3 and Table-4); whereas, degradability was not affected (P>0.05) by supplementation of rosemary oil. Similarly, partitioning factor was found significantly higher (P<0.05) in 600 ppm oregano and garlic oil treated groups (Table-3 and Table-4). Busquet *et al.* [31] showed that the effect of oregano oil was most apparent at 500 mg/l and similar to those by oregano oil and carvacrol at 300 mg/l in combination. Decrease in degradability at higher dose rate of garlic and oregano oil was reflected in total VFA production which was decreased significantly (P<0.05), but molar proportion of acetate was reduced significantly (P<0.05) (Table-3 and Table-4). On the contrary, total VFA content and acetate proportion was increased at higher dose levels of cinnamon oil (P<0.05). The active compounds with phenolic structures, such as thymol and carvacrol, are more effective antimicrobials versus non-phenolic compounds, and the presence of hydroxyl group in their phenolic structure enhances this activity. Therefore, high doses of oregano oil (500 mg/l) seem too strong to show positive effects on rumen fermentation. However, the lowest doses of oregano oil (5 and 50 mg/l) may help improve efficiency of rumen fermen-

tation by increasing VFA concentrations [32]. Similar trend was found in our study where higher dose levels of oregano oil were found to improve rumen fermentation as reflected by increase in partitioning factor. It was consistently reported [4, 5, 31] in *in vitro* fermentation trials that addition of garlic oil reduced the proportions of acetate and increased the proportions of propionate and butyrate. A similar trend was observed in our study where acetate concentration was decreased and a propionate concentration was increased at higher dose rate (600ppm) of garlic oil. Though, it was reported by Castillejos *et al.* [6] that rosemary oil (main compound 1,8-cineole) at 500 mg/l, increased propionate proportion, the present study did not show any significant increase in propionate concentration ($P>0.05$) at any dose rate of rosemary oil.

Effect of essential oils on ammonia nitrogen ($NH_3$-N) concentration: $NH_3$-N concentration was decreased ($P<0.05$) in 300 ppm garlic and 600 ppm oregano, rosemary oil supplemented groups, respectively (Table -3, 4 and 5). It was reported by Cardozo *et al*. [33] that garlic oil, in continuous culture, reduced $NH_3$-N and increased peptide and amino acid nitrogen (AA-N) concentrations, suggesting that deamination was inhibited, others have reported only small and variable effects [4, 5]. Similarly, in our experiment, $NH_3$-N was reduced at higher concentration of garlic oil. In oregano and rosemary oil the decrease in $NH_3$-N might be due to overall decrease in microbial fermentation. It has been previously reported by Ferme *et al.* [34] that garlic oil modified the microbial population profile in a continuous culture experiment, reducing the contribution of *Prevotella* spp. (mainly *P. ruminantium* and *P. bryantii*) to the overall microbial population in the rumen. *Prevotella* spp. is mainly responsible for protein degradation and AA deamination, suggesting a mechanism of action of garlic oil on protein metabolism. Similar results were reported by Vakili *et al.* [35] that no effect of lower dose of cinnamon oil on rumen concentration of NH3-N.

## Conclusion

Based on the present findings, it can be concluded that the supplementation of rosemary, oregano and garlic oil at particular concentration is potent inhibitor of methane production, acetate production and breakdown of amino acids into $NH_3$-N. Thus garlic, oregano and rosemary oil may be supplemented on wheat straw based diet in lactating animals to reduce fermentative losses and improved nutrients utilization.

## Authors' contributions

DR planned and carried out research work to compare different essential oils *in vitro* for his PhD thesis programme in collaboration with advisory members and guide SKT. SKS co-guided and provided facilities in estimating methane and VFA using GLC. VK helped DR in setting overall *in vitro* experiment. MK did statistical analysis. All authors participated in draft and revision of the manuscript. All authors read and approved the final manuscript.

## Acknowledgements

The authors are thankful to National Dairy Research Institute, Karnal, India for providing the financial assistance and facilities to carry out the research.

## Competing interests

The authors declare that they have no competing interests.

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