Determination of the Effects of Some Plant Extracts on Rumen Fermentation and Protozoal Counts by Hohenheim In Vitro Gas Production Technique

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Abstract: The aim of the present study was to determine the effects of some plant extracts on rumen fermentation and protozoal counts by using Hohenheim in vitro gas production technique in cattle. In this study in vitro gas productions at varying doses of thymol, oregano, zingiber and syzygium essence oils were determined at 2, 4, 8, 12, 24, 36 and 48 (h), respectively. For all feed types, high doses (50 ppm) of thymol and oregano supplementations significantly decreased gas production at later hours of incubation (p<0.05). On the other hand, for all feed types, all doses of zingiber and syzygium supplementations significantly increased gas production at later hours of incubation (p<0.05). High total gas production quantity indicates that most of the substrates are converted to gas which results in decreased concentrations of volatile fatty acids and other beneficial end products. Varying doses of all essence oils were assessed within the same incubation periods and it was found that high doses of thymol and oregano supplementations resulted significant decrease in gas production (p<0.05). For all feed types, the highest protozoal counts were identified in Z. officinale 200 ppm group compared to positive and negative control groups, while the lowest protozoal count for TMR was recorded in T. vulgaris, O. vulgaris and S. aromatica groups. These essence oils can be utilized as rumen regulators. Similar effects are anticipated with the supplementation of these oils to ruminant rations (in vivo), which, therefore, will lead to improved ruminant performance.

Keywords: Gas production technique, in vitro, plant extracts, protozoa, rumen fermentation

INTRODUCTION

Feed proteins consumed by ruminants are broken into peptides, amino acids and ammonia by the microorganisms in rumen. Some amount of ammonia passes through rumen epitel and is converted into urea in the liver. While some of the urea is removed by the urine, some enters rumino-hepatic circulation. Removed urea makes up of 20-25% of the nitrogen intake by feed which is the unmetabolized feed protein. Gram-positive bacteria are largely responsible for such losses. Antibiotics have been used since 1970’s to suppress Gram-positive bacteria (Demirtaş et al., 2011). The restrictions posed by medicine and consumers on the use of antibiotics in animal nutrition has evoked exploration of alternatives to antibiotics. Due to this fact, recent studies are concentrated on the use of substances such as probiotics, prebiotics, organic acids, enzymes and plant extracts (Wenk, 2000).

Essence oils are volatile oils obtained from plants or from parts thereof by, for example, steam and or water distillation. Most essence oils consist of mixtures of hydrocarbons (terpenes, sesquiterpenes, etc.), oxygenated compounds (alcohol, esters, aldehydes, ketones, etc.) and a small percentage of non-volatile residues (paraffin, wax, etc.) (Losa, 2000). Essence oils have been used by man for many years. Their main effects in the rumen involve reduction of protein and starch degradation and an inhibition of amino acid degradation, due to selective action on certain rumen microorganisms, specifically some bacteria. One mode of action suggested for essence oils is an effect on the pattern of bacterial colonisation of, in particular starch rich, substrates as they enter the rumen. A second possible mode of action is their inhibition of ‘hyper ammonia producing bacteria’ involved in amino acid deaminaton (Hart et al., 2008). The main antimicrobial mechanisms of essence oils are on cell membrane (Calsamiglia et al., 2007). Chao et al. (2000) have suggested that Gram-negative bacteria tended to have a higher resistance to essence oils than Gram-positive bacteria. Wang et al. (2000) found that including Yucca schidigera (0.5 mg/mL) in the buffer of a rumen simulation system (RESITEC) did not affect the total bacteria numbers. They reported that among the 21 plant extracts tested, Syzigium cumin generated the maximum...
zone in the inhibition of both Gram-negative and Gram-positive bacteria. Enterobacter was found to be the most sensitive bacteria to the experimental plant extracts (Sirohi et al., 2009). Cinnamaldehyde and eugenol, active ingredients of essential oils, have been used safely by a low number of milk manufacturers in United States and by a high number of milk manufacturers in Europe. They do not produce any residue on meat and milk and are reported to have beneficial effects compared to other supplements. Despite the limited number of studies on animals, their effects are reported to be noteworthy (Wall, 2010).

The in vitro gas production technique has proved to be a potentially useful technique for feed evaluation (Menke and Steingass, 1988; Getachew et al., 2004) as it is capable of measuring rate and extent of nutrient degradation. In addition, in vitro gas production technique is less expensive and easier compared to in vivo testing (Getachew et al., 2004). This method also predicts feed intake, digestibility, microbial nitrogen supply and amount of short chain fatty acids, carbon dioxides and metabolizable energy of feed for ruminants (Babayemi, 2007; Maheri-Sis et al., 2008). Hence, the present article demonstrates the effects of some plant extracts (T. vulgaris, O. vulgare, S. aromaticum and Z. officinale) on protozoal counts and rumen fermentation by using Hohenheim In Vitro Gas Production Technique.

### MATERIALS AND METHODS

Three fistulated Holstein dairy cows were used for rumen liquor collection for application of in vitro gas production technique. Four essence oils (T. vulgaris, O. vulgare, S. aromaticum, Z. officinale) were used as plant extracts. T. vulgaris, S. aromaticum and Z. officinale essence oils were obtained from Ege Lokman San. Tic. Company in Manisa Province (Turkey) and O. vulgare essence oil from Aksu Gida San. Tic. Company in Mersin Province (Turkey). All plant extracts were extracted with distilled water. The chemical components of plant extracts were evaluated by gas chromatography-mass spectrometry. For each extract, different doses were tested to determine harmful and usable doses. Incubation run for each regulation in 2, 4, 8, 12, 24, 36 and 48 h time periods. TMR, concentrate and hay were used as substrates. The compositions of TMR, concentrate and hay used in the experiment are presented respectively in Tables 1. Major components of all essence oils were analysed using GC-MC and are given in Table 2.

**In vitro gas production:** Ruminal fluid samples were obtained from three fistulated holstein dairy cows fed twice daily at the maintenance level with a diet containing alfalfa hay (60%) and concentrate (40%). The samples were incubated in vitro in calibrated glass syringes following the procedures of Menke et al. (1979). The 200 mg samples were weighed in triplicate into calibrated glass syringes of 100 mL. The syringes were prewarmed at 39°C before the injection of 30 mL ruminal fluid-buffer mixture into each syringe followed by incubation in a water bath at 39°C. Readings of gas production were recorded before incubation (0) and 2, 4, 6, 8, 12, 24, 36 and 48 h after incubation. Total gas values were corrected for blank incubation. Cumulative gas production data were fitted to the model of Orsko and McDonald (1979):

\[ Y = a + b (1 - e^{-ct}) \]

where,

\[ a = \text{The gas production from the immediately soluble fraction (ml)} \]

\[ b = \text{The gas production from the insoluble fraction (ml)} \]

\[ c = \text{The gas production rate constant for the insoluble fraction (b)} \]

\[ a + b = \text{Potential gas production (ml)} \]

\[ t = \text{Incubation time (h)} \]

\[ Y = \text{Gas produced at time t} \]

a, b, c are gas production parameters described by Orskov and McDonald (1979). Gas production test was carried out in the Laboratory of Animal Nutrition, Hohenheim University, Stuttgart, Germany.

**Protozoal count:** 0.1 mL ruminal fluid samples were collected and fixed by 0.9 mL Methyl green Formal Saline (MFS) solution (100 mL formaldehyde solution (30%), 900 mL distilled water, 0.6 g Methylgreen, 8 g NaCl). Following rinse off, the samples were pipetted into Fuchs-Rosenthal counting chamber (16×16 squares, 0.0625 mm² area, 0.200 mm depth) and total numbers of protozoa were determined using a microscope. The formula below was used in the counts (Boyne et al., 1957):

\[ \text{Cell count per cm}^3 = \frac{\text{Counted cells}}{\text{Total square counts} \times \text{Dilution} \times \text{Volume}} \times 1000 \]
Analyses of variance, Tukey test was conducted to determine any differences between the means of supplements and feed types with respect to the examined parameters. Additionally, Repeated Measures ANOVA was performed to determine any differences with respect to feeds and durations (hours). Following analyses of variance, Tukey test was conducted to determine varying means (Steel and Torrie, 1980). Statistical significance levels of 5 and 1% were adopted in the study and calculations were performed by SPSS (Ver: 13) statistical softwares package.

**RESULTS AND DISCUSSION**

The chemical compositions of all essence oils used in the study are given in Table 1. Analysis results indicate that essence oils compose different main

| Table 2: Major components of all essence oils (%) |
|-----------------------------------------------|
| **Thymus vulgaris** | % | β-Myrcene |
| α-Pinene | 0.46 | β-Phellandrene | 0.14 |
| Camphene | 0.18 | α-Terpinene | 1.41 |
| Geranyl Acetate | 0.17 | Cymol | 7.87 |
| β-Myrcene | 0.75 | Eucalyptol | 0.34 |
| α-Phellandrene | 0.15 | δ-Terpinene | 6.79 |
| α-Terpinene | 1.59 | cis-Sabinene Hydrate | 0.53 |
| Cymol | 8.51 | α-Terpineol | 0.19 |
| δ-Limonene | 0.27 | Linalool | 2.42 |
| Eucalyptol | 0.47 | Bornol | 1.15 |
| δ-Terpinene | 7.3 | 4-Terpinol | 0.47 |
| Linalool | 4.38 | Carvacrol-methyleter | 0.19 |
| Bornol | 0.65 | Carvacrol | 68.46 |
| 4-Terpinol | 0.52 | Trans carvophyllen | 4.80 |
| β-Fenchyl Alcohol | 0.12 | Aromadendrene | 0.33 |
| Thymol | 8.75 | α-Caryophyllen | 0.12 |
| Carvacrol | 57.7 | Ledene | 0.18 |
| Trans-Caryophyllene | 3.10 | β-Bisabolene | 0.11 |
| Aromadendrene | 0.20 | Cadina 3, 9-diene | 0.02 |
| α-Caryophyllene | 0.12 | α-Cadinene | 0.05 |
| α-Murolene | 0.01 | Eremophila-1(10), 11-diene | 0.02 |
| α-Amorphene | 0.08 | Phenol 4-methozy 2, 3, 6 trimethy | 0.22 |
| Ledene | 0.13 | (+) Spalthulnol | 0.03 |
| β-Bisabolene | 2.96 | (+) Spalthulnol | 0.20 |
| Germacrene | 0.10 | Caryophyllen oskit | 0.44 |
| δ-Cadinene | 0.17 | 2-pentadecanone, 6, 10, 14-Trimetil | 0.01 |
| [+] Spathulnol | 0.16 | Perillen | 0.01 |
| Caryophyllene ooxide | 0.24 | Isothymol | 0.01 |
| Cadinol | 0.13 | Cyclooctene, 3-(1-methylethenyl) | 0.09 |
| α-Cadinol | 0.02 | Zingiber officinale | % |
| β-Bisabolol | 0.04 | 2-Pentadecanone 6,10,14-Trimetil | 0.02 |
| Mesitylacetic acid | 0.01 | cis-2-Nonenal | 1.75 |
| Squalene | 0.01 | (E,E) 2, 4-Decadienal | 13.79 |
| Phenol, 2,3,5,6- Tetra methyl | 0.00 | Zingiberene | 8.93 |
| Cyclooctene, 3-(1-Methylenenyl) | 0.11 | α-Farnesene | 3.27 |
| Adamantane | 0.02 | Valancene | 1.29 |
| Acetogenol | 0.09 | β-Bisabolene | 7.68 |
| α-Caryophyllen | 0.48 | β-Sesquiphellandrene | 11.97 |
| α-Caryophyllen | 0.38 | Syzygium aromaticum | % |
| Lanostan | 0.02 | 1, 3, 5-Cyclooctatriene | 0.70 |
| Acetoxydiniol | 0.02 | Zingerone | 4.63 |
| Eucalyptol | 0.02 | Viridiflorol | 0.72 |
| CaryophyllenSynthetic | 0.02 | β-Copanen-4, α ol | 10.98 |
| Oreganum vulgare | % | Squalene | 0.38 |
| α-Phellandrene | 0.44 | 3-(6-Hydroks, 3, 7 Dimethy-octa 2, 7, dienyl)-4-Methozy fenol | 1.73 |
| α-Pinene | 0.96 | Octadecane, 3-ethyl-5-(2-ethylbutyl) | 0.71 |
| Camphene | 0.60 | Lucerin 2 | 0.42 |
| β-Pinene | 0.33 | n-Heptacosane | 0.91 |

Statistical Analysis: The descriptive statistics for the examined parameters were expressed in terms of average and standard errors. Factorial Analysis of Variance (Factorial ANOVA) was conducted to determine any differences between the means of supplements and feed types with respect to the examined parameters. Additionally, Repeated Measures ANOVA was performed to determine any differences with respect to feeds and durations (hours). Following analyses of variance, Tukey test was conducted to determine varying means (Steel and Torrie, 1980). Statistical significance levels of 5 and 1% were adopted in the study and calculations were performed by SPSS (Ver: 13) statistical softwares package.
The effects of types and varying doses of essence oils on in vitro gas production: In vitro gas production quantities of thymol, s oregano, zingiber and syzygium essence oils according to varying doses (for concentrate Table 3, for TMR Table 4 and for hay Table 5) were determined respectively at 2, 4, 8, 12, 24, 36 and 48-h incubation periods. For all three feed types, gas production quantities significantly decreased (p<0.05) with high doses of thymol and oregano.

components. Main components of Thymus vulgaris are carvacrol (57.70%) and thymol (8.75%), main component of Oreganum vulgare is carvacrol (68.46%), main component of Syzygium aromaticum is eugenol (93.43%) and main component of Zingiber officinale is zingerberene (15.77%).

Descriptive statistics and comparative results on gas production quantity for concentrate, TMR and hay are presented respectively in Table 3 to 5. Descriptive statistics and comparative results for the gas production parameters “a” (the gas production from the immediately soluble fraction, ml), “b” (the gas production from the insoluble fraction, ml) and “c” (the gas production rate, ml/h) according to feed type, supplement type and supplement dose are given in Table 6. Descriptive statistics and comparative results on protozoal counts at 24 h for TMR, concentrate and hay according to supplement types are given in Table 7.
### Table 5: Descriptive statistics and comparative results on gas production quantity (ml) for hay

| Incubation Time, hours | 2     | 4     | 8     | 12    | 24    | 36    | 48    |
|------------------------|-------|-------|-------|-------|-------|-------|-------|
|                        | Mean  | Mean  | Mean  | Mean  | Mean  | Mean  | Mean  |
| Zingiber               |       |       |       |       |       |       |       |
| 12.5                   | 12.71 | 50    | 12.5  | 6.25  | 3.73  | 17.98 | 17.94 |
| 25                     | 12.71 | 50    | 12.5  | 6.25  | 3.73  | 17.98 | 17.94 |
| Oregano                |       |       |       |       |       |       |       |
| 12.5                   | 12.71 | 50    | 12.5  | 6.25  | 3.73  | 17.98 | 17.94 |
| 25                     | 12.71 | 50    | 12.5  | 6.25  | 3.73  | 17.98 | 17.94 |

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### Table 6: Descriptive statistics and comparative results for gas production parameters (a), (b) and (c) according to feed type, supplement type and supplement dose

**Parameter** | **Supplement** | **Dose, ppm** | **Concentrate** | **Hay** | **TMR** | $X_{i} \pm S_{X}$ | $X_{i} \pm S_{X}$ | $X_{i} \pm S_{X}$ |
|---------------|---------------|---------------|----------------|---------|---------|------------------|------------------|------------------|
| a             | T             | 6.25          | 12.45 $\pm 0.91$ | 8.31 $\pm 0.62$ | 7.56 $\pm 0.32$ |
|               |               |               | 12.89 $\pm 0.56$ | 7.69 $\pm 0.71$ | 6.86 $\pm 0.43$ |
|               |               |               | 10.30 $\pm 0.57$ | 6.37 $\pm 0.82$ | 6.18 $\pm 0.91$ |
|               |               |               | 10.83 $\pm 1.51$ | 7.39 $\pm 0.82$ | 7.10 $\pm 0.91$ |
|               |               |               | 11.97 $\pm 0.46$ | 9.15 $\pm 0.72$ | 8.92 $\pm 0.91$ |
|               |               |               | 12.84 $\pm 0.68$ | 8.94 $\pm 0.81$ | 8.45 $\pm 0.91$ |
|               |               |               | 10.81 $\pm 0.54$ | 12.29 $\pm 1.34$ | 11.90 $\pm 1.26$ |
| Z             |               | 6.25          | 9.14 $\pm 0.57$ | 8.28 $\pm 0.54$ | 9.98 $\pm 0.32$ |
|               |               |               | 12.70 $\pm 0.60$ | 9.25 $\pm 0.77$ | 9.35 $\pm 0.14$ |
|               |               |               | 12.44 $\pm 0.74$ | 8.21 $\pm 0.70$ | 9.41 $\pm 0.19$ |
|               |               |               | 12.57 $\pm 0.60$ | 8.83 $\pm 0.62$ | 7.70 $\pm 0.50$ |
|               |               |               | 11.17 $\pm 0.91$ | 7.39 $\pm 0.61$ | 7.13 $\pm 0.23$ |
| S             |               | 6.25          | 9.14 $\pm 0.57$ | 8.28 $\pm 0.54$ | 9.98 $\pm 0.32$ |
|               |               |               | 12.70 $\pm 0.60$ | 9.25 $\pm 0.77$ | 9.35 $\pm 0.14$ |
|               |               |               | 12.44 $\pm 0.74$ | 8.21 $\pm 0.70$ | 9.41 $\pm 0.19$ |
|               |               |               | 12.57 $\pm 0.60$ | 8.83 $\pm 0.62$ | 7.70 $\pm 0.50$ |
|               |               |               | 11.17 $\pm 0.91$ | 7.39 $\pm 0.61$ | 7.13 $\pm 0.23$ |
| Control       |               | 6.25          | 9.39 $\pm 0.14$ | 8.71 $\pm 0.54$ | 7.65 $\pm 0.24$ |
|               |               |               | 12.23 $\pm 0.59$ | 7.25 $\pm 1.92$ | 6.04 $\pm 0.63$ |
| b             | T             | 6.25          | 56.86 $\pm 3.20$ | 41.46 $\pm 1.53$ | 57.90 $\pm 2.89$ |
|               |               |               | 51.57 $\pm 3.94$ | 27.53 $\pm 4.34$ | 55.63 $\pm 2.73$ |
|               |               |               | 37.66 $\pm 7.88$ | 21.92 $\pm 2.11$ | 23.78 $\pm 4.92$ |
|               |               |               | 50.00 $\pm 0.00$ | 0.00 $\pm 0.00$ | 0.00 $\pm 0.00$ |
|               |               |               | 50.00 $\pm 0.00$ | 0.00 $\pm 0.00$ | 0.00 $\pm 0.00$ |
| Z             |               | 6.25          | 50.71 $\pm 3.79$ | 50.71 $\pm 3.79$ | 60.72 $\pm 2.93$ |
|               |               |               | 61.96 $\pm 3.59$ | 49.41 $\pm 3.65$ | 60.42 $\pm 0.64$ |
|               |               |               | 61.83 $\pm 3.71$ | 49.10 $\pm 3.44$ | 61.34 $\pm 0.82$ |
|               |               |               | 57.73 $\pm 2.93$ | 45.94 $\pm 0.40$ | 62.07 $\pm 1.10$ |
| S             |               | 6.25          | 60.81 $\pm 2.96$ | 50.96 $\pm 4.07$ | 62.27 $\pm 1.06$ |
|               |               |               | 65.82 $\pm 5.43$ | 48.43 $\pm 3.56$ | 60.12 $\pm 1.73$ |
|               |               |               | 62.19 $\pm 5.52$ | 47.62 $\pm 2.53$ | 63.72 $\pm 1.08$ |
| Control       |               | 6.25          | 64.05 $\pm 5.82$ | 48.55 $\pm 2.92$ | 64.16 $\pm 2.48$ |
| c             | T             | 6.25          | 58.69 $\pm 3.50$ | 47.86 $\pm 3.34$ | 58.69 $\pm 3.50$ |
|               |               |               | 0.09 $\pm 0.01$ | 0.05 $\pm 0.02$ | 0.07 $\pm 0.03$ |
|               |               |               | 0.10 $\pm 0.06$ | 0.02 $\pm 0.01$ | 0.08 $\pm 0.02$ |
|               |               |               | 0.12 $\pm 0.03$ | 0.03 $\pm 0.03$ | 0.12 $\pm 0.09$ |
|               |               |               | 0.04 $\pm 0.00$ | 0.03 $\pm 0.02$ | 0.03 $\pm 0.02$ |
| O             |               | 6.25          | 0.10 $\pm 0.02$ | 0.05 $\pm 0.02$ | 0.08 $\pm 0.01$ |
|               |               |               | 0.11 $\pm 0.03$ | 0.08 $\pm 0.01$ | 0.09 $\pm 0.00$ |
|               |               |               | 0.15 $\pm 0.01$ | 0.03 $\pm 0.03$ | 0.07 $\pm 0.02$ |
|               |               |               | 0.04 $\pm 0.00$ | 0.00 $\pm 0.01$ | 0.03 $\pm 0.00$ |

**LSD: 3.14; * Small letter: is used for comparison of incubation times; * Capital letter: is used for comparison of doses for each supplement type; * Numeral: is used for comparison of supplement types for each dose; * #: The difference from control group is statistically significant (p<0.05); * The deviations are defined at 0.05 significance level**
supplementation (50 ppm) at later periods of incubation. On the other hand, gas production quantities significantly increased (p<0.05) by all varying doses of zingiber and syzygium at later periods of incubation. When all essence oils were compared within the same incubation period according to doses, it was found that high doses of thymol and oregano supplementation significantly decreased (p<0.05) gas production quantities for all three feed types. Identical doses of essence oils were examined within the same incubation period. Accordingly, the lowest gas production quantity (0.00) for concentrate was obtained by 50 ppm doses of thymol and oregano at 24-h incubation, while the highest gas production quantities (74.91 and 72.55, respectively) were obtained by 50 ppm doses of zingiber and syzygium at 48-h incubation. Similarly, carvacrol (Kamalak et al., 2011) and thymol (Önenç, 2008) demonstrated that thyme supplementation to cottonseed meal, timothy grass and barley significantly decreased total gas production quantity at 24-h incubation. Similarly, carvacrol (Canbolat et al., 2011) and thymol (Kamalak et al., 2011) were found to significantly decrease in vitro gas production quantities. Kamalak et al. (2011) attributed the reduction in gas production rate to reduced total volatile fatty acid concentration. On the other hand, Sallam et al. (2011) found that essence oils extracted from A. santolina (25 and 50 µL) and A. judaica (25, 50 and 75 µL) plants significantly increased gas production quantity at 24-h incubation compared to control group, while essence oils extracted from S. terebinthifolius (50 and 75 µL), A. santolina (75 µL) and M. microphylla (25, 50 and 75 µL) plants significantly decreased gas production quantity at 24-h incubation. These findings support the findings of our study demonstrating that high doses of thymol and oregano essence oil supplementations to TMR and hay significantly decrease in vitro gas production quantities. These findings also confirm the antimicrobial activity of essence oils. Low gas production quantity can be attributed to insufficient fermentation of substrates or fermentation generating volatile fatty acids rather than gas (Bunglavan et al., 2010). In contrary to these findings, Bodas et al. (2009) demonstrated that Cardus pycnocephalus, Populus tremula, Prunus avium,

### Table 6: Continue

| Feed Type | Z | Control |
|-----------|---|---------|
| Control   | 6.25 | 0.079±0.002 1aA | 0.038±0.003 2aA | 0.072±0.001 1aA |
| T. vulgaris 12.5 ppm | 12.5 | 0.080±0.001 1aB | 0.038±0.003 2aB | 0.073±0.001 1aA |
| T. vulgaris 25 ppm | 25 | 0.080±0.002 1aB | 0.042±0.004 1aA | 0.078±0.004 1aB |
| O. vulgare 12.5 ppm | 50 | 0.084±0.003 1aB | 0.053±0.006 1aA | 0.083±0.002 1aA |
| S | 6.25 | 0.089±0.005 1aA | 0.036±0.003 #2aA | 0.074±0.002 #2aA |
| T. vulgaris 12.5 ppm | 12.5 | 0.102±0.011 1aAB | 0.041±0.003 2aB | 0.079±0.002 1aA |
| T. vulgaris 25 ppm | 25 | 0.086±0.003 1aAB | 0.051±0.003 1aA | 0.086±0.002 1aB |
| O. vulgare 12.5 ppm | 50 | 0.089±0.005 1aA | 0.058±0.002 1aA | 0.085±0.002 1aA |
| Control | 6.25 | 0.079±0.0011 0.038±0.002 1aA |

T: Thymol; O: Oregano; Z: Zingiber; S: Syzygium; Parameter (a): Immediate Gas Production (ml), Parameter (b): Potential Gas Production (ml), Parameter (c): Gas Production Rate (ml/h); The difference in between means getting different number in the same line is statistically significant (The Comparison of Feed Types) (p<0.01); The difference in between means getting different small letter in the same column and inside feed is statistically significant (The Comparison of Doses) (p<0.01); The difference in between means getting different capital letter in the same column and on the same dose level is statistically significant (The Comparison of Supplement Types) (p<0.01); #: The difference from control group is statistically significant (p<0.01)

### Table 7: Descriptive statistics and comparative results on protozoal counts (X10³/mL) at 24-h for TMR, concentrate and hay according to supplement types

| Treatment group | TMR | Concentrate | HAY |
|-----------------|-----|-------------|-----|
| Negative Control | 693.32c | 339.23d | 464.58b |
| Pozitive Control | 1139.03b | 799.13bc | 596.53ab |
| T. vulgaris 12.5 ppm | 482.45a | 703.37bc | 725.12ab |
| T. vulgaris 25 ppm | 419.43d | 698.35bc | 495.48ab |
| O. vulgare 12.5 ppm | 449.68d | 992.07b | 776.37ab |
| O. vulgare 25 ppm | 406.08d | 689.08bc | 717.92ab |
| Z. officinale 200 ppm | 1569.60a | 1356.88a | 950.88a |
| S. aromaticum 200 ppm | 469.43d | 550.98cd | 370.77b |
| SED | 13.58 | 42.92 | 55.25 |
| Significance (P =) | 0.0001 | 0.0001 | 0.1167 |

*: Means within same column having different letters are significantly significant (p<0.05); SED: Standart Error of Difference between means

Benchaar et al. (2007a) reported that while clove, cinnamon and thymol did not have any effect on gas production rate (h-1), carvacrol and eugenol significantly decreased it. Önenç (2008) demonstrated that thyme supplementation to cottonseed meal, timothy grass and barley significantly decreased total gas production quantity at 24-h incubation. Similarly, carvacrol (Canbolat et al., 2011) and thymol (Kamalak et al., 2011) were found to significantly decrease in vitro gas production rate. Kamalak et al. (2011) attributed the reduction in gas production rate to reduced total volatile fatty acid concentration. On the other hand, Sallam et al. (2011) found that essence oils extracted from A. santolina (25 and 50 µL) and A. judaica (25, 50 and 75 µL) plants significantly increased gas production quantity at 24-h incubation compared to control group, while essence oils extracted from S. terebinthifolius (50 and 75 µL), A. santolina (75 µL) and M. microphylla (25, 50 and 75 µL) plants significantly decreased gas production quantity at 24-h incubation. These findings support the findings of our study demonstrating that high doses of thymol and oregano essence oil supplementations to TMR and hay significantly decrease in vitro gas production quantities. These findings also confirm the antimicrobial activity of essence oils. Low gas production quantity can be resulted by insufficient fermentation of substrates or fermentation generating volatile fatty acids rather than gas (Bunglavan et al., 2010). In contrary to these findings, Bodas et al. (2009) demonstrated that Cardus pycnocephalus, Populus tremula, Prunus avium,
**Quercus robur**, *Rheum nobile* and *Salix caprea* supplementation to 50:40:10 alfalfa:hay:barley sheep rations did not have any significant effect on gas production quantities and fermentation efficiency (mg DM digested/mL gas) at 24-h of incubation in *in vitro* conditions. The varying results obtained from studies can be attributed to varying types and doses of plant extracts and varying ration compositions used in the studies.

For all feed types, all doses of zingiber and syzygium significantly increased gas production quantities at later hours of incubation (p<0.05). High total gas production quantity demonstrates that most of the substrates are converted to gas which results in decreased concentrations of volatile fatty acids and other beneficial end products (Bunglavan et al., 2010).

**The effects of essence oils on the gas production parameters (a), (b) and (c):** The gas production quantity from the immediately soluble fractions (a) was higher in concentrate compared to hay and TMR (Table 6). This finding can be explained by the high quantity of immediately soluble nutrients (raw protein) and low quantity of cell wall components (NDF, ADF, ADL). The lowest (a) value for concentrate (9.390) was recorded in the group with 50 ppm *Syzygium* supplementation while the lowest (a) value for hay (7.395) was identified in the groups supplemented with 50 ppm Thymol and Zingiber. The lowest (a) value for TMR (7.131) was obtained in the group with 50 ppm Zingiber supplementation. Similarly, in their study on the effects of thymol on digestion of alfalfa and rumen fermentation, Kamalak et al. (2011) demonstrated that 200 mg/L thymol supplementation to ruminal fluid resulted a 22.77% reduction in potential gas production value (a).

The gas production quantity from the insoluble fractions (b) was lower in hay compared to concentrate and TMR (Table 6). (b) value significantly decreased with higher doses of thymol and oregano supplementations (p<0.01). For all three feeds (TMR, concentrate and hay), (b) value decreased down to 0.00 with 50 ppm thymol and oregano supplementations.

Similar to (b) value, the gas production rate constant (c) for the insoluble fraction (b) lower in hay compared to concentrate and TMR (Table 6). While the lowest (c) values for concentrate (0.040 and 0.043, respectively) were recorded in the groups supplemented with 50 ppm thymol and oregano, respectively, the lowest (c) value (0.003) for hay was identified in the groups supplemented with 25 ppm thymol and oregano. For TMR, the lowest (c) value (0.032) was also identified in the groups supplemented with 50 ppm thymol and oregano. As a result, the decrease in (a), (b) and (c) values indicate that thymol, oregano, zingiber and syzygium essence oils have an effect on rumen fermentation and demonstrate antimicrobial effect.

**The effects of some essence oils supplemented to TMR, concentrate and hay on 24-h protozoal counts:** For all three feeds (TMR, concentrate and hay), the highest protozoal counts were obtained in the group supplemented with 200 ppm *Z. officinale* compared to positive and negative control groups (Table 7). On the other hand, the lowest protozoa count for TMR was obtained in the groups supplemented with *T. vulgaris*, *O. vulgare* and *S. aromaticium*. For concentrate and hay, the lowest protozoal counts were obtained in the group supplemented with 200 ppm *S. aromaticium* compared to positive control group.

Depending on their dose, many essence oils have bactericidal and bacteriostatic effects on microorganisms like bacteria, fungi, virus and protozoa (Greathead, 2003). The proportion and quantity of ruminal microorganisms vary according to the composition of ration. Protozoal count in rumen microbial ecosystem was reported as 10^4-10^6 cell/mL (Alataş and Umucalılar, 2011). Protozoa, like Gram-positive bacteria in rumen, produce excessive amount of hydrogen and the symbiotic relation between protozoa and methanogenic bacteria leads to increased methane production. Despite their additional positive effects, it has been reported that decreased protozoal counts may improve ruminant performance (Demirtaş et al., 2011).

There are different study findings on the effects of essence oils on rumen protozoal counts. Newbold et al. (2004) tested the effects of EO in sheep. EO (the major components are thymol, guajacol and limonene) had no influence on protozoal numbers. However, EO tended to numerically increase protozoal numbers in the rumen. Wallace (2004) reported that essence oils had no effect on protozoal number and activity. Benchaar et al. (2006) demonstrated that essence oil blend with major components of thymol, eugenol, vanilin and limonene (2 g/day) had no influence on protozoal number of dairy cattle. In another study, Benchaar et al. (2007b) found that CRINA ruminants (the major components are thymol, eugenol, vanilin, guajacol and limonene) did not have any effect on total rumen viable bacteria, cellulolytic bacteria and protozoal number. Demirtaş et al. (2011) reported that 250 mg of rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) supplementations to 50:50 roughage:concentrate rations did not have any significant effect on total protozoal number. In contrary to these researchers, Sallam et al. (2011) demonstrated that essence oils derived from *A. santolina* and *M. microphylla* plants significantly decreased protozoal numbers at 24-h. Öztürk et al. (2012) revealed that 150 mg of olive leaf extract and antibiotic (monensin) supplementations to 50:50 roughage:concentrate rations decreased total protozoal number. Sirohi et al. (2012) reported significant decrease in protozoal numbers generated by *Myristica fragrans* extract. Decreased protozoal count,
increased bacterial and fungal counts, increased propionate production and reduced methanogenesis improve ruminant performance (Sirohi et al., 2009). The essence oils of T. vulgaris, O. vulgare and S. aromaticum tested in this study demonstrated antibacterial effects in in vitro conditions and lead to reduced protozoa counts. These findings are in agreement with the findings of Sallam et al. (2011), Öztürk et al. (2012) and Sirohi et al. (2012). Depending on the active ingredient, plant extracts may have fatal effect on bacteria or protozoa. For example, phenols denature proteins at bacterial cell wall and increase cell wall permeability. As a result of alternation of cell wall permeability, cytosol moves out of the cell and the bacteria eventually die (Kutlu, 1999).

CONCLUSION

There are some positive in vitro studies about the effects of essence oils on rumen fermentation. The varying results demonstrated by the studies can be attributed to the extraction methods of essence oils, the types, properties and cultivation patterns (climate, harvest time etc.) of the plants they are derived from, chemical compositions of rations and tested doses. The existing literature findings need to be utilized at the field in in vivo studies and new studies are required to assess the effects on animal performance and identify any residues in meat and dairy products.

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