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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 pmm group compared to positive and negative control groups, while the lowest protozoal count for TMR was recorded in *T. vulgaris*, *O. vulgare* and *S. aromaticium* 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 deaminotion (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

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Table 1: Composition of TMR, concentrate and alfalfa hay

	Dry matter	Crude protein	MJNEL/kg DM
TMR*	43.00	15.25	6.9
Concentrate **	98.50	20.30	8.7
Alfalfa Hay	95.31	14.46	8.0

*TMR composition: Maize silage 28.9%, Grass silage 31.2%, Hay 4.8%, Haylage 2.3%, Molassed sugar beet pulp 1.3%, Josera-betavit 0.09%, Salt 0.09%, Monosodium phosphate 0.09%, Ca-carbonate 0.35%, Bergophor GM13 0.45%, Bergophor LM07 0.45%, MgO 0.02%, Concentrate-TMR-silage-2, 17.4%, Soybean meals 0.7%, chelates 0.3%, Water 12.1%, Yeast mixture 100g. Mineral g/kg DM: Ca 7.62, P 4.53, Mg 2.52, Na 2.82, K 16.04, Zn 62, Mn 58 and Se 0.1; **Concentrate composition: Maize 20%, sunflower 7.5%, pea 8%, barley 16%, soyabean meal 15%, wheat 30.5%, crib bean 3%

zone in the inhibition of both Gram-negative and Gram-positive bacteria. Enterobacter was found to be the most sensitive bacteria to the experimented plant extracts (Sirohi *et al*., 2009). Cinnamaldehyde and eugenol, active ingredients of essence 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 Holstain 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 Gıda San. Tic. Company in Mersin Province (Turkey). All plant extracts were extracted with distilated 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 holstain 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 = The gas production from the immediately soluble fraction (ml)
b = The gas production from the insoluble fraction (ml)
c = The gas production rate constant for the insoluble fraction (b)
a+b = Potential gas production (ml)
t = İncubation time (h)
Y = 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 mm^2 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):

$$\text{Cell count per cm}^3\ \text{(ml)} = \frac{\text{Counted cells}}{\text{Total square counts X Dilution X Volume}} \times 1000$$

Table 2: Major components of all essence oils (%)

Thymus vulgaris	%	β-Myırcene	1.05
α-Pinene	0.46	β-Phellandrene	0.14
Camphene	0.18	α-Terpinene	1.41
Gerenyl Acetate	0.17	Cymol	7.87
β-Myrcn	0.75	Eucalyptol	0.34
α-Phelandrene	0.15	δ-Terpinene	6.79
α-Terpinene	1.59	Cis-Sabinene Hydrate	0.53
Cymol	8.51	α-Terpinolene	0.19
d-Limonene	0.27	Linalooll	2.42
Eucalyptol	0.47	Borneol	1.15
δ-Terpinene	7.73	4-Terpineol	0.47
Linalooll	4.38	Carvacrol-methyeter	0.19
Borneol	0.65	Carvacrol	68.46
4-Terpineol	0.52	Trans caryophyllen	4.80
β-Fenchyl Alcohol	0.12	Aromadendrene	0.33
Thymol	8.75	α-Caryophllen	0.12
Carvacrol	57.70	Ledene	0.18
Trans-Caryophyllene	3.10	β-Bisabolene	0.11
Aromadendrene	0.20	Cadina 3, 9-diene	0.02
α-Caryopyllene	0.12	ε-Cadinene	0.05
α-Muurolene	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	(-) Spathulnol	0.03
β-Bisabolene	2.96	(+) Spathulnol	0.20
Germacrene	0.10	Caryophyllen oksit	0.44
δ-Cadinene	0.17	2-pentadecanone, 6, 10, 14-Trimetil	0.01
[+] Spathulenol	0.16	Perillen	0.01
Caryophyllene ozide	0.24	İsothymol	0.01
Cadinol	0.13	Cyclooctene, 3-(1-methylethenyl)	0.09
α-Cadinol	0.02		
α-Bisabalol	0.04	*Zingiber officinale*	%
2-Pentadecanone 6,10,14-Trimetil	0.02	Cis 2-Nonenal	1.75
Mesitylacetic asit	0.01	(E,E) 2, 4-Decadienal	13.79
Squalene	0.01	Ar-Curcumene	8.93
Phenol, 2,3,5,6- Tetra methyl	0.00	Zingiberene	15.77
Cyclooctene, 3-(1- Methyethenmyl)	0.11	α-Farnsene	3.27
Adamantane	0.02	Valancene	1.29
Aceteugenol	0.09	β-Bisavolene	7.68
		β-Sesquiphellandrene	11.97
Syzygium aromaticum	%	1, 3, 5-Cyclooctatriene	0.70
Eugenol	93.43	Zingerone	4.63
Trans-Caryophyllen	2.79	Viridiflorol	0.72
α-Caryophyllen	0.48	β-Copanen-4, α ol	10.98
Lanostan	0.02	Linoleic Asit	0.50
Obscurinervidinediol	0.02	Oleic Asit	0.62
Caryophyllen alchol	0.02	n-Hekza Dekonoik Asid	1.04
Caryophyllen oksit	0.13	Retinol	0.54
Humulene oksit	0.02	Monopalmitin	3.19
Benzyl salycilate	2.99	Retinol Acetate	0.22
Aceteugenol	0.09	Stearoik Asit	4.06
		Linoleyl Choloride	4.19
Oreganum vulgare	%	Squalene	0.38
α-Phellandrene	0.44	3-(6-Hydroksi, 3, 7 Dimethy-octa 2 ,7, dieniyl)-4-Methozy fenol	1.73
α-Pinene	0.96	Octadecane, 3-ethy-5-(2-ethylbutryl)	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.

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 3: Descriptive statistics and comparative results on gas production quantity (ml) for concentrate

		Incubation Time, hours						
		2	4	8	12	24	36	48
Supplement	Dose, ppm	Mean	Mean	Mean	Mean	Mean	Mean	Mean
Thymol	6.25	21.72 f1A	30.30 e1A	42.87 d1A	50.61 c1A	62.19 b1A	67.68 a1A	68.86 a1A
	12.5	22.90 f1A	30.67 e1A	41.73 d1A	50.39 c1A	60.23 b1A	62.65 ab1B#	63.86 a1B#
	25	16.59 e1B#	20.77 d1B	25.77 c1B#	29.37 b1B#	32.31 ab1B#	33.54 a1C#	34.85 a1C#
	50	7.66 a1C#	5.87 ab1C#	3.56 bc1C#	2.30 b1C#	0.00 c1C#	0.00 c1D#	0.00 c1D#
Oregano	6.25	22.37 f1A	30.98 e1A	44.26 d1A#	52.46 c1A#	64.13 b1A#	68.56 a1A	69.77 a1A
	12.5	22.72 e1A	30.29 d1A	42.18 c1A	50.81 b1A	60.49 a1B	62.61 a1B#	63.18 a1B#
	25	17.81 d1B	22.37 c1B#	28.60 b1B#	33.00 a1B#	34.55 a1C#	35.14 a1C#	35.80 a1C#--
	50	6.20 a1C	4.76 ab1C#	2.95 abc1C#	1.58 bc1C#	0.00 c1D#	0.00 c1D#	0.00 c1D#
Zingiber	6.25	20.73 g1A	29.48 f1A	41.87 e1A	49.89 d1A	62.55 c1A	69.45 b1A	72.79 a1A
	12.5	20.74 g1A	29.56 f1A	42.80 e1A	50.62 d1A	63.44 c1A	70.51 b1A	74.14 a1A#
	25	20.88 g1A	29.58 f1A	42.75 e1A	50.63 d1A	63.46 c1A	70.45 b1A	74.11 a1A#
	50	20.52 g1A	29.21 f1A	43.39 e1A	51.85 d1A#	64.61 c1A#	71.37 b1A#	74.91 a1A#
Syzygium	6.25	21.57 g1A	30.41 f1A	44.03 e1A#	51.66 d1A#	64.62 c1A#	69.78 b1A	73.02 a1A
	12.5	17.55 g1B	30.22 f1A	43.88 e1A#	52.30 d1A#	63.64 c1A	70.50 b1A#	73.85 a1A#
	25	19.59 g1AB	29.18 f1A	41.87 e1A	50.98 d1A	63.25 c1A	69.33 b1A	72.53 a1A
	50	19.87 f1AB	29.28 e1A	41.41 d1A	52.52 c1A#	65.64 b1A#	70.85 a1A#	72.55 a1A
Control		20.04	28.40	40.58	47.90	60.82	67.25	70.31

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

Table 4: Descriptive statistics and comparative results on gas production quantity (ml) for TMR

		Incubation time, hours						
		2	4	8	12	24	36	48
Supplement	Dose, ppm	Mean	Mean	Mean	Mean	Mean	Mean	Mean
Thymol	6.25	15.83 f2A	22.76 e2A	33.08 d2A	41.57 c2A	55.08 b2A#	58.67 a2A	61.31 a2A
	12.5	15.95 f2A	22.40 e2AB	34.08 d2A	42.37 c2A#	55.74 b2A#	59.77 a1A	61.30 a1A
	25	15.64 e12A	19.43 d1B	24.59 c1B#	28.21 b1B#	33.69 a1B#	33.70 a1B#	33.54 a1B#
	50	7.45 a12B#	6.15 a1C#	2.57 b12C#	0.77 b1C#	0.00 b1C#	0.00 b1C#	0.00 b1C#
Oregano	6.25	18.40 f2A#	25.18 e2A#	37.61 d2A	46.25 c2A#	59.64 b2A#	65.13 a2A#	67.31 a1A#
	12.5	18.19 f2A#	24.24 e2A#	35.22 d2A#	44.03 c2A#	55.38 b2B#	58.52 ab2B	59.52 a2B
	25	15.11 c1B	17.49 c1B	22.04 b1B#	25.05 b1B#	31.08 a2C#	33.06 a1C#	32.79 a1C#
	50	7.28 a1C	5.93 a1C#	2.24 b1C#	1.09 b1C#	0.00 b1D#	0.00 b1D#	0.00 b1D#
Zingiber	6.25	17.26 g2A#	24.13 f2A#	36.82 e2A#	44.78 d2A#	58.20 c2A#	64.99 b2A#	68.96 a2A#
	12.5	16.98 g2A#	24.12 f2A#	36.95 e2A#	44.71 d2A#	57.93 c2A#	64.66 b2A#	68.59 a2A#
	25	16.40 g2A	23.92 f2A#	36.86 e1A#	45.57 d2A#	58.11 c2A#	65.47 b2A#	67.92 a2A#
	50	16.58 g2A#	23.84 f2A#	38.25 e2A#	46.55 d2A#	59.35 c2A#	65.26 b2A#	69.10 a2A#
Syzygium	6.25	17.57 g2A#	25.06 f2A#	37.76 e2A#	46.46 d2B#	59.63 c2B#	66.87 b1A#	70.61 a1A#
	12.5	18.44 g1A#	26.15 f2A#	38.70 e2A#	47.30 d2AB#	59.83 c2B#	66.08 b2A#	69.68 a2A#
	25	18.44 g1A#	26.65 f1A#	40.45 e1A#	49.96 d1A#	62.62 c1AB#	68.46 b1A#	71.88 a1A#
	50	17.77 f1A#	25.89 e2A#	39.61 d1A#	48.63 c2AB#	63.30 b1A#	68.52 a1A#	70.97 a1A#
Control		13.28	20.10	31.57	38.83	51.71	57.99	61.49

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

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 zingiberene (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.

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

Table 5: Descriptive statistics and comparative results on gas production quantity (ml) for hay

		Incubation Time, hours						
		2	4	8	12	24	36	48
Supplement	Dose, ppm	Mean	Mean	Mean	Mean	Mean	Mean	Mean
		11.72 g3A	15.16 f3A	21.46 e3A	25.80 d3A	36.04 c3A	41.14 b3A	45.01 a3A
	12.5	12.08 e3A	15.18 e3A	20.72 d3A	23.98 c3A	30.49 b3B#	32.36 ab1B#	34.23 a2B#
	25	12.71 bc2A	15.13 ab2B	17.40 a2B	17.07 a2B#	13.08 bc2C#	10.81 c2C#	10.14 c2C#
	50	4.62 a2B	2.54 ab2B#	0.66 b2C#	0.07 b1C#	0.00 b1D#	0.00 b1D#	0.00 b1D#
Oregano	6.25	12.90 g3A	16.45 f3A	22.69 e3A	27.25 d3A	37.73 c3A	42.96 b3A	46.91 a2A
	12.5	13.28 e3A	16.54 d3A	21.52 c3A	24.54 c3A	30.28 b3B#	32.01 ab3B#	33.96 a3B#
	25	10.92 bc2A	12.61 abc1B	14.46 a1B#	13.90 ab1B#	10.31 cd3C#	7.55 de2C#	6.82 e2C#
	50	5.42 a1B#	3.41 ab1C#	0.39 b1C#	0.00 b1D#	0.00 b1D#	0.00 b1D#	0.00 b1D#
Zingiber	6.25	12.56 g3A	16.03 f3A	22.40 e3A	27.15 d3A	38.35 c3A#	45.51 b3A#	50.89 a3A#
	12.5	11.41 g3A	14.98 f3A	21.54 e3A	26.00 d3A	36.93 c3A	43.81 b3A	49.32 a3A
	25	12.35 g3A	16.26 f3A	23.15 e1A#	28.03 d3A#	39.08 c3A#	45.91 b3A#	51.12 a3A#
	50	11.61 g3A	15.47 f3A	23.28 e3A#	28.36 d3A#	38.81 c3A#	44.43 b3A	49.17 a3A
Syzygium	6.25	12.73 g3A	16.48 f3A	22.59 e3B	27.16 d3C	38.42 c3C#	45.51 b2B#	51.13 a2A#
	12.5	13.05 g2A	17.20 f3A#	23.63 e3AB#	28.20 d3BC#	39.20 c3BC#	45.89 b3B#	51.19 a3A#
	25	13.00 g3A	17.94 f2A#	25.18 e2AB#	30.62 d2AB#	41.97 c2AB#	47.81 b2AB#	52.74 a2A#
	50	12.90 g2A	17.98 f3A#	25.89 e2A#	31.86 d3A#	44.35 c2A#	49.60 b2A#	53.55 a2A#
Control	10.56	13.80	19.89	24.27	35.17	42.17	46.78	

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

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

			Concentrate	Hay	TMR
Parameter	Supplement	Dose, ppm	$\bar{X}\pm S_{\bar{x}}$	$\bar{X}\pm S_{\bar{x}}$	$\bar{X}\pm S_{\bar{x}}$
a	T	6.25	12.415±0.511aA	8.021±1.6182bA	7.576±0.9312bcA
		12.5	12.839±0.567 1aA	7.694±1.741 2bA	6.866±0.4732cB
		25	10.307±0.571 2aA	16.377±2.828 #1aA	10.417±0.981 #2aA
		50	10.837±1.518 1aA	7.395±0.858 2bA	10.130±0.392 #1abA
	O	6.25	11.978±0.462 1aA	9.153±1.072 2bA	9.372±0.399 #12aA
		12.5	11.840±0.688 1aA	9.343±1.133 12bA	8.771±0.415 #2aAB
		25	10.819±0.541 1aA	12.294±1.344 #1aB	10.900±0.326 #1aA
		50	9.519±1.597 #1aA	8.278±0.545 1bA	9.998±0.932 #1aA
	Z	6.25	12.705±0.630 1aA	9.237±0.773 2aA	9.355±0.146 #2aA
		12.5	12.447±0.474 1aA	8.216±0.760 2aA	9.041±0.194 #2aAB
		25	12.574±0.609 1aA	8.833±0.692 2aC	7.705±0.506 2aB
		50	11.173±0.913 1aA	7.395±0.613 2aA	7.131±0.259 2aB
	S	6.25	12.219±0.710 1aA	9.644±0.582 12aA	9.361±0.279 #2aA
		12.5	7.168±1.878 #2cB	9.868±0.831 #1aA	9.998±0.281 #1aA
		25	10.438±0.682 1abA	8.977±1.239 1aC	8.554±0.446 #1aAB
		50	9.390±1.043 #1bcA	7.813±0.554 1aA	7.651±0.692 1aAB
Control			12.236±0.591	7.252±1.926	6.046±0.843
b	T	6.25	56.862±3.205 1aA	41.461±4.153 2aA	57.930±2.890 1aA
		12.5	51.357±4.984 1aB	27.553±4.434 #2bB	55.653±2.733 1aA
		25	37.686±7.884 #1bB	2.192±2.116 #3cB	23.784±4.694 #2bB
		50	0.000±0.000 #cB	0.000±0.000 #cB	0.000±0.000 #cB
	O	6.25	58.067±2.182 1aA	42.345±4.053 2aA	59.044±1.513 1aA
		12.5	51.790±4.148 1aB	27.064±4.026 #2bB	51.779±2.107 1aA
		25	30.037±3.838 #1bB	7.778±4.090 #2cB	28.578±3.772 #1bB
		50	0.000±0.000 #cB	0.000±0.000 #cB	0.000±0.000 #cB
	Z	6.25	60.568±3.330 1aA	50.713±3.791 2aA	60.722±1.293 1aA
		12.5	61.963±3.592 1aA	49.411±3.365 2aA	60.424±0.643 1aA
		25	61.832±3.717 1aA	49.101±3.434 2aA	61.347±0.826 1aA
		50	63.773±2.953 1aA	45.594±3.404 2aA	62.027±1.190 1aA
	S	6.25	60.814±2.961 1aA	50.964±4.017 2aA	62.271±1.063 1aA
		12.5	65.682±5.435 1aA	48.436±3.556 2aA	60.128±1.731 1aA
		25	62.198±5.525 1aA	47.627±2.453 2aA	63.372±1.088 1aA
		50	64.095±5.823 1aA	48.551±2.929 2aA	64.165±2.481 1aA
Control			58.694 ± 3.501	47.865 ± 3.304	58.694 ± 3.501
c	T	6.25	0.093±0.001 1aA	0.050±0.005 2bA	0.075±0.003 12bA
		12.5	0.109±0.006 1aAB	0.102±0.022 #1aB	0.085±0.002 1bA
		25	0.122±0.039 #1aA	0.003±0.003 2cA	0.121±0.009 #1aA
		50	0.040±0.003 #1bA	0.039±0.002 1bcA	0.032±0.002 #1cB
	O	6.25	0.100±0.002 1bA	0.050±0.005 2bA	0.081±0.001 12aA
		12.5	0.114±0.003 #2bA	0.175±0.086 #1aA	0.094±0.005 #2aA
		25	0.154±0.017 #1aA	0.003±0.003 3cA	0.074±0.021 2aB
		50	0.043±0.002 1cA	0.040±0.001 1bcA	0.032±0.001 #1bB

Table 6: Continue

Z	6.25	0.079±0.002 1aA	0.038±0.003 2aA	0.072±0.001 1aA
	12.5	0.080±0.001 1aB	0.038±0.003 2aB	0.073±0.001 1aA
	25	0.080±0.002 1aB	0.042±0.004 1aA	0.078±0.004 1aB
	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 1aA
	12.5	0.102±0.011 1aAB	0.041±0.003 2aB	0.079±0.002 12aA
	25	0.086±0.003 1aB	0.051±0.003 1aA	0.086±0.002 1aB
	50	0.089±0.005 1aB	0.058±0.002 1aA	0.085±0.002 1aA
Control		0.079±0.0011	0.038±0.002	0.079±0.0011

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 supplement 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 ($X10^3$/mL) at 24-h for TMR, concentrate and hay according to supplement types

	Feeds		
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	482.45d	703.37bc	726.12ab
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. officinele 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 betwen means

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, the lowest gas production quantity (0.00) for TMR was obtained by 50 ppm doses of thymol and oregano at 24-h incubation, while the highest gas production quantities (69.10 and 70.97, respectively) were obtained by 50 ppm doses of zingiber and syzygium at 48-h incubation. The lowest gas production quantity (0.00) for hay was obtained by 50 ppm dose of oregano at 12-h incubation, while the highest gas production quantity (53.55) was obtained by 50 ppm dose of syzygium at 48-h incubation. Varying gas production quantities resulted by essence oils for the same feed type can be attributed to varying main components in each essence oil and varying doses used.

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 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 pynocephalus, 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 part 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, orageno, 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 limonen (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. aromaticium* tested in this study demonstrated antibacterial effects in *in vitro* conditions and lead to reduced protozoal 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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