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Effects of the addition of essential oils cumimaldehyde, eugenol, and thymol on the in vitro gas production and digestibility of alfalfa (*Medicago sativa L.*) silage

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Abstract: The aim of this study was to investigate the in vitro effects of the addition of three essential oils, mainly cuminaldehyde, eugenol, and thymol to alfalfa (*Medicago sativa L.)* silage on gas production, digestibility, and metabolic energy. The essential oils were added at 0 ppm (controls) and 100 ppm (group 1), 200 ppm (group 2), and 300 ppm (group 3) with three replications. The cumulative amount of gas resulting from the addition of the three essential oils at the 96th h of incubation was significantly lower than that of the control group ($P < 0.05$). The cumulative amount of gas production at the 96th h of incubation with thymol ranged from 184.63 mL/g to 217.38 mL/g. In conclusion, this study showed that the addition of different amounts of cuminaldehyde, eugenol, and thymol to alfalfa silage reduced the amount of in vitro gas production. Therefore, these treatments can potentially reduce environmental pollution from ruminal digestion. However, the use of increasing levels of essential oils reduced the amounts of digestible organic matter and metabolic energy. Additional studies are necessary to reveal the effects of these essential oils on in vivo ruminal fermentation by measuring changes in feed consumption and productivity.

Key words: Alfalfa silage, cuminaldehyde, digestibility, eugenol, in vitro gas production, thymol

1. Introduction

Legumes, which are a major feed source worldwide for ruminants and other animals, are richer in proteins, minerals, and vitamins than other roughage (1). The purpose of supplements that mediate the ruminant microbial ecosystem is to increase the efficiency of feed conversion and hence generate productivity gains. In addition to achieving higher yields, the aim should be to reduce nitrogen excretion and methane emissions because they pollute the environment.

Gas production in the rumen depends on feed digestibility and animal performance (2). In order to improve the activity of the rumen microbes and, specifically, ruminal fermentation, there has been a trend towards the use of natural additives such as phytochemicals. Among these, essential oils with antimicrobial effects have attracted attention (3). Ruminal fermentation has some disadvantages, such as methane and ammonia emissions to the environment, with cattle losing 2%–15% of the energy from their feed input through eructation (belching). Total methane emissions by domesticated ruminants are between 65 and 100 million tons per year, which is about 15% of global production (4). Methane production in ruminants is influenced by feed consumption, the digestibility and processing of feeds, and the addition of oils (unsaturated fatty acids) (5–7).

Essential oils are plant metabolites which, due to their antimicrobial and antioxidant properties, are used as feed supplements in animal nutrition (8). The antimicrobial characteristic of essential oils can be used to mediate the activity of rumen microbes and, consequently, lower the level of breakdown of feed protein (9,10). Essential oils and their compounds have a wide spectrum of antibacterial activity but are usually active against bacteria in the rumen only at high levels (11).

The objectives of this study were to determine the in vitro digestibility and metabolic energy values of alfalfa silage when digested with harvested ruminal fluids supplemented with essential oils and also to measure the produced gases.

2. Materials and methods

2.1. Animals and feedstuffs

Ruminal fluid was collected from three Holstein dairy cows slaughtered at Florya slaughterhouse in Samsun, Turkey. The animals had been fed with 60% roughage and

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40% concentrated feed on a dry matter basis. The rumen fluids were collected and transferred to the laboratory in a thermos at 39 °C in the presence of CO_2. The alfalfa was cultivated in the fields of Ondokuz Mayıs University for the purpose of ensilage. The alfalfa was harvested at 10% flowering and was tightly packed in 1-L glass jars. A total of 36 jars were used in the study. The alfalfa-filled jars were opened after 60 days of ensilage at room temperature. The alfalfa silage for analysis was then oven dried at 65 °C for 48 h to achieve a constant weight.

2.2. Chemical analysis

Alfalfa silage samples were ground and passed through a 1-mm sieve (IKA A11 Basic, Guangzhou, China) for chemical analyses and the determination of in vitro gas production. Dry matter content was determined by oven drying for 24 h at 105 °C (Memmert UNE 400, Germany). Crude protein (nitrogen × 6.25) was quantified by the Kjeldahl method (BuchiDigestion Unit K-424, Distillation Unit B-324, Switzerland). Ether extract was calculated following ether extraction in a Soxhlet extraction system (BUCHI extraction system B-811, Switzerland). The amount of organic matter (OM) was calculated as weight loss after combustion at 550 °C for 4 h (Carbolite ELF 11/14, UK) (12). Neutral detergent fiber (aNDFom-NDF), exclusive from residual ash and acid detergent fiber (ADF) acid detergent lignin (ADL) contents were determined using an Ankom 200 Fiber Analyzer (Ankom Technology Corp., Fairport, NY, USA), according to the methodology supplied by the company and following a modification of the procedures of Van Soest et al. (13).

2.3. In vitro gas production method

The ANKOM^{RF} gas collection system of 250 mL glass jars was used to determine the gas production, digestibility, and metabolic energy of the supplemented alfalfa silage. Approximately 1 g of alfalfa ground was used so as to pass through a 1-mm mesh filter, and this amount was weighed and placed in each of three replicates (= modules). The study was conducted with nine treatments, namely 100 ppm, 200 ppm, and 300 ppm for each essential oil (thymol, eugenol, and cuminaldehyde) and controls. The solutions for the gas production and collection systems were prepared according to Goering and Van Soest (14). Anaerobic conditions were maintained through all stages of the experiment. The rumen fluid was filtered to separate the liquid and solid portions using four layers of cheesecloth and then maintained at 39 °C under an atmosphere of carbon dioxide. The ruminal fluid and the prepared solutions were placed in the modules and allowed to incubate for 96 h at 39 °C in a water bath. The average cumulative pressure was recorded at 5-m intervals by an ANKOM gas production system computer program for 96 h. After this, the cumulative pressure values at 0, 2, 4, 8, 16, 24, 48, 72, and 96 h were converted to a mL of gas at standard pressure and temperature. Following that, the data for the cumulative amount of gas at the 24th h was fitted to the model ($y = a + b\ (1 - e^{-ct})$ produced by Ørskov and McDonald (15). Using the quantities of gas produced, the metabolic energy value (ME_{GP}) and organic matter digestibility (OMD) of the silage were calculated with the equations of Menke and Steingass (16), namely, ME (MJ/kg DM) = 2.2 + 0.136 GP (mL/g DM) + 0.057 CP (g/kg DM) + 0.0029 EE (g/kg DM); Digestibility of organic matter (%) = 57.2 + 0.365 GP + 0.304 CP – 1.98 ADL.

2.4. Statistical analysis

Data generated for chemical composition, in vitro OMD, and the ME of each group were subjected to one-way ANOVA for the groups, and Duncan's multiple range test was applied to compare the means. Gas production volume data were obtained by using the records of the computer program of the ANKOM^{RF} gas production system. Differences were considered significant at $P \leq 0.05$ (17).

3. Results

The nutrients of the alfalfa silage used in the study are given in Table 1. The effects of cuminaldehyde, eugenol, and thymol supplementation in different amounts to the silage on in vitro gas production are shown in Tables 2, 3, and 4, respectively. The effects on digestible organic matter and metabolic energy production are given in Tables 5 and 6, respectively.

4.Discussion

Silage preparation of alfalfa has been used for the preservation of its nutritional quality. However, silage production is restricted due to alfalfa's high buffering capacity, as well as its low levels of dry matter and water-soluble carbohydrates (18). Metabolic processes during the fermentation of alfalfa silage have the potential to alter its original nutritive value. Therefore, thymol, eugenol, and cuminaldehyde were added in vitro to alfalfa silage

Table 1. Composition of alfalfa silage.

Feedstuff	Parameters	%	Parameters	%
Alfalfa silage	DM_{asfeed}, %	24.81	Ash %	10.33
	DM, %	95.60	ADF %	35.02
	EE, %	4.10	NDF %	49.25
	CP, %	17.30	ADL %	6.26

Table 2. Effects of adding different amounts of cuminaldehyde to alfalfa silage on its in vitro gas production (mL/g).

Incubation (hour)	Control	Group 1 (100 ppm)	Group 2 (200 ppm)	Group 3 (300 ppm)	P
2	22.18 ± 0.789a	19.37 ± 1.619ab	16.66 ± 1.472b	16.44 ± 1.190b	*
4	49.65 ± 0.787a	46.39 ± 1.619ab	43.90 ± 1.372b	43.16 ± 1.307b	*
8	82.30 ± 1.441a	79.26 ± 2.346ab	78.02 ± 0.333ab	74.686 ± 0.796b	*
16	114.31 ± 1.867a	108.32 ± 3.724ab	107.26 ± 0.578ab	101.49 ± 1.587b	*
24	168.90 ± 2.991a	160.23 ± 6.205ab	160.42 ± 1.227ab	152.21 ± 1.534b	*
48	191.66 ± 2.072a	188.31 ± 3.927a	186.25 ± 1.135ab	178.29 ± 2.166b	*
72	200.00 ± 1.294a	195.94 ± 2.003ab	190.24 ± 0.569b	180.09 ± 5.055c	*
96	201.91 ± 1.253a	197.29 ± 1.406a	190.16 ± 0.540b	182.20 ± 4.186c	*

*Means within the same row with a different letter are significantly different ($P < 0.05$).

Table 3. Effects of adding different amounts of eugenol to alfalfa silage on its in vitro gas production (mL/g)

Incubation (hour)	Control	Group 1 (100 ppm)	Group 2 (200 ppm)	Group 3 (300 ppm)	P
2	17.34 ± 0.318a	15.65 ± 0.283b	15.54 ± 0.779b	16.07 ± 0.189ab	*
4	35.69 ± 0.671	32.65 ± 0.831	35.65 ± 5.293	37.75 ± 4.219	NS
8	79.61 ± 1.597a	73.86 ± 1.571b	71.16 ± 1.964b	72.52 ± 0.687b	*
16	112.96 ± 2.294a	105.75 ± 1.805ab	96.10 ± 5.413b	101.05 ± 1.733b	*
24	175.90 ± 3.746a	171.06 ± 1.869ab	160.65 ± 5.679b	164.11 ± 3.692ab	*
48	198.15 ± 3.413a	196.11 ± 3.328a	185.13 ± 2.990ab	178.38 ± 8.723b	*
72	219.15 ± 1.676a	208.56 ± 1.881a	194.82 ± 5.075b	191.06 ± 8.230b	*
96	232.72 ± 2.043a	219.99 ± 3.038b	203.56 ± 3.241c	197.92 ± 6.214c	*

NS: nonsignificant; *: means within the same row with a different letter are significantly different ($P < 0.05$).

to examine whether they improved its nutritional value. The nutrient contents of the silage used in this research are given in Table 1. The results of the present study were similar to those of many studies in terms of CP (19–21), ADL (19,21,22), ash (21,23,24), EE (25), NDF (19,21), and ADF (19,21,23).

The mean cumulative gas production of 100-ppm, 200-ppm, and 300-ppm concentrations from each cuminaldehyde, eugenol, and thymol addition to alfalfa silage at 2, 4, 8, 16, 24, 48, 72, and 96 h incubation were significantly lower than those of the control group ($P < 0.05$). Reducing the amount of in vitro gas production through supplementation with essential oils, especially the suppressive effect on energy lost as methane, may indicate a more efficient use of energy. In vitro gas production from alfalfa silage increased with incubation times. Values from the 1st and 2nd mowings were 190.1 mL/g and 204.9 mL/g, respectively, at 96 h in the silages of green alfalfa with no additives (24). These results are similar to those of the control group in the current study. Ce et al. (25) reported maximum gas production of 179 mL/g for alfalfa silage in the control group. The results obtained were lower than those of the control group of the present study. Kamalak et al. (20) stated that gas produced in vitro after 96 h of

Table 4. Effects of adding different amounts of thymol to alfalfa silage on its in vitro gas production (mL/ g).

Incubation (hour)	Control	Group 1 (100 ppm)	Group 2 (200 ppm)	Group 3 (300 ppm)	P
2	15.42 ± 0.768	16.21 ± 1.079	17.54 ± 0.746	15.63 ± 0.654	NS
4	43.122 ± 0.498b	42.55 ± 1.288b	50.34 ± 1.787a	45.16 ± 1.699b	*
8	79.15 ± 0.646c	80.11 ± 1.693c	95.77 ± 3.450a	86.93 ± 1.695b	*
16	123.30 ± 1.138a	115.42 ± 2.858ab	114.58 ± 5.284ab	106.58 ± 1.879b	*
24	179.16 ± 1.225a	169.92 ± 3.479a	158.37 ± 4.978b	149.11 ± 1.731b	*
48	198.76 ± 0.980a	187.04 ± 2.285b	170.91 ± 3.023c	160.16 ± 1.457d	*
72	214.86 ± 2.580a	202.35 ± 1.198b	188.62 ± 2.983c	180.96 ± 1.259d	*
96	217.38 ± 2.099a	203.59 ± 1.613b	194.18 ± 1.571c	184.63 ± 1.645d	*

NS: nonsignificant; *: Means within the same row with a different letter are significantly different (P < 0.05).

Table 5. Effects of adding different amounts of cuminaldehyde, eugenol, and thymol to alfalfa silage on the digestibility of organic matter (%).

Groups	Digestibility of Organic Matter		
	Cuminaldehyde	Eugenol	Thymol
Control	62.39 ± 0.218a	62.90 ± 0.273a	63.14 ± 0.089a
Group 1 (100 ppm)	62.20 ± 0.143a	62.55 ± 0.136a	62.63 ± 0.276a
Group 2 (200 ppm)	61.27 ± 0.417b	61.47 ± 0.460b	61.89 ± 0.288b
Group 3 (300 ppm)	61.36 ± 0.209b	62.14 ± 0.213ab	61.00 ± 0.105c
P	*	*	*

*Means within the same column with a different letter are significantly different (P < 0.05).

Table 6. Effects of adding different amounts of cuminaldehyde, eugenol, and thymol to alfalfa silage on its metabolic energy production (ME_{GP}, MJ /kg DM).

Groups	Metabolic energy		
	Cuminaldehyde	Eugenol	Thymol
Control	8.25 ± 0.081a	8.44 ± 0.101a	8.52 ± 0.033a
Group 1 (100 ppm)	8.17 ± 0.053a	8.30 ± 0.050ab	8.27 ± 0.094a
Group 2 (200 ppm)	7.83 ± 0.155b	8.02 ± 0.154b	7.96 ± 0.135b
Group 3 (300 ppm)	7.86 ± 0.077b	8.12 ± 0.100ab	7.71 ± 0.047b
P	*	*	*

*Means within the same column with a different letter are significantly different (P < 0.05).

incubation ranged between 45.33–78.17 mL/200 mg DM. The amount of gas produced at the 96 h of incubation in the present study was between 182.20 mL and 232.72 mL for each gram of dry matter. Obtained mean gas production values were different from the results by Kamalak et al. (20) because of differences in sample amounts.

In the present study, the cumulative in vitro gas amounts of the controls and treatment groups 1, 2, and 3 for eugenol after 96 h of incubation were 232.72 ± 2.043, 219.99 ± 3.038, 203.56 ± 3.241, and 197.92 ± 6.214 mL/g, respectively. Increasing levels by eugenol during fermentation had a repressive effect on rumen microbial biomass. The in vitro gas production values obtained from the study by Sarıçiçek and Kılıç (26) on alfalfa silage using different additives were lower than those of the present study. Likewise, Mokhtarpour et al. (27) reported lower cumulative gas production values at 24 h and 96 h for alfalfa silage treated with different levels of peanut byproducts. However, Benchaar et al. (28) reported that eugenol at the high concentration of 800 mg/L reduced gas production compared to the control, which is similar to the results of our research. In the present study, the mean in vitro cumulative gas amounts of the control and treatment groups 1, 2, and 3 at the 96 h of incubation with added thymol were 217.38 ± 2.099, 203.59 ± 1.613, 194.18 ± 1.571, and 184.63 ± 1.645 mL/g, respectively. In the present study, the addition of 300 ppm of thymol resulted in a 15.1% reduction in the amount of gas produced compared to the control. Evans and Martin (29) reported that thymol (0.4 g/L) sourced from *Thymus* and *Origanum* plants strongly inhibited in vitro gas production, a finding that is complemented by the results of the current study. The in vitro cumulative amounts of gas from alfalfa silage after 24 h of incubation reported by Opsi et al. (23) and Benchaar et al. (28) are similar to the results of the present study. Pour et al. (30) determined gas production to be within the range of 60.89–68.82 mL at the 96th h of the incubation period for each 200-mg sample of alfalfa silage. In the present study, the cumulative amount of gas production at the 96th h of incubation for each 1000-mg sample ranged from 184.63 mL to 217.38 mL. On a 1000-mg basis, the results of Pour et al. (30) ranged from 304.45 mL to 344.10 mL, which is more than 50% higher than those in the present study. There was a significant difference ($P < 0.05$) between all groups in the current study in terms of the amount of in vitro gas production in all periods, except at the 2nd h of the incubation period.

In the present study, digestible organic matter values for the addition of cuminaldehyde to the control and treatment groups 1, 2, and 3 after 96 h of incubation were 62.39 ± 0.218, 62.20 ± 0.143, 61.27 ± 0.417, and 61.36 ± 0.209%, respectively. The in vitro digestibility values for alfalfa silage of the current study were lower than those of Mokhtarpour et al. (27). These differences are likely to have been influenced by factors such as calculation methods, collection method, and conditions of transportation of rumen fluid, animal feeding regime, and different proportions of the same biota. The digestible organic matter values for the addition of eugenol to the control and treatment groups 1, 2, and 3 after 96 h of incubation for the silage were 62.90 ± 0.273, 62.55 ± 0.136, 61.47 ± 0.460, and 62.14 ± 0.213%, respectively. The decrease in gas production appears to have been associated with the inhibition of feed digestion. The in vitro organic digestibility values of the current study were higher than those of Sarıçiçek and Kılıç (26). Benchaar et al. (28) investigated the effects of eugenol on cultures of rumen fluid and buffers. They determined that eugenol had a statistically significant effect on the in vitro digestibility of organic matter, which is in agreement with results for 200 ppm and 300 ppm of eugenol in the present study. However, Castillejos et al. (8) reported that eugenol did not have a statistically significant effect on the in vitro digestibility of organic matter, which is the same result as for the 100-ppm eugenol group in the present study. In the present study, digestible organic matter values for the control and thymol addition to treatment groups 1, 2, and 3 at the 96th h of incubation of the silage were 63.14 ± 0.089, 62.63 ± 0.276, 61.89 ± 0.288, and 61.00 ± 0.105%, respectively. Benchaar et al. (28) reported that thymol added to a total culture liquid attached to a gas production and collection system did not have a significant effect on organic matter digestibility. However, there was a significant effect in the present study. In contrast, Castillejos et al. (8) reported that thymol reduced organic matter digestibility, which is supported by the results of this study.

The mean ME_{GP} values for the control and treatment groups 1, 2, and 3 of cuminaldehyde at 96 h of incubation were 8.25 ± 0.081, 8.17 ± 0.053, 7.83 ± 0.155, and 7.86 ± 0.077 MJ/kg DM, respectively. The ME_{GP} values obtained by Sarıçiçek and Kılıç (26) and Mokhtarpour et al. (27) were lower than those in the current study. The mean ME_{GP} values for the control and treatment groups 1, 2, and 3 of eugenol at 96 h of incubation were 8.44 ± 0.101, 8.30 ± 0.050, 8.02 ± 0.154, and 8.12 ± 0.100 MJ/kg DM, respectively. The mean ME_{GP} values for the control and treatment groups 1, 2, and 3 of thymol at 96 h of incubation were 8.52 ± 0.033, 8.27 ± 0.094, 7.96 ± 0.135, and 7.71 ± 0.047 MJ/kg DM, respectively. The findings of our study on the lowering of the metabolic energy level by thymol additions to alfalfa were similar to the values reported by Kamalak et al. (20).

The present study demonstrated that cuminaldehyde, eugenol, and thymol added at different amounts to alfalfa

silage reduced the cumulative amount of the greenhouse gases CO_2 and CH_4. In addition, increasing levels of these three essential oils reduced the cumulative amount of gas at the 96th h of incubation, which was in parallel with a reduction in the level of digested in vitro organic matter and metabolic energy. However, the effects of these essential oils on in vivo ruminal fermentation, feed consumption, and performance need to be investigated to confirm the findings of the present study.

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