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RESEARCH ARTICLE

Effect of Thymol on *In Vitro* Gas Production, Digestibility and Metabolizable Energy Content of Alfalfa Hay

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Summary

The objective of this study was to determine the effect of inclusion of essential oil thymol on the incubation on gas production kinetics, volatile fatty acids (VFA), organic matter digestibility (OMD) and metabolizable energy (ME) contents of alfalfa hay. Gas productions were determined at 0, 3, 6, 12, 24, 48, 72 and 96 h incubation times. Thymol were added in the ratio of 0, 50, 100 and 200 mg/L. Gas production kinetics were determined using the equation Y = A (1-exp-ct). The thymol addition had a significant effect on the gas production kinetics, OMD and ME of alfalfa hay. Thymol at 200 mg/L resulted in 22.77% of decrease in potential gas production (A). The mean decrease in potential gas production per mg thymol supplementation was 0.0836 ml. The mean decreases in ME and OMD per mg thymol supplementation were 0.0132 (ME unit) and 0.086 (digestibility unit) respectively. The mean decreases in truly digestible dry matter (TDDM) and neutral detergent fibre (NDFD) per mg thymol supplementation were 0.0546 and 0.0748 digestibility units respectively ($P<0.05$; $P<0.001$). As a conclusion, thymol exhibit significant anti-microbial activity causing an inhibition of the overall fermentation process.

Keywords: *Essential oil, Thymol, In vitro gas production, Volatile fatty acid, Digestibility*

Timol'ün Yoncanın Sindirimi, Rumen Fermantasyonu ve *İn Vitro* Gaz Üretimi Üzerine Etkisi

Özet

Bu çalışmanın amacı, esansiyel yağ olan timol'ün yonca otunun in vitro gaz üretimi, uçucu yağ asidi üretimine, organik madde sindirimi (OMS) ve metabolik enerji (ME) içeriğine etkisini belirlemektir. Gaz üretimi 0, 3, 6, 12, 24, 48, 72 ve 96 saatlerinde belirlenmiştir. Rumen sıvısına timol ise sırasıyla 0, 50, 100 ve 200 mg/L oranlarında ilave edilmiştir. Gaz üretimine ait kinetikler Y=A (1-exp-ct) modeli kullanılarak belirlenmiştir. Rumen sıvısına timol eklenmesi yonca otunun gaz üretimine ait kinetikleri ile OMS ve ME değerini önemli derecede etkilemiştir. 200 mg/L oranında timol ilavesi potansiyel gaz üretim (A) değerinde %22.77 oranında azalmaya neden olmuştur. Bir birimlik timol eklenmesiyle potansiyel gaz üretiminde 0.0836 ml'lik azalmaya neden olmuştur. Bir mg timol eklenmesi sonucu ortalama olarak ME'de 0.0132 birim ve OMS'de ise 0.086 birimlik bir azalma olmuştur. Bir mg timol eklenmesi sonucunda gerçek organik madde sindirimi (GOMS)'nde 0.0546 birim ve nötr deterjan lif (NDF) sindiriminde ise 0.0748 birimlik bir azalma meydana getirmiştir ($P<0.05$; $P<0.001$). Sonuç olarak, timol önemli düzeyde anti-mikrobiyal etki göstermiş olup, genel fermantasyon işlemlerini etkilemiştir.

Anahtar sözcükler: *Esansiyel yağ, Timol, İn vitro gaz üretimi, Uçucu yağ asidi, Sindirim derecesi*

INTRODUCTION

After the prohibition of the use of antibiotics as growth promoters considerable effort has been devoted to essential oils (EO) which are aromatic oily liquids obtained from plants and has antibacterial, antifungal and antioxidant properties [1] to manipulate the rumen metabolism [2-8]. Results of these studies showed variable effects of EO and their derivatives on rumen bacteria and ruminal fermentation [9,10]. Discrepancies among studies

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were attributed to difference in type and doses of EO [9] and differences in technique used in these experiments [11].

Thymol (5-methyl-2-isopropylphenol) is a major essential oil component of thyme (*Thymus vulgaris*) and oregano (*Origanum vulgaris*) [10]. Several researchers have tested the effect of thymol on the ruminal metabolism *in vitro* [10,12]. However little is known regarding the effects of thymol on *in vitro* gas production and fermentation end products. Therefore the objective of this study was to determine the effect of thymol on *in vitro* gas production and fermentation end products of alfalfa hay

MATERIAL and METHODS

Alfalfa plant harvested at flowering stage dried at 105°C and grounded to pass through 1 mm sieves. The ground hay samples were stored in plastics bags for further analysis. Alfalfa hay sample milled through a 1 mm sieve were incubated *in vitro* rumen fluid in calibrated glass syringes following the procedures of [13]. Rumen fluid was obtained from three fistulated sheep fed twice daily with a diet containing alfalfa hay (60%) and concentrate (40%). 0.200 gram dry weight of samples was weighed in triplicate into calibrated glass syringes of 100 mL in the presence (50, 100 and 200 mg/L) and in the absence of thymol (2-isopropyl-5-methylphenol) obtained from Fluka. The syringes were prewarmed at 39°C before the injection of 30 mL rumen 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 3, 6, 12, 24, 48, 72 and 96 h after incubation. Total gas values were corrected for blank incubation. At the end of incubation period, serum bottles were opened. The pH was determined in culture fluid and samples for VFA were collected for analyses. Cumulative gas production data were fitted to non-linear exponential model as: $Y= A(1-\exp^{-ct})$

Where *Y* is gas production at time't', *A* is the potential gas production (ml/200 mg DM), *c* is the gas production rate constant (h^{-1}) and *t* is the incubation time (h). The *in vitro* gas production was completed in the laboratory of Department of Animal science, Faculty of Agriculture, Uludag University, Bursa, Turkey.

ME (MJ/kg DM) content of samples was calculated using equation of Menke *et al*.[14] as follows:

ME (MJ/kg DM) = 2.20 + 0.136 x GP + 0.057 x CP, where GP = 24 h net gas production (ml/200 mg); CP = Crude protein

Organic matter digestibility (%) of samples was calculated using equation of Menke *et al*.[14] as follows:

OMD (%) = 14.88 + 0.889 x GP + 0.45 x CP + 0.0651 x A, where A: ash content (%)

In vitro digestibility with the DAISY Incubator was carried out using rumen fluid was obtained from the same fistulated sheep used in *in vitro* gas production experiment. Rumen fluid (400 ml) was transferred to digestion jar of Daisy^{II} Incubator containing 1600 ml buffer solution and heat-sealed bags containing alfalfa hay samples. Buffer solution was made from two other solutions (A and B) in a ratio 1:5 to obtain a final pH of 6.8 at 39°C. The alfalfa hay samples were incubated in triplicate at 39.5°C for 48 h in the presence (50, 100 and 200 mg/L) and in the absence of thymol from Fluka. At the end of 48 h incubation period, the bags were rinsed with cold top water until water is clear. The rinsed bags were dried at 105°C overnight and weighed to determine TDDM of alfalfa hay samples. The dried bags were placed into the ANKOM200/220 Fiber Analyzer and subjected to the normal procedure for determining NDF content of the residue in the bags to determine NDFD of alfalfa samples.

All chemical analyses were carried out in triplicate. Dry matter (DM) was determined by drying the samples at 105°C overnight and ash by igniting the samples in muffle furnace at 525°C for 8 h. Nitrogen (N) content was measured by the Kjeldahl method [15]. Crude protein was calculated as Nx6.25. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) of alfalfa hay sample were analyzed with the ANKOM fiber analyzer using reagents described by Van Soest [16] and Van Soest and Robertson [17] respectively. The VFA contents of cultured fluid were determined using a gas chromatograph with a semi-capillary FFAP column (Agilent Technologies 6890N GC, Stabilwax-DA, 30 m, 0.25 mm ID, 0.25 um df. Max. temp: 260°C. Cat. 11023), over a temperature range of 45-230°C).

One-way analysis of variance (ANOVA) was carried out to determine the effect of thymol treatment on *in vitro* gas production kinetics, VFA production, TDDM and NDFD using General Linear Model [18] of Statistica for windows. Significance between individual means was identified using the Tukey's multiple range tests. Mean differences were considered significant at ($P<0.05$; $P<0.001$). Standard errors of means were calculated from the residual mean square in the analysis of variance [19].

RESULTS

The proximate chemical composition of Alfalfa hay is presented in *Table 1*.

The effects of thymol inclusion on gas production of alfalfa hay are given in *Fig. 1*. Addition of thymol resulted in decreased gas production at all incubation times.

The effects of thymol on gas production kinetics, organic matter digestibility and metabolizable energy of alfalfa hay are given in *Table 2*. The thymol addition had a

significant effect on the gas production kinetics, organic matter digestibility and metabolizable energy of alfalfa hay. Potential gas production (A) decreased with increasing level of thymol *(Fig 1)*. Thymol at 200 mg/L resulted in 22.77% of decrease in potential gas production (A). Thymol at 200 mg/L also resulted in a 29.20% of decrease in gas production rate (c). The mean decreases in potential gas production per mg thymol supplementation were 0.0836 ml.

Table 1. *Chemical composition of alfalfa hay*
Tablo 1. *Yonca kuru otunun kimyasal bileşimi*

Composition (g/kg DM)	Alfalfa hay
Dry matter	909.1
OM	909.1
CP	187.1
EE	30.7
NDF	500.7
ADF	379.6

OM: Organic matter, CP: Crude protein, EE: Ether extract, NDF: Neutral detergent fibre, ADF: Acid detergent fibre

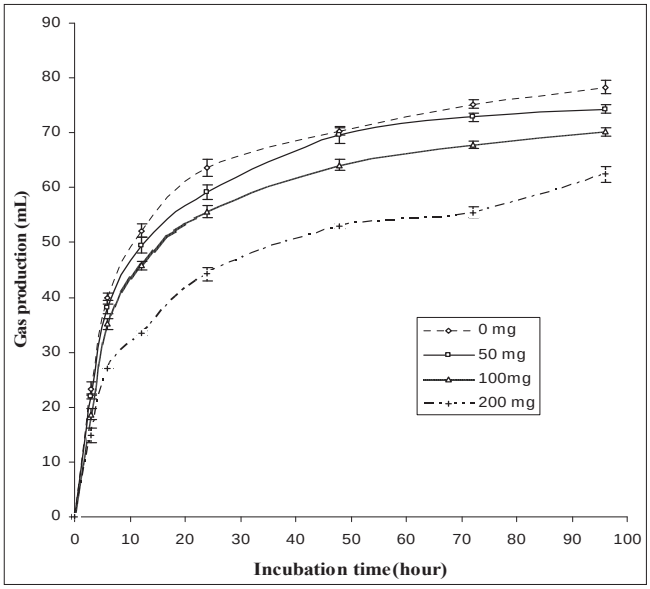

Fig 1. The effect of different doses of thymol on *in vitro* gas production
Şekil 1. Timolun farklı dozlarının *in vitro* gaz üretimi üzerine etkisi

The ME and OMD of alfalfa hay also decreased with increasing level of thymol (*Fig. 3* and *4*). As can be seen from *Table 2* thymol addition at 200 mg/L resulted in

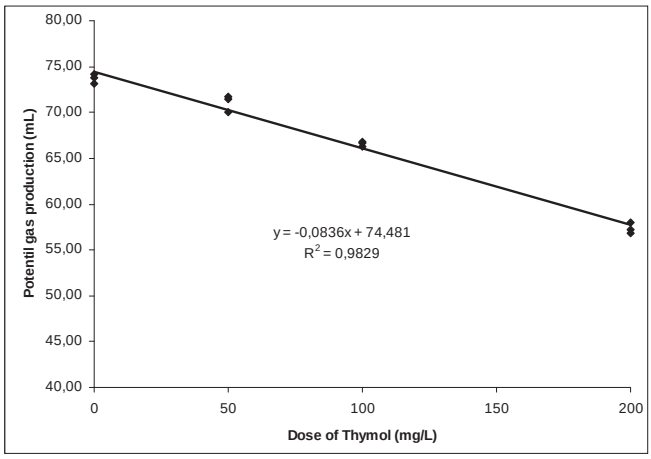

Fig 2. The relationship between different doses of thymol and potential gas production
Şekil 2. Timolun farklı dozları ile potansiyel gaz üretimi arasındaki ilişki

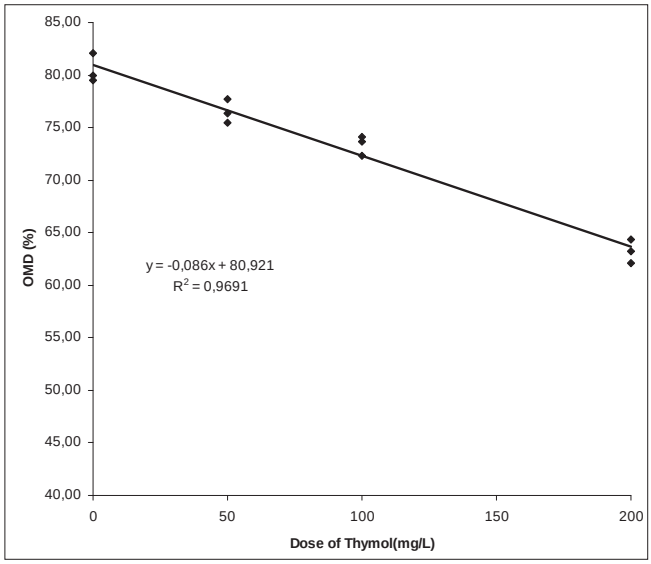

Fig 3. The relationship between different doses of thymol and organic matter digestibility (OMD)
Şekil 3. Timolun farklı dozları ile organik madde sindirimi (OMS) arasındaki ilişki

Table 2. *The effect of different doses of thymol on in vitro gas production kinetics, organic matter digestibility (OMD) and metabolizable energy (ME)*
Tablo 2. *Timolun farklı dozlarının in vitro gaz üretimi kinetikleri, OMS ve ME içeriklerine etkisi*

Parameters	Treatment				SEM	Sig.
	0 mg/L	50 mg/L	100 mg/L	200 mg/L		
A	73.41 [a]	71.18 [b]	66.39 [c]	56.69 [d]	0.371	***
c	0.113[a]	0.107 [a]	0.103 [a]	0.080 [b]	0.004	***
ME	11.93 [a]	11.31 [b]	10.83 [c]	9.28 [d]	0.143	***
OMD	80.49 [a]	76.49 [b]	73.38 [c]	63.23 [d]	0.948	***

*[a b c] Row means with common superscripts do not differ (P>0.05); s.e.m. - standard error mean; Sig. -significance level; c - gas production rate (%); A - potential gas production (mL); OMD - Organic matter digestibility (%); ME- Metabolizable energy (MJ/kg DM); *** P<0.001*

22.21 and 21.44% of decrease in ME and OMD respectively. The mean decreases in ME and OMD per mg thymol supplementation were 0.0132 (ME unit) and 0.086 (digestibility unit) respectively.

The effect of TDDM and NDFD of alfalfa is given in *Table 4*. Thymol had also significant (P<0.001) effect on TDDM and NDFD of alfalfa hay. The TDDM and NDFD of alfalfa hay decreased with increasing level of thymol (*Fig 5* and *6*). The mean decreases in TDDM and NDFD per mg thymol supplementation were 0.0546 and 0.0748 digestibility units respectively.

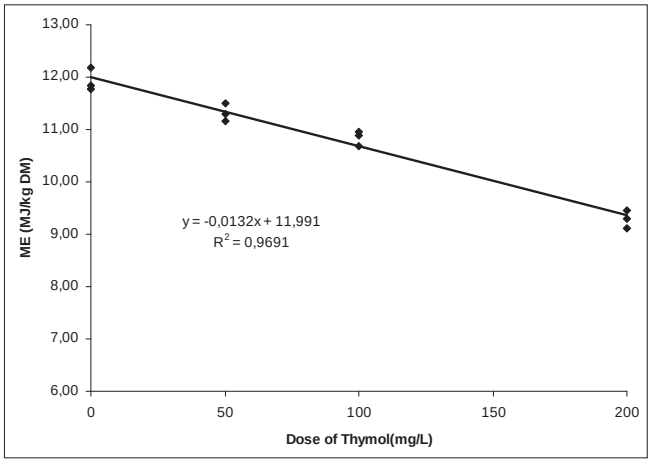

Fig 4. The relationship between different doses of thymol and metabolizable energy (ME)

Şekil 4. Timolun farklı dozları ile metabolik enerji (ME) arasındaki ilişki

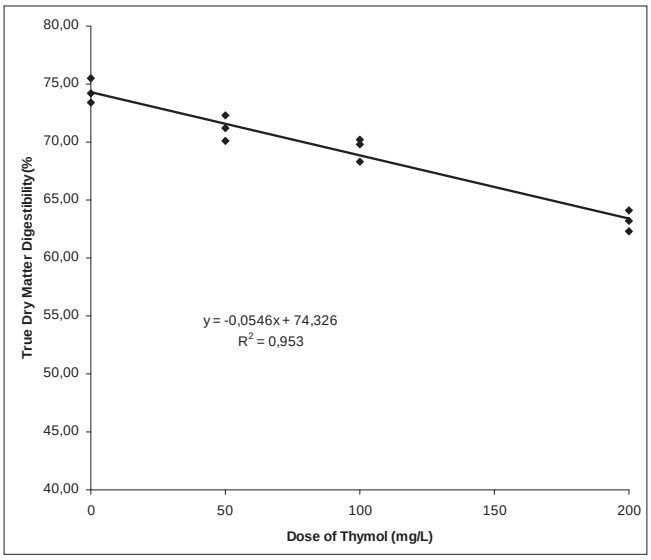

Fig 5. The relationship between different doses of thymol and truly digestible dry matter

Şekil 5. Timolun farklı dozları ile gerçek kuru madde (GKMS) arasındaki ilişki

Table 3. *The effect of different doses of thymol on rumen fermentation*

Tablo 3. *Timolun farklı dozlarının rumen fermantasyon özelliklerine etkisi*

Parameters	Treatments				SEM	Sig.
	0 mg/L	50 mg/L	100 mg/L	200 mg/L		
pH	6.05 ^{c}	6.14 ^{b}	6.17 ^{b}	6.34 ^{a}	0.012	***
Total VFA (mmol/L)	151.92 ^{c}	140.88 ^{b}	135.91 ^{b}	124.90 ^{c}	3.239	***
Acetate (%)	53.61	53.60	53.15	49.91	1.325	NS
Propionate (%)	22.23	22.06	22.03	22.68	0.171	NS
Butyrate (%)	15.16	15.39	15.84	19.00	1.581	NS
Izobutyrate (%)	1.53	2.06	2.08	2.09	0.233	NS
Valerate (%)	3.38	3.38	3.35	3.95	0.189	NS
Izovalerate (%)	3.51	3.46	3.54	2.91	0.250	NS
A:P ratio	2.41 ^{ab}	2.43 ^{b}	2.41 ^{ab}	2.20 ^{a}	0.066	***

*$^{a\,b\,c}$ Row means with common superscripts do not differ (P>0.05); s.e.m. - standard error mean; Sig. - significance level, A:P ratio- acetate/ propionate ratio, *** P<0.001, NS: Non-significant*

Table 4. *The effect of thymol supplementation on true dry matter (TDM) and neutral detergent fiber (NDF) digestibility*

Tablo 4. *Timolun farklı dozlarının gerçek kuru madde (GKMS) ve nötr deterjan lif (NDF) sindirimine etkisi*

Parameters	Treatment				SEM	Sig.
	0 mg/L	50 mg/L	100 mg/L	200 mg/L		
TDDM	74.35 ^{a}	71.19 ^{b}	69.43 ^{b}	63.23 ^{c}	0.812	***
NDFD	66.65 ^{a}	65.64 ^{a}	59.51 ^{b}	52.57 ^{c}	1.017	***

*^{a,b,c} Row means with common superscripts do not differ (P>0.05); s.e.m. - standard error mean; Sig. - significance level; TDDM: Truly digestible dry matter (%); NDF: neutral detergent fiber digestibility (%), *** P<0.001*

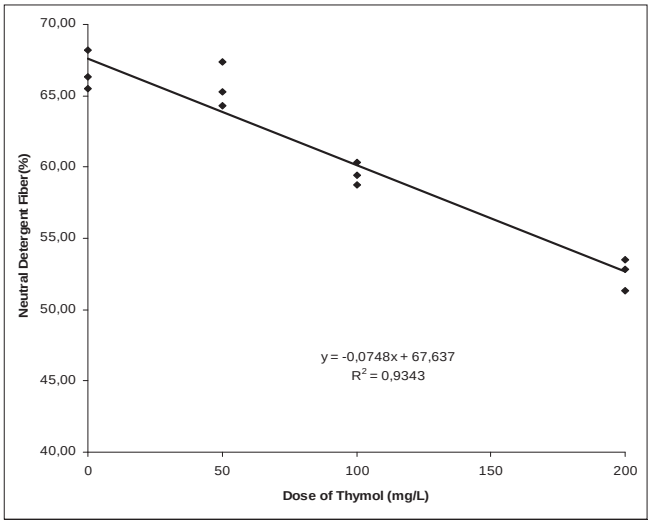

Fig 6. The relationship between different doses of thymol and neutral detergent fiber digestibility

Şekil 6. Timolun farklı dozları ile nötr deterjan lif (NDF) sindirimi arasındaki ilişki

DISCUSSION

Addition of thymol decreased the total gas production with increasing level of thymol. In the current experiment the addition of thymol (200 mg/L) resulted in a 22.77% of decrease in the potential gas production *in vitro*. This result is consistent with finding of Benchaar *et al*.[12]. However Benchaar *et al*.[12] found that addition of thymol (200 mg/L) resulted in only 4% of decrease in total gas production.

In the current experiment the addition of thymol also decreased the gas production rate (h^{-1}). This result is not agreement with finding of Benchaar *et al*.[12] who showed that the addition of thymol had no effect on the gas production rate when administered in a 200 mg/L dose.

It is well known that the gas produced is a sum of direct gas produced as a result of fermentation (CO_2 and methane) and the in direct gas produced from buffering of VFA (CO_2) released from bicarbonate buffer [20]. However gas production is associated with volatile fatty acid (VFA) production following fermentation of substrate so the more fermentation of a substrate the greater the gas production, although the fermentation end products do influence more closely with gas production [20]. In the current experiment the decrease in total gas productions with increasing level of thymol supplementation could be explained by the reduction in total VFA production. Doane *et al*.[21] also found a significant correlation between gas production and VFA production.

The addition of thymol resulted in increase of final pH of *in vitro* batch culture after 96 h incubation times. This result is consistent with those obtained by Castillejos *et al*.[10], Benchaar *et al*.[12] and Evans and Martin [22] also reported that 400, 500 and 200 mg/L of thymol increased final pH in 24 h *in vitro* batch culture incubations. Busquet *et al*.[9] and Castillejos *et al*.[10] suggested that the decrease in pH was resulted from the decrease in VFA concentration, reflecting a reduction in fermentability of diet, which is in agreement with antimicrobial activity of phenolic compounds [11,23]. It was suggested that these phenolic compounds seem to have a detrimental effect on the microbial fermentation when administered at high doses [12]. In the current experiment the addition of thymol decreased total VFA production without affecting the molar proportion of acetate, propionate, butyrate and others. This result is in agreement with finding of Benchaar *et al*.[12] who found that addition of thymol (200 mg/L) decreased the VFA production. The molar proportion obtained in the current experiment was comparable with those obtained by Benchaar *et al*.[12]. In these two experiments the molar proportion of butyrate was considerably higher than the control. On the other hand Castilleous *et al*.[10] suggested that thymol at 500 mg/L decreased the proportion of propionate (-18.4) and increased the proportion of acetate (+1.8). Benchaar *et al*.[12] also showed that thymol at 200 mg/L reduced the molar proportion of propionate compared with the control.

It is well known that the VFA production in the rumen is the main source of energy for ruminant animals. Therefore it was suggested that the decrease in VFA production in rumen may yield adverse nutritional consequences if this same effect was obtained *in vivo* conditions [12]. As can be seen from *Table 2* and *Fig. 2* the estimated ME content of alfalfa hay were decreased with increasing level of thymol supplementation *in vitro* condition. This result consistent with that a decrease in VFA production in rumen may yield adverse nutritional consequences.

The addition of thymol had a significant effect of OMD, ME and TNDFD and DMD of alfalfa hay. Thymol decreased the DMD and OMD *in vitro* possibly due to decrease in fibre digestion and this was attributed to the higher sensitivity of cellulotic bacteria to thymol. As can be seen from *Table 4* and *Fig. 7* NDFD of hay decreased with increasing level of thymol supplementation.

This result is in agreement finding of Castillejos *et al*.[10] who found that addition of thymol (500 mg/L) significantly reduced the DMD, OMD, NDFD and ADFD digestibility.

A decrease in DMD, OMD and NDFD obtained current experiment may be resulted in a decrease in voluntary feed intake of animal when supplemented with thymol. Therefore a reduction in diet digestibility would have an adverse effect on the animal productivity due to low dry matter intake.

In the current experiment, thymol was shown to have antimicrobial activities *in vitro*. This result is in agreement with findings of Özkan *et al*.[8], Benchaar *et al*.[12], Dorman

and Dean [24], Ultee *et al*.[25] and Burt, [26] who found that phenolic compounds such as thymol, carvacol and eugenol had a high microbial activity due to the presence of a hydroxyl group in the phenolic structure. Burt [26] suggested that the antimicrobial activity of these phenolic compounds is through the disturbance of cytoplasmic membrane, disrupting the proton motive force, electron flow active transport, and coagulation of cell contents. It was also shown that two phenolic derivatives, carvacol and thymol, reduced the intracellular ATP pool and increased the extracellular ATP concentration of *E. coli* through the disruptions of the cytoplasmic membrane [27].

These results suggest that thymol exhibit significant anti-microbial activity causing an inhibition of the overall fermentation process. Thymol inclusion had a significant effect on the gas production and such as OMD and ME of alfalfa hay. Thymol inclusion decreased the gas production, VFA production and estimated parameters (OMD and ME contents) of alfalfa hay.

Therefore, before large scale implementation, further investigations are required to determine the effect of thymol on voluntary food intake, animal performance and the profitability of the supplementation *in vivo*.

REFERENCES

1. Cowan MM: Plant products as antimicrobial agents. *Clin Microbiol Rev,* 12, 564-582, 1999.

2. McIntosh FM, Williams P, Losa R, Wallace RJ, Beever DA, Newbold CJ: Effects of a specific blend of essential oil compounds on rumen microorganisms and their protein metabolism. *Appl Envir Micro,* 69, 5011-5014, 2003.

3. Wallace RJ., McEwan NR, McIntosh FM, Newbold CJ: *Proceedings of the 50th Maryland Nutrition Conference on Natural Products for Manipulation of Fermentation in Ruminants,* March 27-28, Timonium, Maryland, pp.116-125, 2003.

4. Newbold CJ, McIntosh FM, Williams P, Loss R, Wallace RJ: Effects of a specific blend of essential oil compounds on rumen fermentation. *Anim Feed Sci Technol,* 114,105-112, 2004.

5. Castillejos L, Calsamiglia S, Ferret A, Losa R: Effects of a specific blend of essential oil compounds and the type of diet on rumen microbial fermentation and nutrient flow from a continuous culture system. *Anim Feed Sci Technol*, 119 (1-2): 29-41, 2005.

6. Garcia V, Catala-Gregori P, Madrid J, Hernandez F, Megias MD, Adrea-Momtemayor HM: Potential of carvacrol to modify *in vitro* rumen fermentation as compared with momensin. *Animal,* 1, 675-680, 2007.

7. Benchaar C, Calsamiglia S, Chaves AV, Fraser GR, Colombatto D, McAllister TA, Beauchemin KA: A review of plant-derived essential oils in ruminant nutrition and production. *Anim Feed Sci Technol*, 145, 209-228. 2008.

8. Özkan O, Sari B, Bayezit M, Doğan A, Akpulat Ha, Erdağ D: Evcil tavşanlarda *Thymus serpyllum*'un koksidiozise karşı etkisi: Oosist atılımı ve canlı ağırlık değişimi. *Kafkas Univ Vet Fak Derg,* 16 (2): 323-327, 2010.

9. Busquet M, Calsamiglia S, Ferret A, Kamel C: Plant extracts affect *in vitro* rumen microbial fermentation. *J Dairy Sci,* 89, 761-771, 2006.

10. Castillejos L, Calsamiglia S, Ferret A: Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in *in vitro* system. *J Dairy Sci,* 89, 2649-2658, 2006.

11. Fraser GR, Chaves AV, Wang Y, McAllister TA, Beauchemin KA, Benchaar C: Assessment of the effects of cinnamon leaf oil on rumen microbial fermentation using two continuous culture systems. *J Dairy Sci,* 90, 2315-2328, 2007.

12. Benchaar C, Chaves AV, Fraser GR, Wang Y, Beuchemin KA, McAllister TA: Effects of essential oils and their components on *in vitro* rumen microbial fermentation. *Can J Anim Sci,* 87, 413-419, 2007.

13. Menke KH, Steingass H: Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Anim Res Develop,* 28, 9-55, 1988.

14. Menke KH, Raab L, Salewski A, Steingass H, Fritzi D, Schneider W: The estimation of digestibility and metabolizable energy content of ruminant feedstuffs from the gas production when they incubated with rumen liquor *in vitro*. *J Agric Sci Camb,* 92, 217-222, 1979.

15. AOAC: Official Method of Analysis. 15th ed., Association of Official Analytical Chemist, Washington, DC. USA, 1990.

16. Van Soest PJ: Use of detergents in the analysis of fibrous feeds. II. A rapid method for the determination of fiber and lignin. *J Assoc off Anal Chem,* 46, 829-835, 1963.

17. Van Soest P, Robertson JB: A Laboratory Manual for Animal Science 612. Cornell University, Ithaca, New York, USA, 1985.

18. Statistica: Minitab Inc: Minitab for Windows, Release 11.1. Minitab Inc., State College, 3081 Enterprise Drive, PA 16801-3008, USA, 1996.

19. Mendeş M, Akkartal E: Comparison of ANOVA F and WELCH tests with their respective permutation versions in terms of type I error rates and test power. *Kafkas Univ Vet Fak Derg,* 16 (5): 711-716, 2010.

20. Blummel M, Orskov ER: Comparison of an *in vitro* gas production and nylon bag degradability of roughages in predicting feed intake in cattle. *Anim Feed Sci Technol,* 40, 109-119, 1993.

21. Doane PH, Schofield P, Pell AN: Neutral detergent fibre disappearance, gas and volatile fatty acids production during the *in vitro* fermentation of six forages. *J Anim Sci,* 75, 3342-3352, 1997.

22. Evans JD, Hrudey SE: A simple apparatus for measuring gas production by methanogenic cultures in serum bottles. *Environ Technol Lett,* 41, 336-340, 1983.

23. Acamovic T, Brooker JD: Biochemistry of plant secondary metabolites and their effects in animals. *Proceedings of the Nutr Society,* 64, 403-412, 2005.

24. Canbolat Ö, Karaman Ş, Filya İ: Farklı kekik yağı dozlarının yemlerin sindirimi ve rumen fermantasyonu üzerine etkileri. *Kafkas Univ Vet Fak Derg,* 16 (6): 933-939, 2010.

25. Dorman DH, Deans SG: Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. *J Appl Microbiol,* 88, 308-316, 2000.

26. Ultee A, Bennik MH, Moezelaar R: The phenolic hydroxyl group of carvacrol is essential for action against the food borne pathogen *Bacillus cereus*. *Appl Envir Micro,* 68, 1561-1568, 2002.

27. Burt S: Essential oils: Their antibacterial properties and potential applications in foods. A review. *Int J Food Microbiol*, 94, 223-253, 2004.

28. Halender IM, Alakomi HL, Latva-Kala K, Mattila-Sandholm T, Pol I, Smid E, Gorris LGM, von Wright A: Characterization of the action of selected essential oil components on gram negative bacteria. *J Agric Food Chem,* 46, 3590-3595, 1998.