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Effects of organic acid treated legume forages on *in-vitro* degradability values

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ABSTRACT: In this context, we aimed to investigate the effects of varying levels of Fumaric Acid (Fu), Malic Acid (Ma), Formic Acid (Fo), and their combinations on in-vitro degradability parameters, organic matter digestibility (OMD), metabolizable energy (ME) and net energy lactation (NEL) of alfalfa (*Medicago polymorphia*), white clover (*Trifolium repens*) and hairy vetch (*Vicia villosa*). Obtained data were analyzed as a completely randomized 3×8 factorial design, with general linear models (GLM) using SAS 9.4. The effects of legume forages and their interaction with organic acid were significant on ME, NEL and OMD values ($p<0.05$, $p<0.01$). In such a way that fumaric acid was led to an increase in NEL. All of the parameters, except ME and OMD, were affected by organic acids ($p<0.01$). To conclude, utilization of organic acids could improve microbial protein synthesis in the rumen.

Keywords: Legume forage, Organic acid, *in-vitro* degradability parameters, ME, NEL and OMD.

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INTRODUCTION

Forages, attained from natural and artificial meadows as well as forage plants, are used ruminant nutrition to improve digestive physiology and reduce the feed cost. Forages are rather nutrient rich and have shown preventive effects against certain metabolic diseases. The feedstuff with high quality forages has a great importance for ruminant nutrition and human economy. The determination of animal forages qualities is important for the pricing of the forages, and appropriate for fulfill specific animal needs. The quality of forage is affected by its type and variety, maturity level at time of harvest, number of forms and height, climate, mass production and storage method. Changes in forage quality, dry matter consumption, ration energy density, addition of concentrated feed, feeding costs, lactation performance have great effect on animal health. According to the ease of applicability in determination of roughage quality; sensory, chemical and biological analysis methods are used (Kaya, 2008; Kilic, 2006; Shaver, 2004; NRC, 2001; Boğa and Ayasan, 2022). In animal feeding, *in-vivo*, *in-sacco* and *in-vitro* techniques are used to determine the value of feed. However, *in-vivo* studies not only affect animal welfare but also are cost- and time-inefficient as well as labor intensive. Therefore, *in-vitro* technique was developed to determine the digestibility of feed by some researchers (Palangi and Macit, 2019). Organic acids have repeatedly shown to eliminate the pathogenic micro- organisms in gastro-intestinal system by lowering pH in silage and increasing the beneficial bacterial population, improve yields in farm animals, preserve animal health by reducing bacterial toxins such as ammonia and amine. Organic acids, in recent years, have been widely used as alternatives to antibiotics especially in ruminant methane mitigation studies (Gul and Tekce, 2017). Organic acids have positive effects on ruminant nutrition and could prevent energy waste of feeds and reduce ruminal methane production (Palangi and Macit, 2021). Yet, information on synergistic, additive, and interactive effects of organic acids are scarce. This study aimed to determine the effects of organic acids on degradability and digestibility values (OMD, ME, NEL contents) of Alfalfa (*Medicago polymorphia*), Vetch (*Vicia villosa*) and Clover (*Trifolium repens*) hay using *in-vitro* gas production technique.

MATERIALS AND METHODS

The ethical approval (protocol no. 75366018- 000-E. 1600251581) for this experiment was taken from Local Ethics Committee of Animal Experiment, Ataturk University.

Animal and feeds

Rumen liquor was collected from two rumen cannulated yearling Awassi Rams. After drying on a feed basis, the samples were ground through a 1 mm screen and were analyzed for a proximate fraction (DM), crude protein (CP), ether extract (EE), crude fiber (CF) and ash according to AOAC (2005).

Gas production

The gas volume of feed samples was measured 0, 3, 6, 12, 24, 48, 72 and 96h of incubation (Palangi, 2019; Taghizadeh et al., 2008). Metabolizable energy (ME) and organic matter digestibility (OMD) of feed raw materials were determined by the following equations reported by Menke and Steingass (1988). Net energy lactation (NE_L) was calculated using the equality reported by Palangi (2019).

$$\text{OMD } \% = 15.38 + 0.8453 \text{ x GP} + 0.0595 \text{ x CP} + 0.0675 \text{ x Ash}$$

$$\text{ME, MJ/kg DM} = 2.20+0.1357\text{xGP} + 0.057\text{x CP} + 0.002859 \text{ x EE}^2$$

$$NE_L \text{ (MJ/kg KM)} = 0.101\text{xGP} + 0.051\text{x CP} + 0.112 \text{ x EE}$$

(GP: 200 mg gas production of 24h, CP: crude protein (%) and EE: Ether Extract (%)).

Statistical Analysis

Statistical analyses were performed using the completely randomized 3×8 factorial design applying a significance level of 0.05 (SAS version 9.2, ANOVA procedure, Duncan's multiple range test).

RESULTS

OMD, ME and NEL contents

The effects of organic acids on OMD, ME and NEL contents of obtained feeds are given in Table 1. The interaction between feed and organic acids had significant effect on all parameters ($P < 0.01$). Formic acid treated alfalfa showed higher amounts of OMD, ME and NEL. In addition, the effect of feed variety on OMD, ME and NEL was significant ($P < 0.01$), while organic acids could only affect NEL ($P < 0.01$). The OMD value was highest and lowest in alfalfa (57.87%) and white clover (52.59%) groups, respectively ($P < 0.01$). According to our results, organic acids had insignificant effect on OMD values of substrate without feed forages (P> 0.01). The lowest and highest OMD values were observed in malic acid treated vetch (49.52%) and formic acid treated white clover groups (61.90%), respectively (P <0.01). The highest NEL and OMD values were observed in Fo, Fu and Fu + Fo acid treated alfalfa groups which were comparable to control group. In vetch samples, while lowest ME, NEL and OMD values were seen in Ma acid treated group, highest ones were observed in Fo acid treated feed and its combinations (Fu + Fo, Fu + Ma, Fo + Ma and Fu + Fo + Ma).

Table 1. Effects of Feed and Organic Acids on Organic Matter Digestibility, Metabolic Energy and Net Energy Lactation Contents

Feeds	OA	ME (MJ/Kg KM)	NE_L (MJ/Kg KM)	OMD (%)
Alfalfa	C (Control)	7.73^{abcd}	5.12^{abc}	59.35^{abcd}
	Fo	8.14^{a}	5.37^{a}	61.90^{a}
	Fu	7.07^{cdefg}	4.28^{def}	52.66^{efg}
	Ma	7.96^{ab}	5.21^{ab}	60.95^{ab}
	Fu+Fo	7.78^{abc}	5.20^{ab}	59.74^{abc}
	Fu+Ma	7.37^{abcde}	4.87^{abcd}	56.89^{abcde}
	Fo+Ma	7.23^{cdef}	4.70^{bcde}	56.02^{bcdef}
	Fu+Fo+Ma	7.10^{cdefg}	4.63^{bcde}	55.48^{cdef}
Vetch	C	6.60^{efg}	4.23^{def}	51.71^{efg}
	Fo	6.83^{efg}	4.46^{def}	53.77^{efg}
	Fu	6.97^{cdefg}	4.52^{cdef}	54.50^{defg}
	Ma	6.24^{g}	3.97^{f}	49.52^{g}
	Fu+Fo	6.79^{efg}	4.38^{def}	53.43^{efg}
	Fu+Ma	$6.92d^{efg}$	4.51^{cdef}	54.11^{efg}
	Fo+Ma	$6.87d^{efg}$	4.48^{def}	53.78^{efg}
	Fu+Fo+Ma	$6.92d^{efg}$	4.51^{cdef}	53.91^{efg}
White Clover	C	6.82^{efg}	4.37^{def}	52.96^{efg}
	Fo	6.73^{efg}	4.29^{def}	52.21^{efg}
	Fu	6.60^{efg}	4.23^{def}	51.64^{efg}
	Ma	6.77^{efg}	4.35^{def}	52.34^{efg}
	Fu+Fo	6.46^{fg}	4.20^{ef}	50.83^{fg}
	Fu+Ma	6.87^{defg}	4.42^{def}	53.44^{efg}
	Fo+Ma	6.91^{defg}	4.54^{cdef}	53.73^{efg}
	Fu+Fo+Ma	6.83^{efg}	4.50^{cdef}	53.59^{efg}
SEM		0.258	0.190	1.604
P		**	**	**
Feeds	Vetch	6.77^{b}	4.38^{b}	53.09^{b}
	White Clover	6.75^{b}	4.36^{b}	52.59^{b}
	Alfalfa	7.55^{a}	4.92^{a}	57.87^{a}
SEM		0.091	0.067	0.567
P		**	**	**
Organic Acids	C	7.05	4.57^{ab}	54.67
	Fo	6.99	4.51^{ab}	54.27
	Fu	7.23	4.71^{a}	55.96
	Ma	6.88	4.34^{b}	52.93
	Fu+Fo	7.05	4.60^{ab}	54.81
	Fu+Ma	7.01	4.59^{ab}	54.67
	Fo+Ma	7.01	4.57^{ab}	54.51
	Fu+Fo+Ma	6.95	4.55^{ab}	54.33
SEM		0.149	0.110	0.926
P		NS	**	NS

$^{a-g:}$ Differences between the averages indicated by different letters in the same column are important.
SEM= Standard Error Means. ** = P <0.01 and NS= P> 0.05

In-vitro gas production parameters

According to the results reported in Table 2, interactions of feeds × organic acids (OA) had significant effect on the in-vitro gas production parameters (P <0.01). Among the gas production parameters related to interactions (feeds × OA), Fu + Ma acid treated alfalfa (in terms of "a" values), and fumaric acid treated (in terms of "b" values) had highest values (P <0.01). Fumaric acid treated alfalfa and malic acid treated vetch showed the lowest "a" values (P <0.01). Moreover, malic acid treated vetch showed the lowest "b" value (P <0.01). The results of *in-vitro* gas production parameters of vetch, clover and alfalfa disagreed with those reported by others.

Table 2. Effects of Feed and Organic Acids on Fermentation Parameters (a, b, c and a+b) of Treated Legume Forages

Feeds	OA	a	b	a+b	c	RSD
Alfalfa	C (Control)	7.22[bcdef]	59.71[cd]	66.93[bcde]	0.070[cde]	3.30
	Fo	6.16[cdefg]	64.26[ab]	70.42[ab]	0.078[bc]	3.36
	Fu	3.80[g]	64.96[a]	68.76[bc]	0.085[ab]	3.06
	Ma	6.10[cdefg]	51.54[hijk]	57.63[jk]	0.063[efghi]	2.58
	Fu+Fo	3.99[g]	60.78[bc]	64.77[def]	0.068[def]	2.63
	Fu+Ma	9.87[a]	50.60[ijk]	60.47[hij]	0.058[ghi]	3.58
	Fo+Ma	8.02[abc]	54.70[efghi]	62.72[fghi]	0.055[hi]	2.42
	Fu+Fo+Ma	8.02[abc]	54.70[efghi]	62.72[fghi]	0.055[hi]	2.42
Vetch	C	5.06[efg]	52.93[ghijk]	57.99[j]	0.075[cd]	2.63
	Fo	0.97[h]	58.48[cde]	59.45[ij]	0.094[a]	2.88
	Fu	1.59[h]	57.88[cdef]	59.47[ij]	0.086[ab]	2.59
	Ma	5.00[fg]	49.15[k]	54.15[k]	0.062[efghi]	1.91
	Fu+Fo	5.70[cdefg]	52.49[hijk]	58.19[j]	0.064[efghi]	2.04
	Fu+Ma	5.70[cdefg]	51.23[hijk]	56.93[jk]	0.057[ghi]	2.23
	Fo+Ma	6.59[cdef]	52.38[hijk]	58.97[ij]	0.044[j]	2.20
	Fu+Fo+Ma	9.51[ab]	50.12[jk]	59.63[ij]	0.046[j]	2.39
White Clover	C	7.71[abcd]	63.85[ab]	71.56[a]	0.067[defg]	3.06
	Fo	7.55[abcde]	58.45[cde]	66.00[cdef]	0.059[fghi]	2.17
	Fu	7.53[abcde]	60.24[bc]	67.77[bcd]	0.061[efghi]	2.42
	Ma	6.62[cdef]	57.07[cdefg]	63.69[efgh]	0.062[efghi]	2.56
	Fu+Fo	6.66[cdef]	53.92[fghij]	60.58[ghij]	0.065[efgh]	2.86
	Fu+Ma	5.48[defg]	57.87[cdef]	64.50[defg]	0.063[efghi]	2.69
	Fo+Ma	6.59[cdef]	57.41[cdef]	62.88[fghi]	0.062[efghi]	1.84
	Fu+Fo+Ma	7.30[bcdef]	55.44[defgh]	62.74[fghi]	0.054[i]	2.67
SEM		0.732	1.315	1.206	0.003	-
P		**	**	**	**	-
Feeds	Vetch	5.02[b]	53.08[b]	58.10[b]	0.066[a]	2.36
	White Clover	6.94[a]	58.03[a]	64.96[a]	0.062[b]	2.53
	Alfalfa	6.65[a]	57.66[a]	64.30[a]	0.067[a]	2.92
SEM		0.259	0.465	0.426	0.001	-
P		**	**	**	*	-
Organic Acids	C	6.66[bc]	58.83[a]	65.49[a]	0.071[b]	3.00
	Fo	5.90[cd]	52.59[c]	58.49[c]	0.062[cd]	2.35
	Fu	4.89[de]	60.40[a]	65.29[a]	0.077[a]	2.80
	Ma	4.31[e]	61.03[a]	65.34[a]	0.078[a]	2.69
	Fu+Fo	7.40[ab]	53.23[c]	60.63[b]	0.059[d]	2.83
	Fu+Ma	5.45[cde]	55.73[b]	61.18[b]	0.066[bc]	2.51
	Fo+Ma	6.70[bc]	54.83[bc]	61.53[b]	0.054[e]	2.15
	Fu+Fo+Ma	8.28[a]	53.42[c]	61.70[b]	0.052[e]	2.49
SEM		0.423	0.759	0.696	0.002	-
P		**	**	**	**	-

[a-k]: Differences between the averages indicated by different letters in the same column are important.SEM= Standard Error Means. ** = P <0.01 and * = P <0.05

a = rapidly soluble fraction (%); b = slowly degradable fraction (%); c = degradation rate constant (%/h) of fraction RSD= Residual Standard Deviation

DISCUSSION

OMD, ME and NEL contents

The low level of organic matter digestibility of feeds with high crude fat content can be explained by the fact that fats are more complex than carbohydrates and are less fermented by bacteria. According to our findings, it can be said that vetch being rich in rumen-soluble nutrients such as NDF, ADF and ADL, reduces the amount of OMD by limiting microbial fermentation. The total amount of daily gas produced during 24 hours of incubation was utilized to calculate metabolic energy and OMD values. Due to the low *in-vitro* gas production values of feeds including high ADF and NDF content, ME and OMD values decreased accordingly. ME, NEL and OMD values determined in this study were higher than those reported by Tunç et al. (2017) and lower than the ones reported by Kamalak et al. (2011), Uslu et al. (2018), Kamalak et al. (2004), Tian et al. (2014), Canbolat and Karaman (2009), Canbolat et al. (2013), Gürsoy and Macit (2015). Nonetheless, our findings are similar to the values reported by Abas et al. (2005) and Hamilton et al. (2005). It was also reported by Kilic and Saricicek (2007) that variety differences among the feeds affect OMD and energy contents. Accordingly, the above-mentioned differences can be attributed to these factors. The *in-vitro* produced gas contains carbon dioxide and methane, which is obtained directly from microbial fermentation and indirectly from the reaction between volatile fatty acids with bicarbonate, accordingly gas production is also linearly related to the volatile fatty acids production and microbial synthesis (Palangi et al., 2022).

In-vitro gas production parameters

Gas production parameters values were higher than the values reported by Sui (2018) (a = 1.51, b = 50.62); Boga et al. (2014) (a = -1.2, b = 47.4); Aydın (2008) (a = 4.05 and b = 67.86); Canbolat and Karaman (2009) (a = 10.2, b = 60.7). Obtained values were lower than those reported by Lei et al. (2018) (a+b = 170.33); Palangi and Safamehr (2016) (a = 21.46, b = 42.66); Kamalak et al. (2011) (a+b = 73.41); and Kamalak (2006) (a+b = 72.12). Nevertheless, our results agreed with the observations of Karabulut et al. (2007) (a+b = 71.37), and Kilic (2005) (a+b = 58.17).

Various studies have considered the rate of gas production (c) as a good indicator of feed digestibility, fermentation and microbial protein synthesis (Elahi et al., 2017; Elghandour et al., 2015; Elahi et al., 2014; Ayasan et al., 2020). Correspondingly, ruminal fermentation and microbial protein synthesis had linear correlation. The rate of fermentation influences microbial protein synthesis per unit volume of gas produced (Palangi, 2019). It has been previously shown that c value is directly related with the activity of rumen microorganisms. Danielsson et al. (2014) showed the effect of microbial papulation on the in-vitro over time parameter and that over time have close relationship with rate of gas production (c).

Residual standard deviation (RSD) is used to express the reliability of the test in *in-vitro* gas production technique studies. The RSD ≤3.5 is an indication of high reliability of data obtained in *in-vitro* gas production technique studies (Kılıç and Sarıçiçek, 2006). Accordingly, we observed a RSD of ≤3.5 meaning our data is reliable. Bruno-Soares et al. (2010) reported a negative relationship (-0.846) between NDF and a, b parameters of feeds. In our study, low values of “a” and “b” parameters for the vetch may be due to its high rate of NDF (45.18%). The difference between our *in-vitro* gas production parameters and others may be due to feed and organic acid types as well as processing techniques applied to the feeds. Yet, additional experiments are required to elucidate the positive effect of legume forage processing with organic acids (alone or in combination) at varying levels on the performance parameters, *in-vitro* gas production and digestibility.

CONCLUSIONS

Per our results adding organic acids does not have any negative effect on ruminal fermentation and could improve ME, OMS and NEL values. In addition, the combination of organic acids leads to increase in ‘a+b’ fraction of gas production, that can improve microbial protein synthesis. It is more appropriate to add fumaric acid to legume roughage from organic acids that are present in individual or combinations. Subsequent *in-vivo* studies in same or higher doses of organic acids are required to confirm the positive effect of organic acids supplementation on ruminal fermentation and feed efficiency. The presence of organic acids in the rumen can improve microbial protein synthesis, which could provide the protein needed by ruminants.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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COMPLIANCE WITH ETHICAL STANDARDS

The ethical approval (protocol no. 75366018- 000-E. 1600251581) for this experiment was taken from Local Ethics Committee of Animal Experiment, Atatürk University.

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REFERENCES

Abas I, Ozpınar H, Kutay HC, Kahraman R, Eseceli H (2005) Determination of the metabolizable energy (ME) and net energy lactation (NEL) contents of some feeds in the Marmara region by *in-vitro* gas technique. Turkish Journal of Veterinary and Animal Sciences 29: 751-757.

AOAC (2005) Official Methods of Analysis of AOAC International. 18th ed. Assoc. Off. Anal. Chem., Arlington.

Ayaşan T, Çetinkaya N, Aykanat S, Çelik C (2020) Nutrient contents and *in-vitro* digestibility of different parts of corn plant. South African Journal of Animal Science 50(2): 302-309.

Aydin D (2008) Fertilizer instead of Romanian liquid in *in-vitro* gas production technique. Master Thesis. K.S.İ.Ü. Science. Ins. Kahramanmaras. Turkey.

Boga M, Ayasan T (2022) Determination of Nutritional Value of Alfalfa Varieties and Lines by Using the *in-vitro* Method and Gas Production Technique. Journal of the Hellenic Veterinary Medical Society 73(1): 3613-3620.

Boga M, Yurtseven S, Kilic U, Aydemir S, Polat T (2014) Determination of nutrient contents and *in-vitro* gas production values of some legume forages grown in the Harran plain saline soils. Asian-Australasian Journal of Animal Sciences 27(6): 825-831.

Bruno-Soares AM, Cadima J, Matos T (2010) Predicting degradability parameters of diets for ruminants using regressions on chemical components. Journal of the Science of Food and Agriculture 90: 949-955.

Canbolat O, Kara H, Filya İ (2013) Comparison of *in-vitro* gas production, metabolic energy, organic matter digestion and microbial protein production of some legume roughages. The Journal of Agricultural Faculty of Bursa Uludag University 27: 71-81.

Canbolat O, Karaman Ş (2009) *In-vitro* gas production, organic matter digestion, relative feed value and metabolic energy contents of some legume roughages. Journal of Agricultural Sciences 15 (2): 188-195.

Danielsson R, Werner-Omazic A, Ramin M, Schnurer A, Griinari M, Dicksved J, Bertilsson J (2014) Effects on enteric methane production and bacterial and archaeal communities by the addition of cashew nut shell extract or glycerol-An *in-vitro* evaluation. Journal of Dairy Science 97: 5729-5741.

Elahi MY, Kargar H, Salehi Dindarlou M, Kholif AE, Elghandour MY, Rojas-Hernandez S, Odongo NE, Salem AZM (2017) The chemical composition and *in-vitro* digestibility evaluation of almond tree (Prunus dulcis D. A. Webb syn. Prunus amygdalus; var. Shokoufeh) leaves versus hulls and green versus dry leaves as feed for ruminants. Agroforestry Systems 91: 773-780.

Elahi MY, Nia MM, Salem AZM, Mansouri H, Olivares-Perez J, Kholif AE (2014) Effect of polyethylene glycol on *in-vitro* gas production kinetics of Prosopis cineraria leaves at different growth stages. Italian Journal Animal Science 13: 363-368.

Elghandour MMY, Kholif AE, Marquez-Molina O, Vazquez- Armijo JF, Puniya AK, Salem AZM (2015) Influence of individual or mixed cellulase and xylanase mixture on *in-vitro* rumen gas production kinetics of total mixed rations with different maize silage and concentrate ratios. Turkish Journal of Veterinary and Animal Sciences 39(4): 435-442.

Gul M, Tekce E (2017) Organic Acids: Organic Acids and Their Use in Animal Nutrition. Animal Nutrition and Nutritional Diseases 3 (1): 57-63.

Gursoy E, Macit M (2015) Determination of *in-vitro* gas production values of some legume forage crops grown naturally in the province of Erzurum. Anadolu Journal of Agricultural Sciences 30 (3): 292-299.

Hamilton MA, Murray BR, Cadotte MW, Hose GC, Baker AC, Harris CJ, Licari D (2005) Life-history correlates of plant invasiveness at regional and continental scales. Ecology Letters 8(10): 1066-1074.

Kamalak A (2006) Determination of nutritive value of leaves of a native grown shrub Glycyrrhiza glabra L. using *in-vitro* and in situ measurement. Small Ruminant Research 64: 268-278.

Kamalak A, Canbolat O, Ozkan CO, Atalay AI (2011) Effect of thymol on *in-vitro* gas production, digestibility and metabolizable energy content of alfalfa hay. Kafkas Universitesi Veteriner Fakultesi Dergisi 17(2): 211-216.

Kamalak A, Canbolat O, Gurbuz Y, Ozay O, Ozkan CO, Sakarya M (2004) Chemical composition and *in-vitro* gas production characteristics of several tannin containing tree leaves. Livestock Research for Rural Development 16 (6).

Karabulut A, Canbolat O, Kalkan H, Gurbuzol F, Sucu E, Filya I (2007) Comparison of *in-vitro* gas production, metabolizable energy, organic matter digestibility and microbial protein production of some legume hays. Asian-Australasian Journal of Animal Sciences 20(4): 517-522.

Kaya S (2008) Relative feed value and relative roughage quality index in evaluation of roughages. Turkish Journal of Scientific Reviews 1(1): 59-64.

Kilic A (2006) Determination of quality in roughages. Assistant Course Book. Hasad Publishing, 159 p.

Kilic U, Saricicek BZ (2006) Factors affecting results in *in-vitro* gas production technique. Animal Products 47 (2): 54-61.

Kilic U, Saricicek BZ (2007) Effects of roughage storage type on *in-vitro* gas production amount, gas production parameters and energy contents. 5. National Animal Science Congress, P.69. 05-08 September, Van.

Kilic U (2005) Determination of Some Fermentation Products and Energy Content by Using *in-vitro* Gas Production Technique of Some Feed Raw Materials Used in Ruminant Feeding. Ondokuz Mayıs Univ. Science Bil. Inst. (Doctoral Thesis) Samsun.

Lei Y, Hannoufa A, Prates LL, Shi H, Wang Y, Biligetu B, Christensen D, Yu P (2018) Effects of TT8 and HB12 silencing on the relations between the molecular structures of alfalfa (Medicago sativa) plants and their nutritional profiles and *in-vitro* gas production. Journal of Agricultural and Food Chemistry 66: 5602−5611.

Menke KH, Steingass H (1988) Estimation of the energetic feed value obtained from chemical analysis and *in-vitro* gas production using rumen fluid. Animal Research and Development 28: 7-55.

NRC (2001) Nutrient Requirements of Dairy Cattle. 7th Rev. Ed. National Academic Sci., Washington, DC.

Ørskov ER, McDonald I (1979) The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. The Journal of Agricultural Science 92: 499-503.

Palangi V, Macit M, Nadaroglu H, Taghizadeh A (2022) Effects of green-synthesized CuO and ZnO nanoparticles on ruminal mitigation of methane emission to the enhancement of the cleaner environment. Biomass Conversion and Biorefinery. 1-9.

Palangi V, Macit M (2021) Indictable mitigation of methane emission using some organic acids as additives towards a cleaner ecosystem. Waste and Biomass Valorization 12: 4825-4834.

Palangi V, Macit M (2019) In situ crude protein and dry matter ruminal degradability of heat-treated barley. Revue de Medecine Veterinaire 170: 123-128.

Palangi V, Safamehr A (2016) The determination of nutritive value of alfalfa in different cuts using nylon bags and gas production techniques. Journal of Research in Animal Nutrition 2(3): 1-7.

Palangi V (2019) Effects of processing legume forages with organic acids on *in-vitro* gas production, rumen fermentation and methane production. Ataturk Uni. Department of Animal Science. (Doctoral Thesis) Erzurum. Turkey.

SAS Institute Inc (2018) SAS/CONNECT® 9.4 User's Guide. Fourth Edition. Cary. NC: SAS Institute Inc.

Shaver RD (2004) Forage quality variation. Mid-South Ruminant Nutrition Conference, Arlington.

Snedecor GW, Cochran WA (1976) Statistical methods (6th ed.). Ames, Iowa: The Iowa State University Press.

Sui MX (2018) Correlation research between gas production characteristics and CNCPS components for roughages. 4th International Conference on Agricultural and Biological Sciences. Earth and Environmental Science 185: 012015.

Taghizadeh A, Safamehr A, Palangi V, Mehmannavaz Y (2008) The determination of metabolizable protein of some feedstuffs used in ruminant. Research Journal of Biological Sciences 3: 804-806.

Tian J, Yu Y, Yu Z, Shao T, Na R, Zhao M (2014) Effects of lactic acid bacteria inoculants and cellulase on fermentation quality and *in-vitro* digestibility of Leymus chinensis silage. Grassland Science 60: 199-205.

Tilley JMA, Terry RAA (1963) Two-stage technique for the *in-vitro* digestion of forage crops. Journal of the British Grassland Society 18: 104-111.

Tunc AE, Cufadar Y, Yaman S (2017) Estimation of relationship between *in situ* and *in-vitro* rumen protein degradability of extruded full fat soybean. Turkish Journal of Agriculture - Food Science and Technology 5(10): 1237-1242.

Uslu OS, Kurt O, Kaya E, Kamalak A (2018) Effect of species on chemical composition, metabolizable energy, organic matter digestibility and methane production of some legume plants grown in Turkey. Journal of Applied Animal Research 46(1): 1158-1161.

Van Soest PJ, Wine RH, Moore LA (1966) Estimation of the true digestibility of forages by the *in-vitro* digestion of cell walls. In: Proceedings of the Xth International Grassland Congress. Finnish Grassl. Assoc., Helsinki, Finland, pp. 438-441.