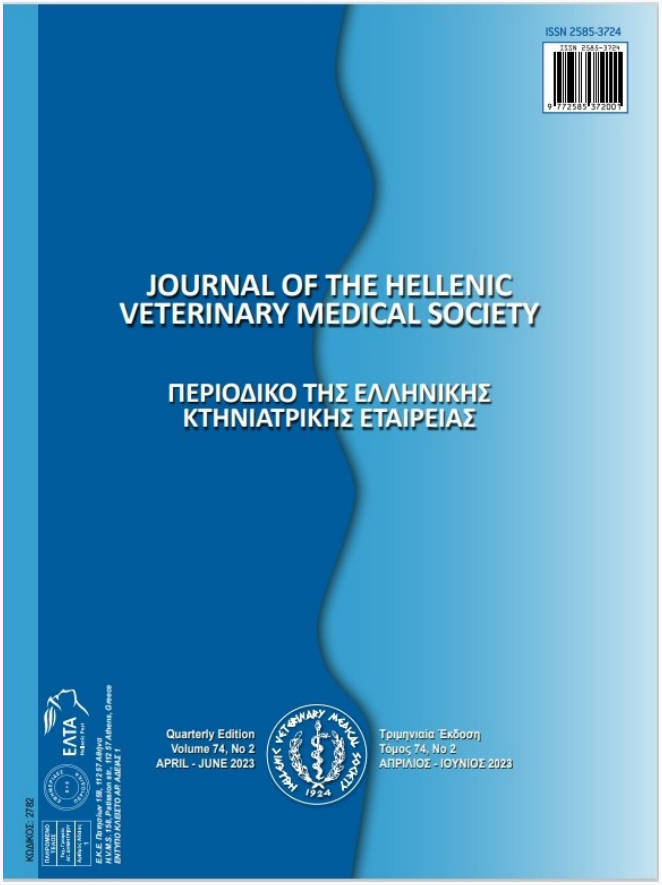
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Research article
Ερευνητικό άρθρο

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).

OMD % = 15.38 + 0.8453 x GP + 0.0595 x CP + 0.0675 x Ash

ME, MJ/kg DM = 2.20+0.1357xGP + 0.057x CP + 0.002859 x EE^2

NE_L (MJ/kg KM) = 0.101xGP + 0.051x CP + 0.112 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, or-

ganic 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, Ataturk University.

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