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Comparison of *In vitro* Gas Production, Metabolizable Energy, Organic Matter Digestibility and Microbial Protein Production of Some Legume Hays

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ABSTRACT : The aim of this study was to compare *in vitro* gas production kinetics, metabolizable energy (ME), organic matter digestibility (OMD) and microbial protein (MP) production of widely used legume hays in ruminant nutrition in Turkey. Gas production were determined at 0, 3, 6, 12, 24, 48, 72 and 96 h and their kinetics were described using the equation $p = a+b\ (1-e^{-ct})$. There were significant differences among legume hays in terms of chemical composition. The crude protein content of legume hays ranged from 11.7 to 18.6% of dry matter (DM); crude fat from 2.1 to 3.5% DM; neutral detergent fiber from 35.6 to 52.0% DM; acid detergent fiber from 32.0 to 35.5% DM and acid detergent lignin 1.7 to 11.0% DM. Total gas production after 96 h incubation ranged between 61.67 and 76.00 ml/0.200 g of substrate. At 24, 72 and 96 h incubation the total gas production for common vetch were significantly ($p<0.01$) higher than those of the other legume hays. The ME, OMD and MP of legume hays ranged from 9.09 to 11.12 MJ/kg DM, 61.30 to 75.54% and 90.35 to 138.05 g/kg DM, respectively. The ME, OMD and MP of common vetch was significantly ($p<0.01$) higher than those of the other hays due to low cell-wall contents and high crude protein. At the end of the experiment, differences in chemical composition of legume hays resulted in the differences in the *in vitro* gas production, gas production kinetics and the estimated parameters such as ME, OMD and MP. Common vetch can be recommended to hay producers and ruminant breeders, due to high ME, OMD and MP production. (**Key Words** : Legume Hay, Gas Production, Digestibility, Metabolizable Energy, Microbial Protein)

INTRODUCTION

Gas production methods has been used to determine the rate and extent of dry matter (DM) degradation and the effect of the some anti nutritive factors such as tannin since *in vitro* methods are less expensive, less time consuming, allow more control of experimental conditions than *in vivo* experiments. Fermentation of substrate by rumen micro-organisms results in production of short chain volatile fatty acids (VFAs) and microbial protein (MP) and gases (Blummel et al., 1997a).

The MP produced in the rumen by micro-organisms is the major source of protein for the ruminants and the prediction of efficiency of MP production is very important in ruminant nutrition (Leng, 1993; Srinivas and Krishnamoorthy, 2005). There are several factors affecting MP production in the rumen. Crude protein (CP) and carbohydrates content of feedstuffs effects of MP yield (Sinclair et al., 1995). It has been also reported that carbohydrate intake, protein intake and carbohydrate source, protein source and fermentation rate in the rumen had a significant effect on the MP production (Rymer and Givens, 1999; Chang et al., 2005).

Legumes have a symbiotic relationship with atmospheric nitrogen (N) fixing bacteria that live in root nodules and that make legumes independent of N fertilization. As a consequence, legume hays commonly have higher concentrations of CP than grasses. In addition, legume hays usually are higher than grasses in their concentrations of pectins, lignin, and calcium, and are lower in neutral detergent fiber (NDF) and cellulose. At comparable stages of maturity, legume and grass hays are about equal in concentrations of acid detergent fiber (ADF) (Duane, 1997). Legumes such as alfalfa, sainfoin, common vetch, pea and white clover are important forages on many dairy cattle farms in Turkey. The perennial and annual forage legumes are widely grown for hay in Turkey. The main reason of this interest is to enhance the ruminants performance, because of their high protein contents.

The aim of this experiment was to compare the gas production kinetics, metabolizable energy (ME), organic

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matter digestibility (OMD) and MP production of widely used legume hays in ruminant nutrition in Turkey.

MATERIALS AND METHOD

Hay samples

The following legume hays were used in this experiment: alfalfa (*Medicago sativa* L.), sainfoin (*Onobrychis sativa* L.), common vetch (*Vicia sativa* L.), pea (*Pisum sativum* L.), white clover (*Trifolium repens* L.) and chick pea (*Cicer arietinum* L.). All legumes were grown in the Experimental Station (40°14' N, 28°50' E) of Agricultural Faculty of Uludag University, Bursa. The sampling area is located at an altitude of 105 m above sea level. The mean annual rainfall and temperature are 729 mm and 15°C. Forages were harvested by hand at flowering maturity stage. Six representative fresh samples (approximately 6×1 kg of each) were taken from each legume and those samples were dried at 60°C for 48 h in a fan-assisted oven.

Chemical analysis

After drying samples were milled through a 1 mm sieve for chemical analysis and *in vitro* gas production procedure. The DM was determined by drying the samples at 105°C overnight and ash was determined by igniting the dry samples in muffle furnace at 550°C for 4 h. The crude fat (CF) content was analyzed using the ether-extraction method. Nitrogen content was measured by the Kjeldahl method (AOAC, 1990). The CP was calculated as N×6.25. The OM was calculated as the difference between DM and ash. The NDF, ADF and acid detergent lignin (ADL) were analyzed using the sodium sulphite addition method without α-amylase and expressed with residual ash (Van Soest et al., 1991).

In vitro gas production

The milled samples were incubated in triplicate in rumen fluid in glass syringes following the procedures of Menke and Steingass (1988). Rumen fluid was obtained from three fistulated Merino sheep fed twice daily with a diet containing alfalfa hay (60%) and concentrate (40%). The concentrate consisted of wheat (74%), sunflower meal (24%), $CaCO_3$ (1.4%), NaCl (0.5%) and mixture of vitamins and minerals (0.1%). The artificial rumen fluid consisted of (added in order) 500 ml H_2O, 0.1 ml solution A, 200 ml solution B, 200 ml solution C, 1 ml resazurin (0.1%, w/v) solution D, and 40 ml reduction solution E. This mixture was then kept under CO_2 in a 39°C water bath and stirred using a magnetic stirrer.

Solution A consisted of 13.2 g $CuCl_2 2H_2O$, 10.0 g $MnCl_2 4H_2O$, 1.0 g $CoCl_2 6H_2O$, 8.0 g $FeCl_2 6H_2O$ and made up to 100 ml with water. Solution B consisted of 35 g $NaHCO_3$ and 4 g NH_4HCO_3 added up to 1,000 ml with water. Solution C consisted of 5.7 g Na_2HPO_4, 6.2 g KH_2PO_4, 0.6 g $MgSO_4 7H_2O$ added up to 1,000 ml with water. The solution D consisted of 0.5 g resazurin up to 100 ml with water. The solution E is the reduction solution consisted of 95 ml H_2O, 4 ml 1 N-NaOH and 625 mg Na_2S9H_2O.

Approximately 0.200 g dry weight of sample was weighed into calibrated glass syringes (Fortuna®, Häberle Labortechnik, Germany) of 100 ml. The syringes were pre-warmed at 39°C before the injection of 30 ml rumen fluid-buffer mixture consisting of 10 ml rumen liquor and 20 ml digestion medium into each syringe followed by incubation in a water bath at 39°C. Readings of gas production were recorded before incubation (0) and after 3, 6, 12, 24, 48, 72 and 96 h of incubation. Total gas values were corrected for blank incubation. Cumulative gas production data were fitted to the model of Ørskov 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 (h)

a+b = the potential gas production (ml)

t = incubation time (h)

y = gas produced at the time "t"

ME (MJ/kg DM) content of legume hays was calculated using equation of Menke et al. (1979) as follows:

$$(MJ/kg\ DM) = 2.20+0.136\ GP+0.057\ CP+0.0029\ CF^2$$

Where

GP: 24 h net gas production (ml/200 mg)

CP: Crude protein

CF: Crude fat

OMD (%) of legume hays was calculated using equation of Menke et al. (1979) as follows:

$$OMD\ (\%) = 14.88+0.889GP+0.45CP+0.0651XA$$

Where

XA = Ash content (%)

Microbial protein synthesis

Approximately 0.500 g dry weight of sample was weighed into calibrated glass syringes of 100 ml. At the end of 24 h incubation, gas volume was read and the contents of the syringe were transferred quantitatively to tubes and syringes were washed twice with distilled water. Contents

Table 1. The chemical composition of legume hays (n = 4)

Legume hays	Chemical constituents (%)							
	DM	OM	CP	CF	Ash	NDF	ADF	ADL
Alfalfa	91.4[a]	90.6[c]	17.1[d]	3.1[c]	9.4[b]	43.4[b]	30.4[c]	8.8[b]
Sainfoin	91.3[a]	93.5[a]	15.9[e]	2.8[d]	6.5[f]	42.0[c]	35.5[a]	11.0[a]
Common vetch	89.2[c]	89.8[d]	18.1[b]	3.5[a]	10.2[a]	35.6[d]	31.0[e]	7.9[b]
Pea	88.4[d]	91.0[e]	18.6[a]	3.3[b]	9.0[d]	35.9[d]	33.3[b]	1.7[d]
White clover	90.2[b]	90.8[c]	16.6[c]	3.2[c]	9.2[c]	37.1[d]	34.3[ab]	5.0[c]
Chick pea	91.0[a]	91.6[d]	11.7[f]	2.1[e]	8.4[e]	52.0[a]	34.8[a]	8.4[b]
SEM	0.02	0.59	1.41	0.26	0.60	4.37	1.59	4.83
Sig.	**	*	**	**	**	**	**	**

Means within columns with unlike superscript differ significantly. * $p<0.05$; ** $p<0.01$.
SEM = Standard error mean, Sig. = Significant level.
DM = Dry matter, OM = Organic matter, CP = Crude protein, CF = Crude fat, NDF = Neutral detergent fiber, ADF = Acid detergent fiber, ADL = Acid detergent lignin.

were centrifuged at 21,000 g for 30 min at 4°C. The supernatant fraction was removed with a pipette and stored frozen until analyzed for ammonia-N using a Kjeldahl method (Blummel et al., 1997b). Ammonia-N was determined in the supernatant fraction by steam distillation; 2 ml 1 M NaOH was added to 5 ml of the supernatant fraction diluted with 30 ml water and the solution was directly distilled and ammonia-N evolved was collected into boric acid (30 g/L). The distillate was titrated with 0.05 M H_2SO_4. The microbial pellets were washed, lyophilized in the centrifuge tubes, and the tubes containing the pellets were weighed. The N in this residue was also determined. For determination of neutral detergent insoluble N (NDIN), syringe contents after incubation were digested with ND, filtered using crucibles (porosity = 1), and the residue on the crucibles was dried and subjected to N analysis. The N in the syringe at the beginning of the incubation was added the amount of N in the buffered rumen fluid added to the syringe, and N in the buffered rumen fluid was determined similar to that for supernatant (Makkar, 2005).

Statistical analysis

The data were analyzed as a completely randomized design and subjected to one-way analysis of variance by the General Linear Model of Statistica for windows (1993). Significance between individual means was identified using the Tukey's multiple range tests (Pearse and Hartley, 1966). Mean differences were considered significant at $p<0.05$ and $p<0.01$. Standard errors of means were calculated from the residual mean square in the analysis of variance.

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of legume hays is given in Table 1. There were significant ($p<0.05$; $p<0.01$) differences among hays in terms of chemical composition. The CP content of legume hays ranged from 11.7 to 18.6% DM. The CP content of pea was significantly ($p<0.01$) higher than those for the other hays. The CP contents of hays are consistent with the findings of Ensminger et al. (1990) and Filya et al. (2002). The ash content of legume hays ranged from 6.5 to 10.2% DM. The ash content of common vetch was significantly ($p<0.01$) higher than the other hays. The ash content of legume hays studied in this experiment was considerably higher than those obtained by Morrison (1956) whereas the ash content of alfalfa was comparable with that reported by Kamalak et al. (2005a).

There were significant ($p<0.01$) differences in cell-wall contents among legume hays. The NDF, ADF and ADL contents of hays ranged from 37.1 to 52.0, 30.4 to 35.5 and 1.7 to 11.0% DM, respectively. The NDF content of chick pea was significantly ($p<0.01$) higher than those for the other hays. However, legume hays such as pea, common vetch and white clover had similar NDF contents. The NDF content of legume is consistent with findings of Ensminger et al. (1990). The NDF content of alfalfa hay was in agreement with the findings of Kamalak et al. (2005a) and Ozturk et al. (2006). The ADF and ADL contents of sainfoin were significantly ($p<0.01$) higher than those for the other hays. The ADF and ADL contents of legume hays were comparable with the findings of Ensminger et al. (1990).

In vitro gas production

Data of gas production during the incubation periods are given in Table 2. Significant differences among the legume hays were recorded. The cumulative gas production increased during the incubation period. Gas produced after 96 h incubation ranged between 61.67 and 76.00 ml per 0.200 g of substrate. At 3 h incubation the gas production for common vetch was significantly ($p<0.01$) higher than those of the other hays. At 6 h incubation gas production for common vetch was significantly ($p<0.01$) higher than those of alfalfa, sainfoin and chick pea hays. At 24, 72 and 96 h incubation the gas production for common vetch were significantly ($p<0.01$) higher than those of the other hays. The gas production from quickly soluble fraction (a) of

Table 2. Gas production (ml/200 mg) and some estimated parameters of legume hays when incubated with rumen fluid

Item	Legume hays						SEM	Sig.
	Alfalfa	Sainfoin	Common vetch	Pea	White clover	Chick pea		
Incubation time (h)								
3	14.67^{cd}	14.33^{d}	20.67^{a}	17.67^{b}	16.67^{bc}	11.33^{e}	0.707	**
6	24.67^{b}	22.33^{c}	27.33^{a}	26.33^{ab}	25.67^{ab}	19.33^{d}	0.745	**
12	36.67^{b}	32.00^{c}	41.67^{a}	37.33^{b}	40.00^{a}	32.00^{c}	0.816	**
24	53.33^{b}	51.00^{c}	58.33^{a}	51.33^{bc}	50.00^{c}	45.67^{d}	0.720	**
48	63.67^{b}	60.33^{bc}	69.00^{bc}	61.67^{a}	59.67^{cd}	56.33^{d}	1.097	**
72	69.33^{b}	64.67^{c}	73.67^{a}	66.33^{bc}	64.77^{c}	59.33^{d}	1.027	**
96	72.67^{b}	67.67^{c}	76.00^{a}	70.33^{c}	67.00^{d}	61.67^{e}	0.692	**
Estimated parameter								
c	0.056^{a}	0.051^{b}	0.056^{a}	0.049^{b}	0.055^{a}	0.057^{a}	0.061	**
a	6.36^{bc}	5.60^{c}	10.56^{a}	10.70^{a}	8.80^{ab}	2.43^{d}	0.942	**
b	65.00^{a}	61.43^{b}	64.83^{a}	58.27^{bc}	56.60^{c}	58.47^{bc}	1.083	**
a+b	71.37^{b}	67.03^{cd}	75.40^{a}	68.97^{bc}	65.40^{d}	60.90^{e}	1.083	**

Means within rows with unlike superscript differ significantly. * $p<0.05$; ** $p<0.01$.
SEM = Standard error mean. Sig. = Significant level.
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 (h).
a+b = potential gas production (ml).

Table 3. Correlation coefficients (r) between gas production and chemical composition

	Chemical constituents					
	CP	CF	Ash	NDF	ADF	ADL
Incubation times (h)						
3	0.809**	0.869**	0.565*	-0.833**	-0.418^{NS}	-0.377^{NS}
6	0.893**	0.909**	0.605**	-0.862**	-0.394^{NS}	-0.450^{NS}
12	0.717**	0.800**	0.784**	-0.768**	-0.638*	-0.437^{NS}
24	0.701**	0.796**	0.497*	-0.611*	-0.109^{NS}	-0.040^{NS}
48	0.643*	0.736**	0.552*	-0.547*	-0.040^{NS}	-0.038^{NS}
72	0.722**	0.798**	0.558*	-0.610*	-0.085^{NS}	-0.011^{NS}
96	0.772**	0.833**	0.532*	-0.623*	-0.015^{NS}	-0.069^{NS}
Estimated parameters						
c	0.344^{NS}	0.266^{NS}	0.120^{NS}	0.155^{NS}	-0.503*	0.035^{NS}
a	0.858**	0.855**	0.506*	-0.859**	-0.389^{NS}	-0.555*
b	0.197^{NS}	0.284^{NS}	0.212^{NS}	-0.006^{NS}	0.384^{NS}	0.486*
a+b	0.723**	0.786**	0.498*	-0.581*	-0.027^{NS}	-0.008^{NS}

* $p<0.05$; ** $p<0.01$. NS = Non-significant.
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 (h).
a+b = potential gas production (ml).
CP = Crude protein, CF = Crude fat, NDF = Neutral detergent fiber, ADF = Acid detergent fiber, ADL = Acid detergent lignin.

common vetch and pea was significantly ($p<0.01$) higher than alfalfa, sainfoin and chick pea hays. Gas production from slowly fermentable fraction (b) for alfalfa and common vetch was significantly ($p<0.01$) higher than the other hays. However, the potential gas production (a+b) for common vetch was significantly ($p<0.01$) higher than the other hays.

The protein content of forages is very important and limiting factor for growth of micro-organism (Cone and Van Gelder, 1999; Blummel et al., 2003). However, the CP contents of legume hays studied in this experiment were higher than that required by micro-organisms in the rumen to support optimum activity (Gutteridge and Shelton, 1994). As can be seen Table 3 there was significant ($p<0.01$) correlation between the CP content and gas production. This result is in agreement with findings of Parissi et al. (2005). Kamalak et al. (2005b). The gas production of alfalfa hay was similar to those of reported by Filya et al. (2002) and Ozturk et al. (2006). It can be seen from Table 3 there was a negative correlation between gas production and NDF or ADF contents of legume hays. This result is in agreement with Kamalak et al. (2005b) and Parissi et al. (2005). The use of forages for ruminant nutrition is essentially limited by its low digestibility and voluntary intake. The major factor affecting the voluntary feed intake is the cell-wall contents and digestibility of forages (Buxton, 1996).

Table 4. The metabolizable energy, organic matter digestibility, microbial protein production and 24 h gas production of legume hays

Item	Legume hays						SEM	Sig.
	Alfalfa	Sainfoin	Common vetch	Pea	White clover	Chick pea		
ME	10.46^{b}	10.06^{c}	11.12^{a}	10.27^{bc}	10.03^{c}	9.09^{d}	0.022	*
OMD	70.60^{b}	67.78^{c}	75.54^{a}	69.47^{b}	67.83^{c}	61.30^{d}	0.161	**
MP	126.36^{b}	113.36^{c}	138.05^{a}	110.37^{c}	119.95^{b}	90.35^{d}	1.135	**
GP^{y}	122.76^{b}	115.78^{c}	138.53^{a}	128.32^{b}	125.17^{b}	100.33^{d}	1.540	**

Means within rows with unlike superscript differ significantly. * $p<0.05$; ** $p<0.01$.
SEM = Standard error mean. Sig. = Significant level.
ME = Metabolizable energy (MJ/kg DM). OMD = Organic matter digestibility (%).
MP = Microbial protein g/kg DOM. GP^{y} = Gas production at 24 h incubation time/0.500 g DM.

Table 5. The correlation coefficient (r) among some estimated parameters and chemical constituents of legume hays

	Chemical constituents					
	CP	CF	Ash	NDF	ADF	ADL
ME	0.802**	0.874**	0.510*	-0.710**	-0.160^{NS}	-0.067^{NS}
OMD	0.814**	0.886**	0.522*	-0.722**	-0.170^{NS}	-0.087^{NS}
MP	0.770**	0.855**	0.528*	-0.688**	-0.286^{NS}	-0.159^{NS}
GP^{y}	0.928**	0.976**	0.610*	-0.906**	-0.394*	-0.376*

* $p<0.05$; ** $p<0.01$. NS = Non-significant.
ME = Metabolizable energy (MJ/kg DM), OMD = Organic matter digestibility (%), MP = Microbial protein g/kg DOM. GP^{y} = Gas production at 24 h incubation time/0.500 g DM, CP = Crude protein, CF = Crude fat, NDF = Neutral detergent fiber, ADF = Acid detergent fiber, ADL = Acid detergent lignin.

The ME, OMD and MP production and 24 h gas production are given in Table 4. As can be seen from Table 4 there were significant ($p<0.05$, $p<0.01$) differences among legume hays in terms of ME, OMD, MP and gas production. The ME, OMD, MP and gas production at 24 h of legume hays ranged from 9.09 to 11.12 MJ/kg DM, 61.30 to 75.54%, 90.35 to 138.05 g/kg DM, 100.33 to 138.53 ml/0.500 g DM respectively. The ME, OMD, MP and gas production at 24 h incubation of common vetch was significantly ($p<0.01$) higher than the other hays due to low cell-wall contents and high CP. The ME content of alfalfa was consistent with the findings of Getachew et al. (2004) and Kamalak et al. (2005a) but higher than that obtained by Kamalak et al. (2005b).

The OMD of alfalfa is in agreement with the finding of Blummel et al. (2003) and Kamalak et al. (2005b). However, the OMD of alfalfa was considerably higher than that reported by Kamalak et al. (2005b). The MP production of legume hays obtained in this experiment is consistent with findings of Ranilla et al. (2001) but lower than Cone and Van Gelder (1999) and Blummel et al. (2003).

The MP synthesis is defined as grams of microbial CP/kg or 100 grams of OM digested in the rumen (Hoover and Stokes, 1991; Stern and Hoover, 1979). There are several factors which may affect the microbial synthesis. However, the average efficiency of MP synthesis is 14.8 ranging from 7.0 to 27.9 g MCP/100 g of OM truly digested in the rumen (Karsli and Russel, 2001). Efficiency of the MP production obtained in this experiment fell into this range.

The correlation coefficient (r) among some estimated parameters and chemical constituents are given in Table 5. As can be seen from Table 5 ME, OMD, MP and gas production were negatively correlated with cell-wall contents whereas the same parameters were positively correlated with CP, CF and ash contents. This result is in agreement with the findings of Tolera et al. (1997) and Parissi et al. (2005) who found positive correlation between CP and ME or OMD. This result is also in consistent with the findings of Kamalak et al. (2005a) and Parissi et al. (2005) who found negative correlation between cell-wall content and the estimated parameters such as ME, OMD and MP.

IMPLICATIONS

There were significant differences among the legume hays in terms of chemical composition. The differences in chemical composition of legume hays resulted in the differences in the *in vitro* gas production, gas production kinetics and the estimated parameters such as ME, OMD and MP. The cell-wall contents especially NDF and ADF had a detrimental effect on the gas production and estimated parameters. Common vetch can be recommended to hay producers and ruminant breeders, especially dairy cattle breeders, due to high ME, OMD and MP production. Other legumes such as pea, white clover and alfalfa are very important forages for ruminants, also. However, *in vivo* experiment is needed to be more informative about these legume hays.

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