IJAS Iranian Journal of Applied Animal Science

Fenugreek Seed (*Trigonella foenum-graecum*) and Asparagus Root (*Asparagus officinalis*) Effects on Digestion and Kinetics of Gas Production of Alfalfa Hay Using *in vitro* Technique

Research Article

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Received on: 24 Apr 2014
Revised on: 25 Aug 2014
Accepted on: 15 Nov 2014
Online Published on: Sep 2015

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Online version is available on: www.ijas.ir

ABSTRACT

The aim of this paper was to study of the effect of fenugreek seed (*Trigonella foenum-graecum*) and asparagus root (*Asparagus officinalis*) on *in vitro* digestibility and kinetics of gas production of alfalfa hay. Fenugreek seed (FS) and asparagus root (AR) were added at different levels (0, 5, 10, 15 and 20% of DM). Total phenolic components of alfalfa hay (AH), fenugreek seed (FS) and asparagus root (AR) were 5.9, 10 and 8.3 g/kg DM, total tannins 0.4, 3.8 and 1.5 g/kg DM and saponin 10.4, 27.3 and 40.3 g/kg DM, respectively. *In vitro* dry matter (DM), organic matter (OM) and neutral detergent fiber (NDF) digestibility of alfalfa decreased (P<0.05) by addition of different levels of FS, but were not affected by adding AR. Addition of FS did not affect alfalfa crude protein (CP) digestibility, but AR at levels of 5 and 20% DM decreased (P<0.05) it. Metabolizable energy (ME) increased (P<0.05) due to addition of FS, but it decreased (P<0.05) by incorporation of AR. Addition of FS (at 10% DM level) and AR (at 5% DM level) decreased (P<0.05) potential gas production. The rate constants (*c* and *d*) were not affected by addition of FS and AR. FS and AR at level of 5% DM decreased (P<0.05) lag time (*l*). FS did not affect fermentation rate (h^{-1}), but AR at level of 10% DM increased (P<0.05) it. Results suggest that fenugreek seed and asparagus root due to secondary metabolites content's may have potential to improve the nutritive value of alfalfa hay.

KEY WORDS alfalfa, asparagus root, digestibility, fenugreek seed, gas production, *in vitro*.

INTRODUCTION

The major limitation to ruminant production under Iranian farming system is poor nutrition, which is characterized by low nitrogen and high fiber content in native grasses and legumes. Alfalfa (*Medicago sativa*) is one of the conventional forages because of its high protein content and digestibility compared to many other types of forage. So, it has been used as basal diet in ruminant rations. The incorporation of antibiotics as feed additive is one of the methods to improve nutritive value of animal feed. But, this one has faced reduced social acceptance in many European countries, on grounds of meat and milk product quality and safety. In particular, the use of antibiotics as feed additives in the EU has been banned since January 2006 (EU 1831/2003). However, natural plant products have been considered as one of possible alternatives to improve the nutritive value of ruminant diets. Plants contain various secondary compounds which protect them from infected with fungi, bacteria, herbivorous insects and vertebrates. Classes of compounds known to act in this way include saponins and tannins (Makkar *et al*. 1995; Pell *et al*. 2001), which are present in many herbal plants. The effects of some of these plants and their constituents on nutrients digestibility and rumen fermentation have been documented (Alexander, 2005; Alexander *et al*. 2007; Naseri *et al*.

2012). For example, at appropriate doses, herbal plants and their constituents, have suppressed protozoal populations, increased bacterial and fungal populations, propionate production, microbial yield and EMPS, increased dietary DM, OM and NDF degradation, reduced dietary CP degradation and methanogenesis to improve performance in ruminants.

In vitro methods to determine nutritional quality of feeds are important to nutritionists. These methods are less expensive, less time consuming and allow more control of experimental conditions than *in vivo* experiments. A number of gas measurement techniques and *in vitro* gas methods have been used by several groups to evaluate the nutritional value of feedstuffs (Getachew *et al*. 1998; Getachew *et al*. 2004; Makkar, 2005; Mirzaei-Aghsaghali *et al*. 2011). The *in vitro* gas method appears to be most suitable for use in developing countries where resources may be limited (Makkar, 2004). This method can also provide useful data on digestion kinetics of both the soluble and insoluble fractions of feedstuffs. The ease of measuring fermentation end products makes this method more efficient than other *in vitro* methods for studies on phytochemicals, plant secondary metabolites and feed additives (Makkar, 2005). This work aims to measure the *in vitro* digestibility and kinetics of gas production of lucerne hay following direct incorporation of fenugreek seed (*Trigonella foenum-graecum*) and asparagus root (*Asparagus officinalis*).

MATERIALS AND METHODS

Selection of plants

Two annual medicinal plants, fenugreek seed (*Trigonella foenum-graecum*; FS) at mature stage and asparagus root (*Asparagus officinalis*; AR) at vegetative stage, were selected for the investigation (Naseri *et al*. 2012).

Preparation of plants

All plant parts used in this study were supplied from the botanical garden of agriculture faculty, Razi University, Kermanshah (longitude 47.1 ˚E, latitude 34.23 ˚N and subtropical climate), Iran. The plant parts were cut or crushed into small pieces, oven dried (at 39 ˚C for 72 h) and ground to pass a 1-mm screen (Naseri *et al*. 2012).

Experimental treatments

Alfalfa hay based diet used as control group, and FS or AR was added at different levels meaning 5%, 10%, 15% and 20% of DM to the base diet (Naseri *et al*. 2012).

Chemical analysis

Samples were analysed for dry matter (DM) (24 h at 103 ˚C), ash and organic matter (OM) (4 h at 550 ˚C), crude protein (CP) content adapted for an automatic distiller Kjeldahl apparatus (Kjeltec Auto 1030 Analyzer; Tecator, Hoganas, Sweden) and using $CuSO_4$/Se as catalyst instead of $CuSO_4/TiO_2$, ether extract using petroleum ether for distillation instead of diethyl ether (AOAC, 1990). The neutral detergent fiber (NDF) contents were determined as described by Van Soest *et al*. (1991).

Plant secondary metabolites

Soluble phenolics and tannins

Four replicates of 200 mg oven-dried sample were extracted in glass tubes using 10 ml of aqueous acetone (70/30 v/v) in an ice bath by ultra-sonication for 10 min. The tubes were centrifuged (Hettich-Rotina 46, Tuttlingen, Germany) at 1400 g at 4 ˚C for 30 min. The supernatant was kept on ice and used as the 'original extract'. Total extractable phenols (TEPH) were determined according to Julkunen-Titto (1985).

Saponin contents

Ground sample (200 mg) was weighed into a 250 mL conical flask and water was added (1:10) as solvent. The extraction was completed by placing the flasks in a shaker at 30 ˚C and 120 rpm for 24 h. Contents of the flask were squeezed through four layers of muslin cloth and the filtrate from aqueous extract was centrifuged (Hettich-Rotina 46, Tuttlingen, Germany) at 17000 g for 20 min at 4 ˚C. Steroidal saponins were determined using a colorimetric method described by Baccou *et al*. (1977) after minor modifications according to Alexander *et al*. (2007).

In vitro digestibility

To assess the influence of FS or AR on DM, OM, CP and NDF digestibility, alfalfa hay was used as a basal substrate, and FS or AR was added at different levels (5%, 10%, 15% and 20% DM) to the media under *in vitro* condition according to the method of Tilley and Terry (1963). Rumen fluid was collected from three rumen cannulated rams before the morning feeding. The rumen fluid was mixed on volume basis then it was bubbled with CO_2 for approximately 2 min and filtered through four layers of cheesecloth. The incubation inoculum was prepared by diluting the fluid inoculum with the buffer (Tilley and Terry, 1963) in a 1:4 (V/V) ratio and stirring in a water bath at 39 ˚C with purging CO_2 until its use.

Approximately 500 mg of ground materials were weighed into 100 mL sterile bottles and 50 ml of the incubation inoculum was added. The bottle was stoppered with a Bunsen valve and incubated in the incubator for 48 h at 39 ˚C. Bottles were gently shaken four times every 8 h and each sample was incubated in four replicates. At the end of the 48 h incubation period, bottle contents were acidified by adding 6 *M* HCL to reach pH 1.3-1.5.

After a few seconds, when the foam subsided, pepsin powder (EC 3.4.23.1) was added to a final concentration of 0.2% (wt/vol.). The bottles were reincubated for an additional 48 h. Two sets of bottles were incubated: one set was used to determine *in vitro* DM, OM and CP digestibility and another set was used to estimate NDF to calculate NDF digestibility.

A blank set comprised of buffered rumen fluid without samples was taken to correct for the presence of feed particles and microbial biomass into rumen liquor. After 48 h incubation, the bottles were centrifuged (Hettich-Rotina 46, Tuttlingen, Germany) at 1100 g for 15 min and the supernatant was discarded.

In vitro dry matter, organic matter and crude protein digestibility were calculated, respectively as DM, OM and CP which disappeared from the initial weight inserted into the bottles. Bottle contents from the parallel set were transferred to 600 mL spoutless beakers, by rinsing twice with 25 mL of neutral detergent (Blümmel and Becker, 1997) and contents were transferred through narrow outlet into the respective beakers for NDF determination. DM, OM, CP and NDF digestibility of AH after the addition of FS or AR at different levels (0%, 5%, 10%, 15% and 20% DM) were measured according to the difference method of Church and Pond (1988).

In vitro gas production

The method used for gas production measurements was as described by Theodorou *et al*. (1994). Approximately 150 mg/DM of ground materials were weighed into tubes kept at 39 ˚C.

Each sample was incubated in five replicates. Fifteen milliliters of buffered rumen fluid (in the proportion of 20% rumen fluid+80% medium) prepared (as described in *in vitro* digestibility) and by flushing CO_2 before was anaerobically dispensed in each tube at 39 ˚C. All the tubes were crimped, placed in an incubator at 39 ˚C and shaken at regular times.

The pressure of gas produced in each tube was recorded using a pressure transducer (Manometer Digital testo 512; Testo, Lenzkirch, Germany) at 2, 4, 6, 8, 12, 18, 24, 48, 72 and 96 h after the start of the incubation. To estimate the kinetics of gas production, data on cumulative gas volume produced were fitted using the generalized Mitscherlich model proposed by France *et al*. (1993):

$$G= A(1-e^{-c(t-L) - d(\sqrt{t}-\sqrt{L})})$$

Where:
G (mL) denotes cumulative gas production at time t.
A (mL) is asymptotic gas production.
c (h^{-1}) and *d* ($h^{-1/2}$): rate constants.
L (h): lag time.

The half-life ($t_{1/2}$, h) of the degradable fraction of each substrate was calculated as the time taken for gas accumulation to reach 50% of its asymptotic value. The fractional degradation rate at $t_{1/2}$ ($\mu_{1/2}$, h^{-1}) was calculated as:

$$\mu_{1/2}= c + (d/^2\sqrt{t_{1/2}})$$

All gas volumes were adjusted to a common sample weight of 200 mg DM (Lopez *et al*. 2007). The volume of gas produced (GP) (mL/200 mg DM) after 24 h of incubation was used with crude protein (CP) content to estimate ME concentration (MJ/kg DM) based on the following equation reported by Menke and Steingass (1988) for roughage feeds:

$$ME= 2.2 + 0.1357\ GP + 0.057\ XP + 0.005829\ XP^2 (R^2=94\%;\ n=200)$$

Where:
ME: metabolizable energy (MJ/kg DM).
GP: gas production after 24 h (mL/200 mg DM).
XP: crude protein (%).

Statistical analysis

Data on *in vitro* digestibility and gas production parameters were subjected to one way analysis of variance using SAS (9.1) for windows (SAS, 1996) and significance between individual means was identified using Duncan (1955) multiple-range test.

RESULTS AND DISCUSSION

Chemical composition of substrates

Chemical composition data are presented in Table 1. Alfalfa hay contained (g/kg DM) a high level of NDF (596) and a moderate level of CP (137). NDF content of AR was higher than that of FS. But the CP content of FS was higher than that of AR. The DM and OM content of three substrates was similar.

Plant secondary metabolite composition of substrates

Phenolic compounds and saponins content of alfalfa hay, fenugreek seed and asparagus root are shown in Table 2. Alfalfa hay had both total tannins and saponin at lower levels. Fenugreek seed and asparagus root contained substantial quantities of saponin (27.3 and 40.3 g/kg DM plant). The total tannins content of fenugreek seed and asparagus root was 3.8 and 1.5 g/kg DM plant, respectively. The non-tannin phenol compounds content of the three substrates was similar.

Table 1 Chemical composition (g/kg DM) of alfalfa hay, fenugreek seed and asparagus root (AR)

	Alfalfa hay	Fenugreek seed	Asparagus root
OM	906	975	926
CP	137	314	73
EE	12	67	6
NDF	596	257	346

OM: organic matter; CP: crude protein; EE: Ether extract and NDF: neutral detergent fibre.

Table 2 Plant secondary metabolites (g/kg DM) composition of alfalfa hay, fenugreek seed and asparagus root

	Alfalfa hay	Fenugreek seed	Asparagus root
Total phenols	5.9	10.0	8.3
Non-tannin phenols	5.5	6.2	6.8
Total tannins	0.4	3.8	1.5
Steroidal saponins	10.4	27.3	40.3

Effect of fenugreek seed and asparagus root on *in vitro* nutrients digestibility and metabolizable energy

In vitro DM, OM and NDF digestibility of alfalfa hay were decreased due to addition of FS, but were not influenced by adding AR (Tables 3 and 4). Addition of fenugreek seed at different levels did not affect (P>0.05) CP digestibility, but *asparagus root* at levels of inclusion (5 and 20% DM) decreased (P<0.05) it. Metabolizable energy (MJ/kg DM) was increased (P<0.05) by addition of FS at levels of 10, 15 and 20% DM, but incorporation of AR at different levels decreased (P<0.05) ME. These agreed with the results of Naseri *et al*. (2012), who found that the patterns of FS or AR effect on *in vitro* nutrient digestibility of lucerne hay at different incubation times (12, 18, 24 and 48 h) were different. So, it can be concluded that FS and AR because of their secondary metabolites content mostly influence ruminal digestion than that post ruminal. Also, in agreement with these results, Alexander *et al*. (2007) showed that the extract of *Moringa oleifera* seed, *Picrorhiza kurroa* root, *Plumbago zeylanica* root, *Terminalia bellerica* (Gaertn) Roxb fruit and *Zingiber officinale* Roscoe rhizome affected *in vitro* apparent DM degradability differently.

In other hand, the apparent rate of nutrient degradation was not affected by plant extracts rich in saponin and tannin (Sliwinski *et al*. 2002).

Generally, medicine plants or their extracts yield complex mixtures of biochemicals so that identification of the phytochemical fractions involved in the effects we observed is not possible (Scehovic, 1999). However, three hypotheses are possible individually, or in combination: (1) the inhibitory or stimulatory action of saponins and tannins on some rumen microorganisms; (2) the effect of the degradation products of saponins and tannins and (3) the direct action of other secondary metabolites. Thereupon, in this study, our observations possibly resulted due to the inhibitory or stimulatory action of saponins and tannins on some rumen microorganisms.

Effect of fenugreek seed and asparagus root on *in vitro* gas production

Gas production kinetic parameters of alfalfa hay plus different levels of FS or AR are presented in Tables 5 and 6 and graphically illustrated in Figure 1 and 2. Potential gas production (A) and rate constant (d) were decreased due to addition of FS (at level of 10% DM) or AR (at level of 5% DM) that could be attributed to decreased OM digestibility and possibly inhibitory effects of saponins and tannins on protozoa and H_2-producing cellulolytic bacteria (Wang *et al*. 2000), as well as decreased NDF digestibility (Tables 1 and 2).

It has been demonstrated that 9-25% of ruminal methanogens associate with ciliate protozoa (Newbold *et al*. 1995) and ruminal ciliate protozoa provide H_2 as substrate for methanogens (Stumm and Zwart, 1986). Lag time (h) was decreased (P<0.05) due to addition of FS at levels of inclusion (5 and 10% DM) and AR at level of inclusion (5% DM). So, decrease in both potential gas production and lag time at the presence of FS and AR (at low levels) are suggesting their potency to improve nutritive value of alfalfa hay and ruminal fermentation efficiency.

Table 3 *In vitro* digestibility (%) of DM, OM, CP and NDF; and estimated metabolizable energy (MJ/kg DM) of alfalfa hay plus different levels (0, 5, 10, 15 and 20%) of fenugreek seed

Alfalfa hay	*In vitro* digestibility (%)[a]				ME (MJ/kg DM)
	DM	OM	CP	NDF	
	45.64[a]	42.08[a]	76.86	33.95[a]	6.09[d]
AH plus different levels of FS					
5%	44.74[ab]	39.99[b]	77.70	32.07[ab]	6.09[d]
10%	43.13[b]	38.25[b]	77.27	30.36[b]	6.15[c]
15%	43.09[b]	40.11[b]	78.11	30.00[b]	6.44[b]
20%	42.71[b]	39.84[b]	77.46	30.51[b]	6.61[a]
SEM	0.35	0.50	0.40	0.68	0.04
P-value	< 0.0001	< 0.0001	0.6115	0.0087	< 0.0001

DM: dry matter; OM: organic matter; CP: crude protein; NDF: neutral detergent fibre; AH: alfalfa hay; FS: fenugreek seed and ME: metabolizable energy (MJ/kg DM) estimated according to Menke and Steingass (1988).
SEM: standard error of the means.
[a] Data are presented after correction according to Church and Pond (1988).
The means within the same columns with at least one common letter, do not have significant difference (P>0.05).

Table 4 *In vitro* digestibility (%) of DM, OM, CP and NDF; and estimated metabolizable energy (MJ/kg DM) of alfalfa hay plus different levels (0, 5, 10, 15 and 20%) of asparagus root

Alfalfa hay	*In vitro* digestibility (%)[a]				ME (MJ/kg DM)
	DM	OM	CP	NDF	
	43.98	40.92	74.60[a]	40.02	6.09[a]
AH plus different levels of AR					
5%	43.92	41.42	72.92[b]	38.59	5.79[c]
10%	44.54	40.93	75.07[a]	37.24	5.77[d]
15%	44.41	40.99	74.59[a]	36.74	5.51[e]
20%	43.97	40.38	71.43[c]	34.84	6.05[b]
SEM	0.23	0.13	0.42	0.65	0.05
P-value	0.7354	0.1334	0.0001	0.0618	< 0.0001

DM: dry matter; OM: organic matter; CP: crude protein; NDF: neutral detergent fibre; AH: alfalfa hay; AR: asparagus root and ME: metabolizable energy (MJ/kg DM) estimated according to Menke and Steingass (1988).
SEM: standard error of the means.
[a] Data are presented after correction according to Church and Pond (1988).
The means within the same columns with at least one common letter, do not have significant difference (P>0.05).

Table 5 Parameters estimated by fitting generalized Mitscherlich model to gas production profile recorded for alfalfa hay plus different levels (0, 5, 10, 15 and 20% DM) of fenugreek seed

Alfalfa hay	Kinetics parameters					
	A (mL/g DM)	c (h^{-1})	d ($h^{-1/2}$)	L (h)	Half-life (h)	Fermentation rate (h^{-1})
	300.01ab	0.030	0.044[bc]	1.156[a]	17.126	0.036
AH plus different levels of FS						
5%	304.32[a]	0.032	0.050[ab]	1.032[b]	16.476	0.039
10%	293.42[b]	0.034	0.024[c]	0.788[c]	17.164	0.037
15%	296.46ab	0.032	0.074[a]	1.154[a]	16.462	0.038
20%	304.46[a]	0.028	0.072[a]	1.220[a]	16.496	0.038
SEM	1.61	0.001	0.004	0.174	0.124	0.001
P-value	0.1064	0.4672	0.0011	< 0.0001	0.1296	0.7124

A: asymptotic gas production; c and d: constant rates; L: lag time; AH: alfalfa hay; FS: fenugreek seed.
SEM: standard error of the means.
The means within the same columns with at least one common letter, do not have significant difference (P>0.05).

Table 6 Parameters estimated by fitting generalized Mitscherlich model to gas production profile recorded for alfalfa hay plus different levels (0, 5, 10, 15 and 20% DM) of asparagus root

Alfalfa hay	Kinetics parameters					
	A (mL/g DM)	c (h^{-1})	d ($h^{-1/2}$)	L (h)	Half-life (h)	Fermentation rate (h^{-1})
	299.64[b]	0.030[b]	0.052[b]	1.108[a]	17.126[b]	0.036[bc]
AH plus different levels of AR						
5%	285.02[c]	0.036[ab]	0.038[b]	0.946[b]	17.00[bc]	0.038[abc]
10%	297.34[b]	0.038[a]	0.034[b]	1.026[ab]	16.736[bc]	0.040[a]
15%	296.36[b]	0.030[b]	0.044[b]	1.010[ab]	18.140[a]	0.035[c]
20%	309.66[a]	0.030[b]	0.070[a]	1.070[ab]	16.106[c]	0.039[ab]
SEM	1.78	0.001	0.003	0.020	0.181	0.001
P-value	< 0.0001	0.0186	0.0033	0.0946	0.0022	0.0155

A: asymptotic gas production; c and d: constant rates; L: lag time; AH: alfalfa hay; AR: asparagus root.
SEM: standard error of the means.
The means within the same columns with at least one common letter, do not have significant difference (P>0.05).

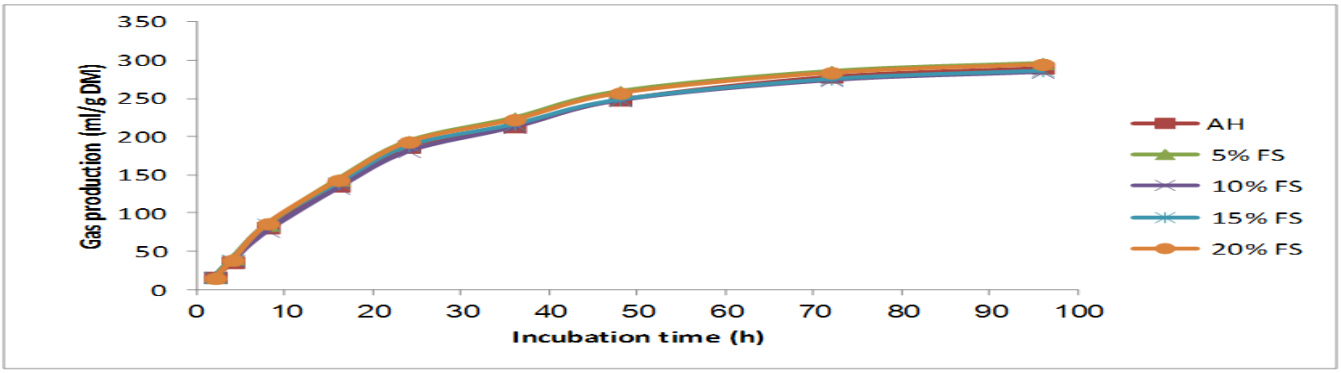

Figure 1 Pattern of *in vitro* gas production of alfalfa hay (AH) plus different levels (0, 5, 10, 15 and 20% DM) of fenugreek seed (FS)

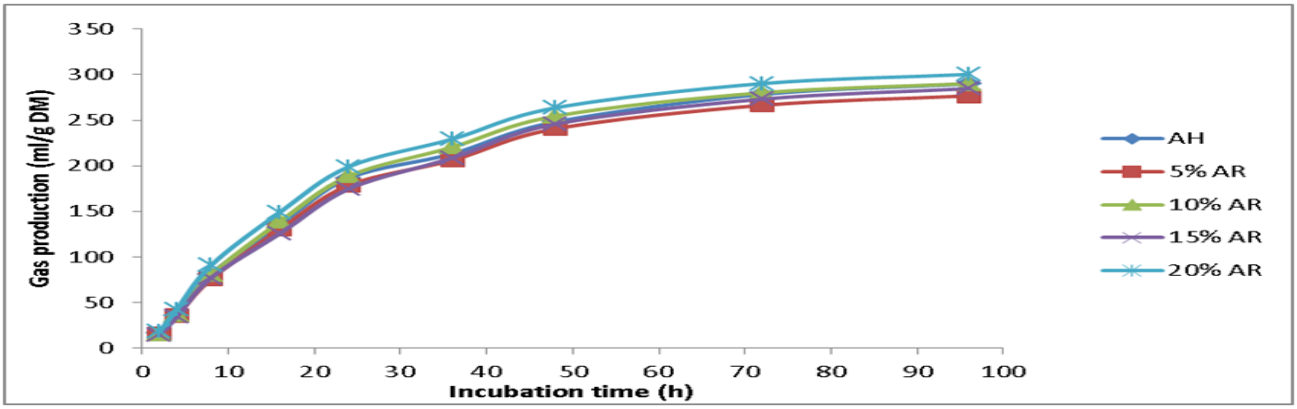

Figure 2 Pattern of *in vitro* gas production of alfalfa hay (AH) plus different levels (0, 5, 10, 15 and 20% DM) of asparagus root (AR)

Naseri *et al*. (2012) showed the decrease in both TVFA and gas production and increase in PF (PF=mg true degradability of organic matter/mL gas) value of lucerne hay at the presence of AR or FS (at levels of 5 and 10% DM) and also they suggested that FS and AR had potential to promote partitioning of degraded DM towards microbial cells at the expense of gas production. Fermentation rate was not affected by the addition of FS at different levels, but it increased ($P<0.05$) due to addition of AR at level of 10% DM that could be attributed to increased rate constant (c).

CONCLUSION

In vitro results of incorporation of FS and AR at different levels to the alfalfa hay and their effects on nutrients digestibility and gas production kinetic parameters suggested that FS and AR may improve kinetics of digestion and gas production of Lucerne hay, especially at low levels. However, these findings should be considered primarily and further investigation using more accurate *in vitro* methods as well as *in vivo* is needed to determine the value of these herbal plants as feed additives to improve animal performance.

ACKNOWLEDGEMENT

The authors are thankful to the University of Razi (Kermanshah, Iran) for the financial support. We are also grateful to Mr. Ali Shemshadi for assisting in the laboratory analysis.

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