THE USE OF *IN VITRO* GAS PRODUCTION TECHNIQUE TO INVESTIGATE THE ASSOCIATIVE EFFECT OF A DESERT PLANT WITH HIGH QUALITY FORAGE

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ABSTRACT

In vitro gas production technique was used to investigate the associative effect of mixing Rhodes grass (RG-Chloris gayana) with alfalfa (AA-Medicago sativa) by various proportions (3:1, 1:1, 1:3) on gas production and ruminal fermentation. A gas test technique was performed using fistulated sheep rumen fluid. Cumulative gas production was recorded at 3, 6, 9, 12, 24, 48, 72 and 96 hr of incubation. The neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were slightly higher in RG than AA while crude protein (CP) was significantly higher in AA than RG. The secondary compounds analyses resulted in negligible contents of these feeds from total phenol, total tannins and condensed tannins. The potential gas volume was highest for AA, and significant associative response in gas production (P <0.05) was found when the RG was mixed with AA by different ratios, while the lowest gas production was observed with RG. The maximum rate of gas production (c) was highest in AA and lowest for RG and the same trend was observed with gas production from soluble fraction (GPSF) and gas production from non soluble fraction (GPNSF). Mixing RG with AA improved significantly (P<0.05) the rate of gas production and decreased the lag time (L). There were significant (P<0.05) differences in terms of predicted dry matter intake (DMI), short chain fatty acids (SCFA), organic matter digestibility (OMD), microbial protein (MP), metabolizable energy (ME) and net energy (NE). The inclusion of RG by 25% with 75% of AA showed the best results and improved the DMI, SCFA, OMD, MP, and energy content in comparison with RG. It was concluded that RG nutritive value can be improved by mixing with high quality roughages.

Key words: Alfalfa, Rhodes grass, associative effect, in vitro gas production.

INTRODUCTION

In tropical and sub-tropical developing countries, there is a gap between available and required animal feeds. Ruminants in these regions are often fed lignified forages and crop residues that are low in available energy and nitrogen. In these plants, a large proportion of structural carbohydrates is protected by lignin and thus not utilized by rumen bacteria which are unable to degrade lignin (Akin and Benner, 1988; McSweeney *et al.*, 1994). The productivity of ruminants given forage and cereal straw diets is low due to low intake and digestibility and the presence of anti-nutritional factors such as silica, tannins, lignin, phenolics, steroids and alkaloid (Russel and Michael, 1992; D'Mello, 2000). There is growing interest in the use of feeds with a high content of rapidly degradable fiber as supplements to ruminants consuming poor quality forage diets. Silva and Ørskov (1988) reported that supplementation of barley straw with feeds providing digestible fiber improved the degradation of straw. Also, Manyuchi *et al.* (1996) found that supplementation of poor quality natural pasture (veld) hay with Napier and peanut hays increased the intake of veld hay. *In vitro* gas production measurements confirmed positive associative effects between both supplements and veld hay (Wood and Manyuchi, 1997). Liu *et al.* (2003) noted that positive associative effects on *in vitro* gas production occurred more consistently when rice straw was incubated in mixtures with hay or mulberry leaves than when incubated in mixtures with chemically treated rice straw. The *in vitro* gas production technique has been used as a measure of ruminal degradation of feeds (Menke and Steingass, 1988; Getachew *et al.*, 1998) and as an indicator of digestible dry matter intake (DMI) and growth rate of cattle fed cereal straws (Bümmel and Ørskov, 1993; Williams *et al.*, 1996). Gas production technique also has potential to investigate associative effects between feeds (Wood and Manyuchi, 1997; Liu et al., 2000) and has been widely used to evaluate the energy value of several classes of feeds (Getachew et al., 1998), particularly straws (Makkar et al., 1999) and agro-industrial by-products (Krishnamoorthy et al., 1995; Sallam et al., 2008). However, feeds are commonly evaluated as single entities despite the fact that most of the time an animal is fed a mixture of ingredients. Therefore, the objective of the present study was to investigate the associative effects of mixing RG with AA by different proportions using the gas production technique *in vitro*.

MATERIALS AND METHODS

The present study was carried out at the Laboratory of Rumen Microbiology, Animal Production Department, Faculty of Agriculture, Alexandria University, Egypt.

Feedstuffs:

Two green fodder species (alfalfa and Rhodes grass) grown at Hada Al-Sham Experimental Station, Department of Arid Land Agriculture- King Abdulaziz University, Saudi Arabia are harvested during the winter season (2008) and mixed according to the next combinations.

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I- Rhodes grass.
II- Rhodes grass : Alfalfa (3 : 1).
III- Rhodes grass : Alfalfa (1 : 1).
IV- Rhodes grass: Alfalfa (1: 3).
V- Alfalfa.

The samples were chopped to either 6 or 8 mm length and the dry matter content were determined according to (AOAC,1995), then ground in mills to pass a 1 mm sieve prior to chemical analyses and *in vitro* gas production measurements.

Chemical Analyses:

Plant samples were analyzed according to AOAC (1995) for dry matter (DM); organic matter (OM); crude protein (CP) – as 6.25 x N; and acid-detergent fibre (ADF); neutral-detergent fibre (NDF) and acid-detergent lignin (ADL) (Mertenz, 2002). Sodium sulphite and alpha amylase were not added to the solution for the NDF determination. Samples were also analyzed for extractable total phenols (TP), total tannins (TT) and condensed tannins (CT). Dried plant material (200 mg) was extracted with acetone: water (10 ml; 70:30 v/v) in an ultrasonic bath for 20 minutes. The contents were centrifuged (4°C, 10 min, 3000 *g*) and the supernatant was kept on ice until analysis. Total phenols were determined with the Folin-Ciocalteau reagent (Makkar et al.; 1993; Makkar, 2003). Extractable tannins were determined as the differences in total phenols (measured by Folin-Ciocalteau reagent) before and after treatment with insoluble polyvinyl polypyrrolidone (PVPP), as this polymer binds strongly to tannins (Makkar et al., 1995). TP and TT were expressed as tannic acid equivalents. Condensed tannins were measured by the HCl-butanol method and results were expressed as leucocyanidin equivalent (Makkar, 2003).

***In vitro* gas production:**

In vitro gas production was completed according to the procedure described by Menke and Steingass (1988). Buffer and mineral solution were prepared and placed in a water bath at 39°C under continuous flushing with CO_2. Rumen fluid was collected from fistulated sheep fed on berseem hay and commercial concentrate mixture diet into a pre-warmed thermos flask. The rumen fluid was filtered and flushed with CO_2, and the mixed and CO_2-flushed rumen fluid was added to the buffered mineral solution (1:2 v/v), which was maintained in a water bath at 39°C, and combined. Samples (200 ± 10mg) of the air-dry feedstuffs were accurately weighed into syringes fitted with plungers. Buffered rumen fluid (30 ml) was pipetted into each syringe containing the feed samples, and the syringes were immediately placed into water bath at 39°C (Blümmel and Ørskov, 1993). Three syringes with only buffered rumen fluid were incubated and considered as blank. The syringes were gently shaken every 2 h, and the incubation was terminated after recording the 72 h gas volume. The gas production was recorded after 3, 6, 9, 12, 24, 48 and 72 h of incubation. Cumulative gas was expressed as milliliter of gas produced per 200 mg of dry matter and corrected for blanks.

Cumulative gas production GAS (Y) at time (t) was fitted to the exponential model of Ørskov and McDonald (1979) as modified by Dhanoa (1988) as follows: Gas (Y) = a + b (1-exp^{-ct}), where; a = the gas production from the immediately soluble fraction, b = the gas production from the insoluble fraction, c = the gas production rate constant for the insoluble fraction (b), t = incubation time.

Estimated parameters:

As a new approach to evaluate feeds from these parameters, gas production caused by fermentation of the soluble fraction (GPSF) was estimated by gas production after 3 h (GP3) of incubation. Gas production caused by fermentation of the nonsoluble fraction (GPNSF) could be estimated from the gas production between 3 h (GP3) and 20 h (GP20) of incubation according to Van Gelder et al. (2005).

The energy values (metabolizable energy (ME) and net energy (NE)) of the investigated feedstuffs calculated from the amount of gas produced at 24 h of incubation with supplementary analyses of crude protein, ash and crude fat. This approach was developed by the research group in Hohenheim (Germany) and is based upon extensive *in vitro* incubation of feedstuffs (Menke et al. 1979; Menke and Steingass, 1988).

ME (MJ /kg DM) = 1.06 + 0.157GP + 0.084 CP + 0.22CF - 0.081CA

OMD (%) = 14.88 + 0.889 GP + 0.45CP + 0.0651 CA

Where: OMD is organic matter digestibility
GP is 24 h net gas production (ml/200 mg DM)
CP is crude protein (% of DM)
CA is ash (% of DM)

NE (Mcal/lb) = (2.2 + (0.0272*GP) + (0.057*CP) + (0.149*CF)) /14.64

Where: GP is 24 h net gas production (ml/g DM)
CF is crude fat (%of DM)

Then the NE unit was converted to be MJ/kg DM, microbial protein (MP, g/kg DOM) was calculated according to Czerkawski (1986). Short chain fatty acids (SCFA) were calculated according to the Getachew et al. (2002) and dry matter intake (DMI, kg/d) was calculated according to Blummel and Ørskov (1993).

Statistical analyses:

Data were subjected to analysis of variance (ANOVA) using the General Linear Model. Significant differences between individual means were identified using least significance difference (LSD) (SAS, 2002).

RESULTS AND DISCUSSION

The chemical composition and tannins contents of the investigated plants are presented in Table 1. Results showed wide variations in the chemical composition of the feeds. The neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were slightly higher in RG (49.0, 34.8 and 16.2 %, respectively) than those of AA (46.9 and 31.5, and 11.9 %, respectively), while crude protein (CP) was significantly higher in AA (17.2 %) than that of RG (8.1 %). The secondary compounds analyses resulted in negligible contents of these feeds of total phenol, total tannins and condensed tannins.

The gas production profiles for the associative effect of mixing RG with AA incubated 96hr *in vitro* are showed in Fig.1. A significant associative response in gas production, corrected for blank ($P < 0.05$) was found when the RG was mixed with alfalfa by different ratios, while the lowest gas production was observed with RG. This may be attributed to the low NFE content for RG, which has a positive correlation with gas production. On the other hand, cell wall content (NDF and ADF) were negatively correlated with gas production at all incubation times and estimated parameters. This seems to reduce the microbial activity through increasing the adverse environmental conditions as incubation time progresses. Present results are consistent with those of De Boever et al. (2005), who reported that gas production was related negatively with NDF content and positively with starch. Also, the relatively high level of ADL in RG as shown in Table 1 explains in part the limited *in vitro* degradation and therefore the lower amount of gas produced.

However, since gas production upon incubation of feeds in buffered rumen fluid is associated with feed fermentation and carbohydrate fractions, the low gas production from RG could be related to low feeding value of these feeds. Upon incubation of feedstuff with buffered rumen fluid *in vitro*, the carbohydrates are fermented to short chain fatty acids (SCFA), gases mainly CO_2 and CH_4, and microbial cells. Gas production is basically the result of fermentation of carbohydrates to acetate, propionate and butyrate (Wolin, 1960; Blummel and Ørskov, 1993), and substantial changes in carbohydrate fractions are reflected by total gas produced (Deaville and Givens, 2001). Gas production from protein fermentation is relatively small as compared to carbohydrate fermentation, while contribution of fat to gas production is negligible (Wolin, 1960). The maximum rate of gas production (c) was highest in AAH (0.084) and lowest for RG (0.029). Mixing of RG with AA improved ($P<0.05$) the gas production rate. The same trend was observed with gas production from soluble fraction (GPSF) and gas production from non soluble fraction (GPNSF). Cone et al. (1997) showed that gas production profiles could be divided into three phases, representing gas production caused by fermentation of the water-soluble fraction, the non-soluble fraction and microbial turnover. Kinetics of gas production is dependent on the relative proportions of soluble and insoluble particles of the feed (Cone et al., 1997).

Mathematical descriptions of gas production profiles allow analysis of data, evaluation of substrate and media-related differences, and fermentability of soluble and slowly fermentable components of feeds. Mixing RG with AA improved significantly ($P<0.05$) the rate of gas production and decreased the lag time (L). The lag time (time from incubation to start of gas production) is a very important digestibility parameter. The longest lag time for RG could be due to high whole non-structural carbohydrate content, while the shortest lag time for AA is related to the high content of fermentable carbohydrates in AA which is rapidly fermented. Kinetics of gas production is dependent on the relative proportions of soluble and insoluble particles of the feed. These results suggested that gas production profiles are related to the degradation or fermentation of substrates.

Liu, *et al.* (2003) showed that positive associative effects on the *in vitro* gas production occurred more consistently when rice straw was incubated in mixtures with hay or mulberry leaves than when incubated in mixtures with chemically treated rice straw. Significant positive associative effects on *in vitro* gas production were also observed with untreated finger millet straw at different levels of peanut cake supplementation after 12, 52, and 166 h of incubation (Sampath *et al.*, 1995). Similar interactions were observed for cottonseed cake supplementation to urea-treated straw, although statistical significance was not achieved for all supplementation levels. Wood and Manyuchi (1997) observed statistically significant positive associative effects of veld hay and napier hay or peanut hay fermented in both N-rich and N-free media as assessed by gas production and DM disappearance.

The energy values of forage were calculated from the amount of gas produced at 24 h of incubation with the supplementary analysis of crude protein and fat. The predicted dry matter intake (DMI, kg/d), short chain fatty acids (SCFA, mM), organic matter digestibility (%, OMD), microbial protein (MP, g/kg DOM), metabolizable energy (ME, MJ/kg DM), and net energy (NE, MJ/kg DM) were given in Table 3. Significant ($P<0.05$) differences were noted in terms of predicted parameters in RG, AA and inclusion of RG with AA. The inclusion of RG with AA improved (0.05) the DMI, short chain fatty acids (SCFA), organic matter digestibility (OMD), microbial protein (MP), metabolizable energy (ME) and net energy (NE). The inclusion of RG by 25% with 75% of AA showed the best results and improved the DMI, SCFA, OMD, MP, and energy content compared with RG.

The complex interactions between mixed rumen microbial populations leading to the conversion of plant components to gas production and hence SCFA can be predicted from gas volume (Wolin, 1975; 1979; Russell and Wallace, 1988; Van Soest, 1994).

One of the main reasons for the low degradability of RG was the presence of lignin which protects carbohydrates from attack by rumen microbes. Kamalak et al. (2002) noted a significant correlation between SCFA and gas production. Getachew et al. (2002) reported the close association between SCFA and gas production *in vitro*, which is used to estimate the SCFA production from gas values as an indicator of energy availability to the animal. Menke and Steingass (1988) and Chenost et al. (1997) concluded that the prediction of ME is more accurate when based on gas production and chemical constituents measurements as compared to calculations based on chemical constituents only.

Menke and Steingass (1988) noted a positive correlation between metabolizable energy calculated from *in vitro* gas production together with CP and fat content with metabolizable energy value of conventional feeds measured *in vivo*. *In vitro* gas production and *in vitro* apparent and true degradability were found to be highly correlated (Blümmel *et al.*, 1997 and Romney *et al.*, 1997). The variable *in vitro* digestibility values could be due to variable levels of phenolics, tannin activity and cell wall content among the plants. Getachew et al. (2002) reported a close association between SCFA and *in vitro* GP, and used the relationship between SCFA and GP to estimate the SCFA production from gas values, which is an indicator of energy availability to the animals. The lower SCFA predicted from GP for RG could be due to a high concentration of the insoluble fraction in such plants and thus producing little amounts of gas during the first 24 h of incubation. Tavendale et al. (2005) attributed the increase in total volatile fatty acid production from AA to the inhibition of methanogenisis and fermentation of organic matter.

In conclusion RG nutritive value could be improved by mixing it with high quality roughages (positive associative effect), and can be used as an alternative feed resource.

Table 1: The proximate analyses and tannins content on dry matter basis of the investigated substrates.

	CP	Ash	EE	NFE	NDF	ADF	ADL	TP	TT	CT
AA	17.2	12.0	1.3	56.0	49.0	34.8	16.2	8.8	4.2	1.26
AA+RG(3:1)	16.9	8.3	1.4	51.1	49.5	34.4	15.7	5.1	1.4	1.49
AA+RG(1:1)	16.8	12.6	1.6	57.0	45.8	27.9	10.9	5.8	1.8	1.21
AA+RG(1:3)	10.5	9.7	1.3	59.6	47.5	30.8	11.4	5.7	2.1	2.07
RG	8.1	11.9	1.0	64.4	46.9	31.5	11.9	3.3	0.3	1.23

RG: Rhodes grass; AA: Alfalfa; CP: crude protein; EE: ether extract; NFE: nitrogen free extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; TP: total phenols (eq-g tannic acid/100g DM); TT: total tannins (eq-g tannic acid/100g DM), CT: condensed tannins (eq-g leucocyanidin /100g DM).

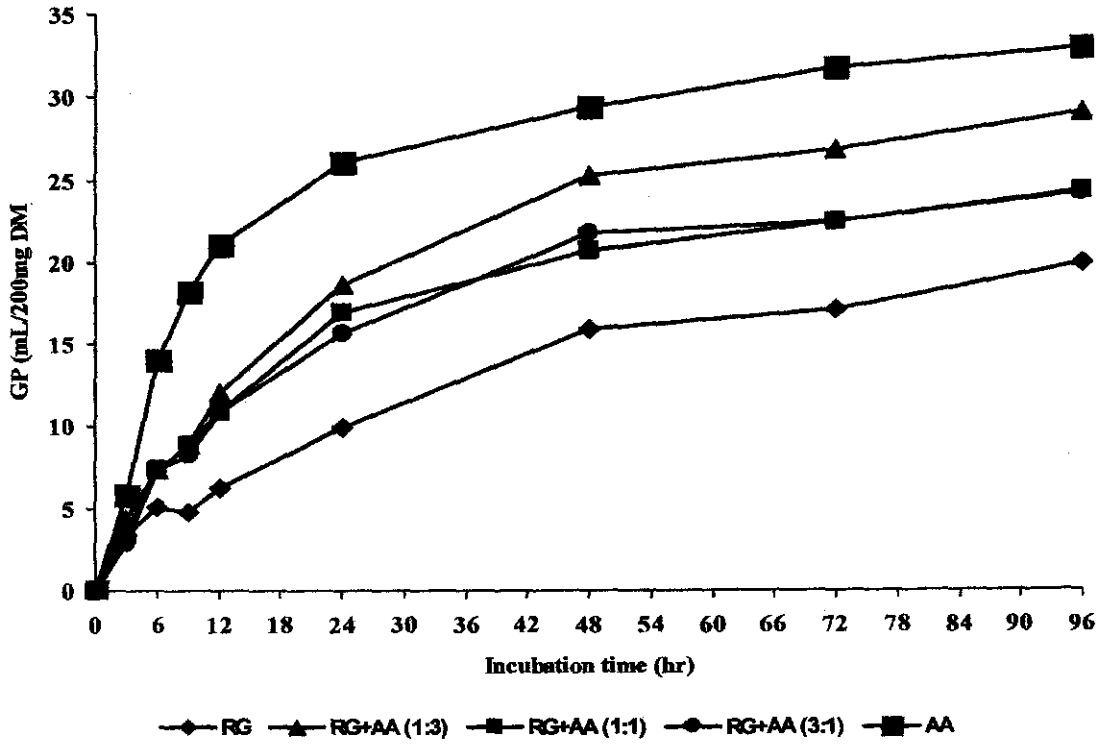

Figure 1. The gas production (GP) profiles for the associative effect of mixing Rhodes grass (RG) with alfalfa (AA) incubated *in vitro* for 96hr.

Table 2: The effect of the associative effect of mixing rhodes grass (RG) with alfalfa (AA) on gas production kinetics, gas production from soluble fraction (GPSF, ml/gDM) and gas production from non soluble fraction (GPNSF, ml/gDM) incubated 96 hr in vitro.

	a+b	c	L	GPSF	GPNSF
RG	17.2^{c}	0.029^{c}	2.294^{a}	8.2^{d}	40.5^{d}
RG+AA (1:3)	28.81^{a}	0.042^{b}	1.507^{bc}	18.2^{b}	96.8^{b}
RG+AA (1:1)	23.25^{b}	0.053^{b}	1.676^{ab}	14.0^{c}	88.2^{bc}
RG+AA (3:1)	23.65^{b}	0.049^{b}	1.702^{b}	11.1^{cd}	82.0^{bc}
AA	30.83^{a}	0.084^{a}	1.466^{c}	26.0^{a}	115.6^{a}

Means within a column bearing different superscripts differ (P<0.05).

Table 3. The effect of the associative effect of mixing rhodes grass (RG) with alfalfa (AA) on predicted dry matter intake (DMI, kg/d), short chain fatty acids (SCFA, mM), organic matter digestibility OMD,%), microbial protein (MP, g/kg DOM), metabolizable energy (ME, MJ/kg DM) and net energy (NE, MJ/kg DM).

	DMI	SCFA	OMD	MP	ME	NE
RG	2.116^{c}	16.3^{d}	29.9^{c}	57.6^{d}	4.26^{c}	2.74^{c}
RG+AA (1:3)	2.566^{a}	40.9^{b}	39.9^{a}	76.9^{a}	5.75^{a}	3.69^{a}
RG+AA (1:1)	2.128^{c}	37.1^{c}	38.1^{ab}	73.5^{ab}	5.50^{ab}	3.55^{ab}
RG+AA (3:1)	2.188^{c}	34.4^{c}	34.4^{b}	66.3^{c}	4.98^{b}	3.20^{b}
AA	2.468^{ab}	49.0^{a}	39.0^{a}	75.3^{a}	5.74^{a}	3.65^{a}

Means within a column bearing different superscripts differ (P<0.05).

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الملخص العربي

إستخدام طريقة إنتاج الغاز معمليا لدراسة التأثير المصاحب لخلط النباتات الصحراوية مع الأعلاف المالئة عالية القيمة الغذائية

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أجري هذا البحث لدراسة التأثير المصاحب لخلط حشيشة الرودس مع البرسيم الحجازي بنسب مختلفة (١:١ ، ١:٣ ، ٣:١) باستخدام طريقة إنتاج الغاز معمليا على إنتاج الغاز وتخمرات الكرش. تم إجراء هذه التقنية بإستخدام ثلاثة كباش بها فتحة مستديمة فى الكرش كمصدر لسائل الكرش وتم تسجيل الغاز الناتج معمليا في أوقات ٣ ، ٦ ، ٩ ، ١٢ ، ٢٤ ، ٤٨ ، ٧٢ ، ٩٦ ساعة من تحضين السرنجات فى حمام مائى على درجة حرارة ٣٩ ° م.

أوضحت نتائج التحليل الكيميائي أن محتوى حشيشة الرودس كان مرتفعا عن البرسيم الحجازى من حيث الـ NDF , ADF , ADL بينما دريس البرسيم الحجازى كان اعلى فى محتواه من البروتين الخام. محتوى حشيشية الرودس و البرسيم الحجازى من الفينولات الكلية والتانينات الكلية والتانينات المكثفة كانت منخفضة جدا.

أعطى البرسيم الحجازى أعلى إنتاج للغاز ومعدل إنتاج الغاز مقارنة بحشيشة الرودس ولكن كان هناك تأثير مصاحب إيجابى لخلط حشيشة الرودس مع البرسيم الحجازى وحدوث زيادة في إنتاج الغاز و معدل إنتاج الغاز وإنخفاض فى فترة الكمون Lag time. كذلك خلط حشيشة الرودس مع البرسيم حسن من هضم المادة العضوية والبروتين الميكروبى والمحتوى من الطاقة الميتابولزمية والصافية والمأكول اليومى مقارنة بحشيشة الرودس دون خلط.

وتوصي نتائج الدراسة بخلط أوراق حشيشة الرودس مع البرسيم الحجازى وذلك نتيجة إيجابية التأثير المصاحب للخلط على تخمرات الكرش الميكروبية.