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Developing a Modified *in vitro* Gas Production Technique to Replace the Nylon Bag Method of Evaluating Protein Degradation of Alfalfa Hay in Ruminants

Research Article

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ABSTRACT

The present study was conducted to investigate the possibility of using a modified *in vitro* gas production technique in place of the nylon bag method to estimate protein degradability of alfalfa hay in ruminants. In the *in situ* experiment dacron bags were filled with 3 g alfalfa hay. This was incubated in the rumen of three ruminally cannulated Ghezel rams for the periods of 0, 2, 4, 8, 12, 16, 24, 36 and 48 h. At the end of the experiment, dry matter, organic matter and crude protein degradability (CPD) were calculated. In the *in vitro* experiment, buffered rumen fluid was prepared in a solution of 19:1 artificial saliva to rumen fluid, which was pre-incubated by rapidly fermentable carbohydrates for 4 h. After pre-incubation, 30 mL of the buffered rumen fluid were added to 100 mL syringes, containing the alfalfa sample, with 7.5 mg N. The samples were incubated for 2, 4, 6, 8, 12, 16, 24, 36, 48, 60 and 72 hours, after which net gas production was computed. In the third experiment, 25 grams of faeces were mixed with 50 mL of artificial saliva, which was then made up to 1 liter by adding more artificial saliva and filtered. Then, the suspension was pre-incubated for 4 h. After pre-incubation, the same steps used to measure gas production were conducted. Results showed that there were significant differences between gas production with rumen liquor and faeces suspension at 2, 4, 24, 36 and 48 h of incubation, while no significant differences were found at other incubation times. There was a close relationship between crude protein degradation at different times and amount of gas production using rumen liquor [CPD= 58.93 + 0.32 gas (r^2=0.76, n=18)] and fecal suspension [CPD= 58.38 + 0.27 gas (r^2=0.60, n=18)]. The results indicated that faeces suspension can be used instead of rumen liquor as the medium in the gas production method. Development of the gas production technique could result in reducing the need to use fistulated animals in feed evaluation studies.

KEY WORDS alfalfa, faeces, gas production, nylon bag.

INTRODUCTION

Ration formulation for ruminant animals requiring rumen undegradable protein is based on accurate estimation of rumen degradable crude protein of the feeds. The nylon bag technique is commonly used to determine extent of protein degradation in the rumen (Orskov and McDonald, 1979; Michalet-Doreau and Ould-Bah, 1992; England *et al.* 1997; Cone *et al.* 2009). This method is the standard against which to compare different methods of protein degradability estimation (Michalet-Doreau and Ould-Bah, 1992). Besides the advantages, there are disadvantages in the method such as time-consumption, labor intensity and cost(England *et al.* 1997; Cone *et al.* 2009). Other constraints include underestimation and in some cases, overestimation of protein degradability (Getachew *et al.* 1998) and the necessity

for fistulated animals. Therefore, simple, cheap and reliable methods for measuring crude protein degradability (CPD) are required.

Another method to determine degradability of crude protein in the rumen involves measuring rumen ammonia nitrogen production from feed fermentation. However, measuring crude protein degradation through ammonia release is complex because crude protein degradation and ammonia uptake for microbial protein synthesis occur simultaneously (Karlsson *et al.* 2009).

The *in vitro* gas production technique is another method that can be used to evaluate feed. Compared to the nylon bag technique, the gas production method is fast, simple, cheap and many data can be collected in one run (Bani *et al.* 1999; Cone *et al.* 2009; Mirzaei-Aghsaghali *et al.* 2011). The gas production technique which was introduced by Menke *et al.* (1979), was used to determine the fermentation characteristics of organic matter, but in the limited research this method developed to estimate protein fermentation in the rumen (Cone *et al.* 2009; Karlsson *et al.* 2009). This method has the disadvantage of requiring rumen liquor from fistulated animals. The necessity for fistulated animals creates a number of problems, e.g. surgical facilities, constant care to avoid infections and long-term maintenance (that can be expensive) of these animals (Mauricio *et al.* 2001). In addition, there are ethical considerations with regard to animal welfare. Thus, introducing an *in vitro* method that does not require rumen liquor from fistulated animals has many advantages.

Similarities between the environments found in the rumen and hindgut include bacterial action on, cellulase, protease, deaminase and urease. Fermentation products include volatile fatty acids (VFA), NH_3, CH_4 and microbial cells in both sections and bacterial concentrations (10^{10} to 10^{12}/mL) are similar with greater than 95% anaerobes (Gressley *et al.* 2011). This makes faeces a potentially suitable alternative inoculum in place of rumen liquor for *in vitro* gas production techniques.

A faecal suspension from sheep was first used as a source of micro-organisms for the estimation of the digestibility of forages by Balfe (1985) and was subsequently tested by El-Shaer *et al.* (1987). A few years later, Omed *et al.* (2000) stated that faeces can be used as an alternative inoculum for the Tilley and Terry (1963) *in vitro* digestibility technique. More recently, Fon and Nsahlai (2012) showed that faecal suspension is a better alternative for rumen liquor for *in vitro* feed evaluation, as demonstrated by the small differences observed in their true digestibility and gas production. Experiments of Parand and Taghizadeh (2009) found a strong relationship (r^2=0.9) between gas production from the two sources of inocula, which suggests that faeces can be used successfully in the gas production technique.

In several studies, some feedstuffs such as maize stover (Fon and Nsahlai, 2012), oat forage, Italian ryegrass, perennial ryegrass and maize forage (Borba *et al.* 2001), meadow hay and wheat straw (Varadyova *et al.* 2005) and dehydrated alfalfa (Bani *et al.* 1999) were evaluated using *in vitro* methods with liquor from sheep or cows, both from the rumen and faeces. Since alfalfa hay was offered as dietary roughage and was available for the present study, it was considered as a test feed in this research.

The present research aims to investigating the possibility of using the *in vitro* gas production technique (with rumen liquor or faeces suspension) in place of the nylon bag technique to estimating protein degradability of alfalfa hay for ruminants.

MATERIALS AND METHODS

Chemical analysis

Alfalfa hay (Hamedani variety) samples were collected from the Agricultural Research Centre of Islamic Azad University, Shabestar Branch, Shabestar, Iran. The samples were milled through a 1 mm sieve for chemical analysis and the *in vitro* gas production procedure, and a 3 mm screen for the nylon bag method. Dry matter was determined by drying the samples at 105 ˚C overnight and ash content by igniting the samples in a muffle furnace at 525 ˚C for 8 h. Ether extract (EE; protocol no. 920.39) and nitrogen (N; protocol no. 955.04) content of the samples were determined by soxhlet extraction and the Kjeldahl method, respectively (AOAC, 1990). Crude protein (CP) was calculated as N × 6.25. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to Van Soest *et al.* (1991). Non-fibrous carbohydrate (NFC) was calculated using the equation of NRC (2001):

NFC % = 100 – (% NDF + % CP + % EE + % Ash).

In situ degradation procedures

Three Ghezel rams (about 55 kg BW), with rumen cannulas were used to determine *in situ* degradation characteristics. Rams were housed in individual stalls bedded with straw. Rams were fed diets containing alfalfa hay (65%) and a concentrate mixture (35%) at the maintenance levels (NRC, 2007). Diets were offered to the animals twice daily at 08.00 and 16.00 in equal sized meals. The animals had free access to fresh water. Dacron bags (14×7 cm; about 50 micron pore size) were filled with 3 g dried and ground test material and then incubated in the rumen for 0, 2, 4, 8, 12, 16, 24, 36 and 48 h. After the removal of bags from the rumen, they were automatically washed in cold tap water until the rinse was clear and dried at 60 ˚C for 48 h (Orskov and McDonald, 1979; Aghajanzadeh-Golshani *et al.* 2012).

Remaining residues were analyzed for organic matter (OM) and crude protein (CP) concentrations. Rumen degradation kinetics of dry matter (DM), OM and CP were calculated by the equations described by Orskov and McDonald (1979) using FITCURVE software version 6. Effective degradability was calculated based on passage rates of 0.02, 0.05 and 0.08 per hour (Chen, 1995):

$P= a + b (1-e^{-ct})$
$ED= a + (b\times c) / (c+k)$

Where:
P: percentage of degradability for response variables at time t.
ED: effective degradability for response variables (%).
a: highly soluble and readily degradable fraction (%).
b: insoluble and slowly degradable fraction (%).
c: rate constant for degradation of the fraction b (/h).
e: 2.7182 (natural logarithm base).
t: time relative to incubation (h).
k: rate constant of passage (/h).

***In vitro* gas production**

Rumen liquor as microbial origin

Rumen fluid was collected in a pre-warmed flask from the three fistulated Ghezel rams (described above) before the morning feed.

The fluid was strained through two layers of cheesecloth and kept at 39 ˚C under CO_2. The test samples were incubated in rumen fluid in calibrated glass syringes according to the procedure outlined by Cone *et al.* (2009). Solutions required for the gas technique (Menke *et al.* 1979) used in the experiment are given below:

Macro mineral solution

Na_2HPO_4: 5.7 g; KH_2PO_4: 6.2 g and $MgSO_4$ $7.H_2O$: 0.6 g; make up to 1 L with distilled water.

Buffer solution

$NaHCO_3$: 39 g; make up to 1 L with distilled water.

Reducing solution

Distilled water: 47.5 mL, 1N NaOH: 2 mL and Na_2S $7.H_2O$: 285 mg.

Micro mineral solution

$CaCl_2$ $2.H_2O$: 13.2 g, $MnCl_2$ $4.H_2O$: 10.0 g, $CoCl_2$ $6.H_2O$: 1.0 g and $FeCl_3$ $6.H_2O$: 0.8 g; make up to 100 ml with distilled water.

Resazurin aqueous

100 mg; make up to 100 mL with distilled water.

Preparation of artificial saliva

Distilled water: 474 (mL), macro mineral solution: 237 (mL), buffer solution: 237 (mL), micro mineral solution: 0.12 (mL), resazurin: 1.22 (mL), reducing solution: 49.5 (mL) and final volume: 1000 (mL).

To avoid a high input of N from the rumen fluid, buffered rumen fluid was prepared from the ratio of 19 parts (artificial saliva) to 1 part (rumen fluid). To ensure that nitrogen was the limiting factor to fermentation, 10 g/L rapidly fermentable carbohydrates (3.33 g each of glucose, xylose and soluble starch) were added to the buffered rumen fluid and incubated at 39 ˚C. This pre-incubation was performed in a 2 liter bottle with continuous flushing of CO_2 for 4 h. During this pre-incubation, it was assumed that all available nitrogen from the rumen fluid was incorporated into bacterial nitrogen components, in order to make nitrogen limiting to microbial growth. After pre-incubation, 30 mL of the buffered rumen fluid, with the rapidly fermentable carbohydrates, were added with a dispenser to a 100 mL calibrated glass syringe, already containing the alfalfa sample, with 7.5 mg N. All laboratory procedures were carried out under continuous flushing with CO_2. The samples were incubated in triplicate in a shaker incubator. Three syringes with only buffered rumen fluid, with the rapidly fermentable carbohydrates (blanks) were also incubated with each run. Amounts of gas production were recorded at 2, 4, 6, 8, 12, 16, 24, 36, 48, 60 and 72 hours after incubation and then net gas production was computed (Cone *et al.* 2009).

Modified faeces suspension as microbial origin

Fresh faeces samples were collected into a warmed flask from the rectums of the three fistulated Ghezel rams (described above) before the morning feed. Twenty five grams of collected faeces were pooled and then mixed with 50 ml of artificial saliva previously saturated with CO_2. The mixture was subsequently filtered after being made up to 1 liter by adding artificial saliva (see Table above) and pH of the suspension was adjusted to 6.8. Then, 10 g/L rapidly fermentable carbohydrates (3.33 g each of glucose, xylose and soluble starch) were added to the suspension and incubated at 39 ˚C. This pre-incubation was performed in a 2 liter bottle with continuous flushing with CO_2 for 4 h. After pre-incubation, 30 mL of the suspension, with the rapidly fermentable carbohydrates, was added with a dispenser to a 100 mL calibrated glass syringe, already containing the alfalfa sample, with 7.5 mg N. All laboratory procedures were carried out under continuous flushing with CO_2. The samples were incubated in triplicate in a shaker incubator. Three syringes with only suspension, with the rapidly fermentable carbohydrates (blanks) were incubated for each

run. Amounts of gas production were recorded at 2, 4, 6, 8, 12, 16, 24, 36, 48, 60 and 72 hours after incubation and then net gas production was computed.

Statistical analysis

Calculations and statistical analysis of data (three replicates) were conducted based on a completely randomized design using GLM procedure of SAS (2001) software. Means were separated by Duncan's multiple range tests. Regression equations between *in situ* rumen CP degradation and gas production were derived also using SAS (2001) software.

RESULTS AND DISCUSSION

The chemical composition of alfalfa hay is given in Table 1. The CP content of alfalfa hay (191.4 g/kg DM) is in line with findings of Bueno *et al*. (2010); (190.8 g/kg) and NRC (2007); (190 g/kg), but higher than reported by Mirzaei-Aghsaghali *et al.* (2008); (156 g/kg) and Jahani-Azizabadi *et al.* (2009); (183 g/kg) and lower than findings of Williams *et al*. (2010); (212 g/kg).

The ash contents of alfalfa hay (102.9 g/kg) is similar to those reported by Kamalak *et al.* (2005); (103.7 g/kg) and Bueno *et al*. (2010); (99.7 g/kg) and lower than reported by Paya *et al.* (2008); (131 g/kg). The ADF (340.2 g/kg DM) and NDF (441.7 g/kg DM) content of alfalfa hay are within the range of values reported by Biagi *et al.* (2005); (234-360 and 310-453 g/kg, respectively), while they are lower than that of Jahani-Azizabadi *et al.* (2009); (400 and 490 g/kg, respectively) and higher than obtained by Kamalak *et al*. (2005); (273.6 and 424 g/kg, respectively).

The variation in chemical composition of alfalfa hay can be caused by differences in variety, fertilization, cutting stage, environmental condition and laboratory procedures of feed evaluation (Maheri-Sis *et al.* 2012; Paya *et al.* 2008).

The mean values of rumen dry matter (DM), organic matter (OM) and crude protein (CP) disappearance of alfalfa hay at different incubation times are shown in Table 2. Based on the results, the soluble fraction of alfalfa hay is high (about 40% of crude protein is soluble) and about 90% of the crude protein is degraded within 24 h of incubation time.

The DM degradability of the alfalfa hay at 24 h of incubation time (73.60%) was higher than that reported by Jalilvand *et al.* (2008); (61.1%). The CP disappearance value at 24 h incubation (89.11%) was within the range reported by Alvir *et al*. (1999); (77.7-89.7%) and Jiri *et al.* (1996); (86.79%).

The parameters for DM, OM and CP degradation and effective degradability of alfalfa hay are given in Table 3.

Dry matter degradation parameters including the immediately soluble fraction (*a*) with 31.43% and insoluble and slowly degradable fraction (*b*) (44.00%), agree with findings of Julier *et al.* (2003), who reported 32.8% and 47.8% for *a* and *b* fractions, respectively, of 16 alfalfa cultivars. The *a* and *b* fractions of alfalfa CP are similar to those reported by Julier *et al.* (2003) who obtained 38.8% and 51.8% for the *a* and *b* fractions, respectively and are also in the range of findings of Alvir *et al*. (1999). The effective degradability (ED) of CP for alfalfa hay (80.10%) with an outflow rate of 5% in the current study is higher than reported by Alvir *et al*. (1999); (62.8 to 74.7%).

A review of the literature revealed that there are some differences in the degradation characteristics among alfalfa hays. Aghajanzadeh-Golshani *et al.* (2012) and Maheri-Sis *et al*. (2011) stated that the major variation sources of *in situ* degradability of feeds include, chemical composition, bag pore size, sample size, washing method and procedures, particle size, diet offered to animals, species of animal, sample preparation, incubation times and differences in methods and apparatuses used in laboratories.

In Table 4 presents average net gas production values at different incubation times of alfalfa hay, containing 7.5 mg N, in a N-free medium (rumen liquor and faeces suspension) after a pre incubation period. These values were corrected for that of the blank. There were significant differences between gas production with rumen liquor and faeces suspension at 2, 4, 24, 36 and 48 h of incubation and values for faeces suspension were higher, while no significant differences were found between gas production with either rumen liquor or faeces suspension at 6, 8, 12, 16, 60 and 72 h of incubation.

Since there are many similarities between the conditions of the rumen and hindgut in ruminants, similar results were predicted from both sources of inocula. However, there were some differences between the two sources in micro-organisms for gas production, which can cause differences at some incubation times.

The main differences between the rumen and hindgut in ruminants is that protozoa are absent in the hindgut, and the buffering capacity of the hindgut is lower than that in the rumen (Gressley *et al.* 2011). Due to differences in the variety and number of micro-organisms in both sections, the amount and composition of the gas produced can also be different.

There was a relationship between the gas production from the method described by Cone *et al*. (2009) and the modified method with faeces suspension and protein degradability in the nylon bag technique. Prediction equations of CPD using the modified gas production method with rumen liquor and with faeces suspension are show in Table 5.

Table 1 Chemical composition of alfalfa hay on dry matter basis (g/kg)

DM	OM	CP	EE	NDF	ADF	NFC
917.8	897.1	191.4	30.1	441.7	340.2	233.9

DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fibre; ADF: acid detergent fibre and NFC: non fibrous carbohydrate.

Table 2 Rumen dry matter (DM), organic matter (OM) and crude protein (CP) degradation of alfalfa hay at different incubation times

Incubation time (h)	DM disappearance (%)	OM disappearance (%)	CP disappearance (%)
0	34.83±0.49	30.34±0.58	41.42±1.46
2	39.16±1.57	35.13±1.30	49.58±4.05
4	50.75±5.18	47.24±5.40	66.09±0.56
8	67.43±1.63	64.67±1.88	81.96±0.41
12	71.84±0.93	69.66±1.01	87.33±1.45
16	73.03±0.46	71.05±0.30	88.36±0.48
24	73.60±0.59	72.49±0.39	89.11±0.55
36	74.43±0.09	73.08±0.46	ND
48	74.51±0.02	73.98±0.29	ND

ND: not determined.

Table 3 Rumen dry matter (DM), organic matter (OM) and crude protein (CP) degradation parameters and effective degradability of alfalfa hay

Items	DM	OM	CP
a (%)	31.43	26.97	38.70
b (%)	44.00	47.43	52.97
a + *b* (%)	75.43	74.40	91.67
c (/h)	0.169	0.160	0.180
Lag time (h)	0.50	0.47	0.30
ED (%); out flow rate 0.02 /h	70.67	69.10	86.33
ED (%); out flow rate 0.05 /h	65.27	63.13	80.10
ED (%); out flow rate 0.08 /h	61.20	58.60	75.37

a: highly soluble and readily degradable fraction; *b*: insoluble and slowly degradable fraction and *c*: rate of degradation of fraction b (/h).
ED: effective degradability.

Table 4 Cumulative gas production volume (mL) at different incubation times of alfalfa hay, containing 7.5 mg N, in N-free medium (rumen liquor and faeces suspension) after pre incubation

Incubation time (h)	With rumen liquor	With faeces suspension	SEM	P-value
2	2.05	15.05	0.64	0.0001
4	19.05	31.97	1.83	0.0076
6	34.50	39.61	1.34	0.0541
8	44.42	48.61	1.89	0.1922
12	66.47	65.97	2.10	0.8744
16	91.08	89.86	2.80	0.7734
24	118.11	144.72	2.91	0.0029
36	131.33	162.03	7.42	0.0429
48	138.00	165.03	6.67	0.0456
60	141.33	167.36	7.26	0.0643
72	143.58	169.05	6.80	0.0570

SEM: standard error of the means.

Table 5 Regression equation of *in situ* crude protein degradability estimated from gas production using rumen liquor and faeces suspension as inocula for alfalfa hay in different incubation times

	Equations	r^2	n
Rumen liquor			
1	Y= 58.93 + 0.32 gas	0.76	18
2	Y= 60.88 + 0.91 gas - 3.21 t	0.85	18
3	Y= 69.77 + 0.32 gas - 0.57 CP	0.77	18
4	Y= 135.44 + 1.12 gas - 3.88 CP - 4.36 t	0.88	18
Faeces suspension			
1	Y= 59.38 + 0.27 gas	0.60	18
2	Y= 62.84 - 0.89 gas + 6.62 t	0.74	18
3	Y= 42.33 + 0.27 gas + 0.89 CP	0.61	18
4	Y= 75.33 - 0.88 gas + 0.29 CP + 6.60 t	0.74	18

Y: crud protein degradability (%); gas: mL/7.5 mg N, t: time of incubation and CP: crud protein (%).
n: number of observation.

Prediction equations of CPD after 24 h incubation using the modified gas production methods with rumen liquor and faeces suspension are as below:

Using rumen liquor:
CPD= 108.32 X
X= (gas 24 h/gas 72 h), r^2= 0.99.

Using faeces suspension:
CPD= 103.18 X
X= (gas 24 h/gas 72 h), r^2=0.99.

According to the literature, no equation was found for estimating *in situ* CPD of alfalfa hay from gas production using the method of Cone *et al*. (2009). Nikkhah and Mahdavi, (2006) presented an equation (Y=47.136+0.854x) that estimated *in situ* CPD of alfalfa hay from the gas production method of Menke *et al*. (1979), which was a technique originally developed to evaluate the fermentation kinetics of organic matter in rumen fluid rather than rumen fermentability of specific nutrients. In their experiments, amount of gas production was expressed as per unit dry matter (200 mg DM), instead of per unit nitrogen of feed. They did not considered nitrogen as a limiting factor for microbial growth, thus their prepared buffer and rumen liquor was not free of nitrogen. In the current study, we have considered a pre-incubation in order to remove nitrogen from the media, (in order to prepare N-free rumen liquor or faeces suspension). Finally gas production was expressed based on 7.5 mg N. Moreover, we have used $NaHCO_3$ in place of NH_4HCO_3 for removing nitrogen from the buffer.

The regression equations presented from the current research data in order to estimate *in situ* CPD of alfalfa hay from gas production by the modified method (faeces suspension) are an innovation that should be corroborate.

CONCLUSION

Since, nitrogen is a limiting factor for microbial growth in both the gas production method described by Cone *et al*. (2009) and the modified method using faeces suspension, there is a close relationship between *in situ* crude protein degradation at different incubation times and amount of gas produced at similar incubation times using either rumen liquor or fecal suspension. Thus, the method it can be used to estimate the amount of crude protein degradation based on gas production. The results indicated that faeces suspension can be used instead of rumen liquor in the gas production method. Developing the current method should result in removing need to fistulated animals. However, there is need of further research using other feedstuffs.

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توسعه روش تولید گاز آزمایشگاهی تغییر یافته به جای روش کیسه‌های نایلونی برای ارزیابی تجزیه‌پذیری پروتئین یونجه خشک در نشخوارکنندگان

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چکیده

این مطالعه به منظور بررسی امکان استفاده از روش تولید گاز آزمایشگاهی تغییر یافته به جای روش کیسه‌های نایلونی برای برآورد تجزیه‌پذیری پروتئین یونجه خشک در نشخوارکنندگان به اجرا در آمد. در آزمایش کیسه‌های نایلونی مقدار سه گرم از نمونه یونجه داخل کیسه‌های داکرونی ریخته شد. کیسه‌ها به مدت صفر، ۲، ۴، ۸، ۱۲، ۱۶، ۲۴، ۳۶ و ۴۸ ساعت در شکمبه سه رأس گوسفند نر قزل کانوله‌دار انکوبه شدند. در پایان آزمایش تجزیه‌پذیری ماده خشک، ماده آلی و پروتئین محاسبه شدند. در آزمایش تولید گاز، شیرابه شکمبه بافری شده به نسبت ۱۹ قسمت بزاق مصنوعی و یک قسمت شیرابه شکمبه تهیه و با کربوهیدرات سریع قابل تخمیر به مدت ۴ ساعت پیش انکوبه شد. بعد از پیش انکوباسیون مقدار ۳۰ میلی‌لیتر از این محلول داخل سرنگ ۱۰۰ میلی‌لیتری که درون آن مقداری نمونه یونجه که دارای ۷/۵ میلی‌گرم نیتروژن بود، ریخته شد. نمونه‌ها در زمان‌های ۲، ۴، ۶، ۸، ۱۲، ۱۶، ۲۴، ۳۶، ۴۸، ۶۰ و ۷۲ ساعت انکوبه شدند و بعد از آن گاز خالص تولیدی محاسبه شد. در سومین آزمایش ۲۵ گرم از نمونه مدفوع با ۵۰ میلی‌لیتر بزاق مصنوعی مخلوط شده و با اضافه کردن بزاق مصنوعی، حجم آن به یک لیتر رسانده شده و صاف گردید. سپس این سوسپانسیون به مدت ۴ ساعت پیش انکوبه شد. پس از پیش انکوباسیون مراحل تولید گاز همانند روش معمول انجام شد. نتایج نشان داد که اختلاف معنی داری بین تولید گاز با شیرابه شکمبه و مدفوع در زمان‌های ۲، ۴، ۲۴، ۳۶ و ۴۸ ساعت انکوباسیون وجود داشت در حالی‌که در بقیه ساعت‌های انکوباسیون اختلاف معنی داری وجود نداشت. رابطه نزدیکی بین تجزیه‌پذیری پروتئین خام در ساعت‌های مختلف انکوباسیون و مقدار گاز تولیدی با استفاده از شیرابه شکمبه (n=۱۸ و r^2=۰/۷۶ و گاز ۰/۳۲+۵۸/۹۳=تجزیه‌پذیری پروتئین خام) و سوسپانسیون مدفوع (n=۱۸ و r^2=۰/۶۰ و گاز ۰/۲۷+۵۸/۳۸=تجزیه‌پذیری پروتئین خام) وجود داشت. نتایج نشان داد که سوسپانسیون مدفوع می‌تواند به جای شیرابه شکمبه در روش تولید گاز آزمایشگاهی استفاده شود. توسعه این روش تولید گاز آزمایشگاهی می‌تواند باعث کاهش نیاز به دام‌های فیستوله‌گذاری شده در آزمایش‌های ارزیابی مواد خوراکی شود.

کلمات کلیدی یونجه، مدفوع، تولید گاز، کیسه‌های نایلونی.