Kafkas Univ Vet Fak Derg
23 (2): 325-332, 2017
DOI: 10.9775/kvfd.2016.16527

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Utilization of Cryopreserved Ruminal Liquor in *In Vitro* Gas Production Technique for Evaluating Nutritive Value of Some Feedstuffs [1]

Nurettin GÜLŞEN [1] Huzur Derya ARIK [1] Armağan HAYIRLI [2]
Mustafa Selçuk ALATAŞ [1] Melih AKSOY [3]

[1] *The Scientific and Technological Research Council of Turkey funded this project (#108O451)*

[1] Department of Animal Nutrition and Nutritional Disorders, Faculty of Veterinary Medicine, Selcuk University, TR-42100 Konya - TURKEY
[2] Department of Animal Nutrition and Nutritional Disorders, Faculty of Veterinary Medicine, Atatürk University, TR-25700 Erzurum - TURKEY
[3] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Adnan Menderes University, TR-09500 Aydın - TURKEY

Article Code: KVFD-2016-16527 Received: 20.07.2016 Accepted: 20.10.2016 Published Online: 20.10.2016

Citation of This Article

Gülşen N, Arık HD, Hayırlı A, Alataş MS, Aksoy M: Utilization of cryopreserved ruminal liquor in *in vitro* gas production technique for evaluating nutritive value of some feedstuffs. *Kafkas Univ Vet Fak Derg,* 23 (2): 325-332, 2017. DOI: 10.9775/kvfd.2016.16527

Abstract

In vitro gas production technique (IVGPT) is a routine method in nutritional sciences to determine energy content, organic matter (OM) digestibility, and fermentation kinetics of feedstuffs. After collecting from two ruminally cannulated Holstein heifers (350 kg), rumen liquors were used either fresh or cryopreserved form in the inoculums for IVGPT. Starch- (barley, wheat, and corn) and protein-rich (sunflower meal, cotton seed meal, and soybean meal) feedstuffs were evaluated for gas production kinetics, fermentation pattern, and energy content in 5 replicates. pH, NH_3-N concentration and volatile fatty acids (VFA) profile, gas production, and fermentation kinetics parameters were measured. Data were analyzed by 2-way ANOVA. Viable protozoa rate was found to be 70.8% in cryopreserved rumen liquor after thawing. Decrease in pH in thawed rumen liquor was less than fresh rumen liquor as the incubation period advanced. Utilization of frozen rumen liquor after thawing in IVGPT was associated with lower VFA and NH_3-N concentration, cumulative gas production, and metabolisable energy estimate for all feedstuffs. In conclusion, despite high correlation between in vitro data obtained from fresh and thawed rumen liquors to predict gas production, further experiments should cope with improving cryopreservation protocol for rumen liquors in order to optimize microbial activity for maintaining fermentation pattern.

Keywords: *Cryopreserved rumen liquor, Energy prediction, Fermentation, Gas production, In vitro gas test, Volatile fatty acid*

Bazı Yemlerin Besin Değerlerini Değerlendirmek için *In Vitro* Gaz Üretim Tekniğinde Dondurulmuş Rumen Sıvısının Kullanımı

Özet

In vitro gaz üretim tekniği (IVGPT), besleme çalışmalarında yem maddelerinin enerji içeriklerini, organik madde (OM) sindirilebilirliğini ve fermentasyon kinetiklerini belirlemede kullanılan rutin bir yöntemdir. İki adet rumen kanüllü Holstein düveden (350 kg) elde edilen rumen sıvıları taze veya dondurulmuş formda IVGPT için inoculum olarak kullanıldı. Nişasta (arpa, buğday ve mısır) ve protein (ayçiçeği küspesi, pamuk tohumu küspesi ve soya fasülyesi küspesi) bakımından zengin yem maddelerinin gaz üretim kinetikleri, fermentasyon profile ve enerji içerikleri 5 tekerrlü belirlendi. pH, NH_3-N konsantrasyonu, uçucu yağ aside (UYA) profili, gaz üretimi ve fermentasyon kinetik parametleri ölçüldü. Veriler 2-yönlü ANOVA ile analiz edildi. Dondurulduktan sonra çözdürülen rumen sıvısındaki canlı protozoa oranı %70.8 bulundu. Inkübasyon periyodu devam ederken çözünmüş rumen sıvısındaki pH düşüşü taze rumen sıvısındaki düşüşten daha azdı. IVGPT'de dondurulup çözdürülmüş rumen sıvısı kullanılmasıyla yem maddeleri için UYA düzeyleri, NH_3-N konsantrasyonu, kümülatif gaz üretimi ve tahmini metabolik enerji değeri düşük bulundu. Sonuçta, gaz üretim tahmini için taze rumen sıvısı ve dondurulup çözdürülmüş rumen sıvısı kullanılmasıyla elde edilen in vitro veriler arasında önemli bir benzerlik olmasına rağmen, optimum mikrobiyal aktiviteyi, dolayısıyla fermentasyonu sürecini sağlamak için gelecekte yapılacak çalışmalar rumen sıvılarının kriyoprezervasyon protokolünü geliştirmeye odaklanmalıdır.

Anahtar sözcükler: *Dondurulmuş rumen sıvısı, Enerji tahmini, Fermentasyon, Gaz üretimi, In vitro gaz testi, Uçucu yağ asitleri*

İletişim (Correspondence)
+90 332 2233566
ngulsen@selcuk.edu.tr

INTRODUCTION

Nutrient content and digestibility are important feedstuff parameters to formulate cost-effective rations. Because of being less costly and laborious as well as reliable while producing a large number of data with a low variability in a short time in a controllable environment, *in vitro* gas production technique (IVGPT, also known as Hohenheimer Futter Test) has become common [1].

Many researchers have employed IVGPT to determine OM digestibility (OMD) [2,3], energy [4,5], fermentation kinetics [6], the adverse effects of anti-nutritional factors [7], and methane emission [8]. Nevertheless, there are inconsistencies among *in vitro* outcomes from different laboratories. It appears that availability of uniform and continuous rumen liquor supply is one of the major factors in yielding variable results [9].

Cryopreservation may offer some advantages for IVGPT, in terms of availability of constant rumen liquor from defined donors to eliminate variability in microbial inoculum source [10]. Controlling cell density, cryoprotectant concentration, equilibrium period, suspension temperature, and time to hold cells in suspension are shown to succeed in maintaining viability of rumen microorganisms [11]. However, studies coping with cryopreserved rumen liquor usage as a source of inoculum in IVGPT are limited.

This study was therefore conducted to evaluate fermentation characteristics and nutritive value of commonly used concentrate feedstuffs in ruminant nutrition by IVGPT employing fresh and cryopreserved rumen liquors in comparison with *in vivo* experimentation.

MATERIALS and METHODS

Animals and Management

The experimental protocol (#2008/028) was approved by the Selçuk University Ethic Committee on Animal Experimentation (Konya, Turkey). Two ruminally cannulated Holstein heifers weighing an average of 350 kg were served as rumen liquor donors. Heifers fed a ration consisting of 60% forage (13.43% CP and 2320 kcal/kg ME on DM basis) and 40% concentrate (17.32% CP and 2810 kcal/kg ME on a DM basis) twice daily at 08:00 and 17:00 h, delivering 5 kg 3rd cut alfalfa hay plus 3.5 kg concentrate per day, to meet nutrient requirement for maintenance and ~0.5 kg daily weight gain [12]. The ration contained 95.2% dry matter (DM), 15% crude protein (CP), 41.2% aNDF (neutral detergent fiber of organic matter with sodium sulphite and heat stable α-amylase), 26.7% acid-detergent fiber (ADF), 50.1% nitrogen-free extract (NFE), 3.1% ether extract (EE), and 9.8% crude ash (CA). Fresh water was available *ad libitum*.

Experimental Feedstuffs

Commercially available barley grain (BG), wheat grain (WG), corn grain (CG), sunflower seed meal (SFM), cotton-seed meal (CSM), and soybean meal (SBM) samples (n = 5) were ground to pass a 1-mm screen (Retsch, SM100 Comfort, Germany) and conserved for experimentations in plastic containers *(Table 1)*.

Rumen Liquor Collection and Cryopreservation Protocol

Before morning feeding, rumen liquors collected from different spots in both heifers were mixed and then poured into a prewarmed container. Liquor was filtered through a double-layer cheese cloth under CO_2 pump. A part liquor was separated for the cryopreservation protocol. Samples were put into 50 ml plastic containers and centrifuged at 4.640 *g* for 30 min (Allegra 64R, Beckman Coulter, Brea, CA) [13]. After removing supernatant, pellet was added with dimethyl sulfoxide (DMSO, 5%, vol/vol), a cryoprotectant and let stand at 25°C for 5 min (equilibration time). For the two-step cryopreservation protocol, treated aliquots (1 ml) were transferred into cryotubes for freezing in a computer-controlled freezer (Ice Cube 14S, Sy-Lab, Neupurkersdorf, Austria), from 25 to -2°C (extracellular ice nucleation temperature) at a rate of 7°C/min (the first step). Then, the suspension was continued to freeze from -2 to -30°C, at a rate of 1.4°C/min and kept at -30°C for 45 min (holding temperature, the second step) [14] before placing them into N tank (-196°C) (Taylor Wharton, Theodore, AL) until IVGPT experimentation. On the day of IVGPT, frozen rumen liquors were thawed at 39°C for 5 min in water bath. In the tests, thawed rumen liquors (1 ml) were added into incubation media (29 ml).

In vitro Gas Production Technique

For the *in vitro* gas production, the media were prepared using the Hohenheim Gas Test as outlined by Menke and Steingass [4]. Each of 0.2 g feedstuff samples, in triplicates, was incubated with the mixture (20 ml medium solution and 10 ml fresh rumen liquor; 29 ml medium solution + 1 ml cryopreserved rumen liquor after centrifugation) in a pyrex bottle (100 ml) a digital manometer (Keller Leo 1, Switzerland) was used for determination of gas production [15]. In each assay, blank bottle without a feed sample were run in triplicates.

Gas production was measured at 6, 12, 24, and 48 hrs post-incubation. Gas production kinetics parameters were calculated using NEWAY software (Version 5.0) as described by Ørskov and McDonald [16], which was as follows: $P = a + b*(1-e^{-c*t})$, where P = corrected gas production at time t relative to incubation (ml), a = gas production from soluble fraction (ml), b = gas production from insoluble but slowly fermentable fraction (ml), c = gas production rate from the fraction b (ml/h), and t = incubation time (h). The effective gas production (EGP) was calculated using the following

Table 1. *Nutrient contents (%) of the experimental feedstuffs **

Nutrient††	Feedstuffs†						P
	BG	WG	CG	SFM	CSM	SBM	
DM	91.2±0.3^{ab} (0.7)	91.4±0.3^{ab} (0.6)	89.2±0.4^{c} (1.1)	91.2±0.4^{ab} (0.9)	92.2±0.7^{a} (1.60)	90.8±0.4^{b} (0.9)	0.001
CP	13.7±0.5^{c} (8.3)	12.5±0.9^{c} (15.9)	7.8±0.5^{d} (13.6)	30.6±2.5^{b} (18.1)	29.0±2.3^{b} (18.0)	46.8±0.6^{a} (3.0)	0.0001
EE	2.44±1.09^{b} (11.1)	2.40±1.07^{b} (25.6)	4.16±1.86^{a} (12.1)	1.38±0.62^{b} (25.5)	4.30±1.92^{a} (67.7)	2.02±0.90^{b} (30.18)	0.006
CA	2.19±0.18^{b} (18.6)	1.46±0.33^{b} (50.8)	1.37±0.41^{b} (66.5)	6.61±0.27^{a} (9.1)	6.46±0.38^{a} (13.3)	6.78±0.14^{a} (4.5)	0.0001
CF	6.97±0.43^{bc} (13.7)	4.73±0.41^{c} (19.4)	4.89±0.15^{c} (7.0)	24.03±1.23^{a} (11.5)	22.78±1.31^{a} (12.8)	8.77±0.40^{b} (10.1)	0.0001
NFE	74.7±0.9^{b} (2.8)	78.9±1.6^{ab} (4.5)	80.3±2.4^{a} (6.8)	37.4±1.7^{c} (10.3)	37.4±1.5^{c} (9.0)	35.7±0.3^{c} (1.97)	0.0001
aNDF	21.5±0.4^{b} (4.3)	17.3±1.0^{bc} (13.4)	13.6±0.4^{cd} (6.6)	46.9±4.2^{a} (20.1)	51.1±1.9^{a} (8.2)	10.8±0.4^{d} (8.7)	0.0001
ADF	6.71±0.32^{b} (10.6)	4.08±0.25^{b} (13.9)	3.61±0.14^{b} (8.5)	32.3±2.7^{a} (18.8)	35.0±2.7^{a} (17.1)	6.77±0.47^{b} (15.7)	0.0001

** Data are LSM±SE (% CV),* ***n*** *= 5; Different superscripts within the same rows differ (P<0.05);* ***†BG*** *= barley grain;* ***WG*** *= wheat grain;* ***CG*** *= corn grain;* ***SFM*** *= sunflower seed meal;* ***CSM*** *= cottonseed meal;* ***SBM*** *= soybean meal;* ***††DM*** *= dry matter;* ***CP*** *= crude protein;* ***EE*** *= ether extract;* ***CA*** *= crude ash;* ***CF*** *= crude fiber;* ***NFE*** *= nitrogen–free extract;* ***aNDF*** *= amylase-treated neutral detergent fiber;* ***ADF*** *= Acid detergent fiber*

formula: EGP, ml = a + b*c/(c + k), where k = ruminal flow rate (5%).

Using the cumulative gas production (CGP) as well as nutrients such as CP, EE, and CA, *in vitro* ME (IVME) values were calculated using following formula as defined by Menke and Steingass [4]:

IVME, Mcal/kg DM = [(1.06 + 0.157 x CGP + 0.084 x CP + 0.022 x EE - 0.0081 x CA) x 1000]/4.186.

In vitro NE_L ($IVNE_L$) value of feedstuffs was determined using the same variables as defined by Steingass [17] as follows: $IVNE_L$, kcal/kg DM) = [(1.64 + 0.0269 x CGP + 0.00078 x CGP^2 + 0.0051 x CP + 0.01325 x EE) x 1000] /4.186.

In vitro digestible OM (IVDOM) was calculated as defined by Öğretmen [18] as follows: IVDOM, % DM = 0.7602 x CGP + 0.6365 x CP + 22.53.

Measurements and Laboratory Analyses

Feedstuffs were subjected to wet chemistry for DM, CP, CF, EE, and CA [19] as well as for aNDF and ADF [20] using The Ankom^{200} Fiber Analyzer.

On the day of using frozen rumen liquors after thawing at 39°C for 5 min, 3 cryotubes were subjected to viable protozoa enumeration by the same two individuals. Viability was determined immediately after mixing rumen liquors (15 µl) with 15 µl ml 0.5% trypan blue [21]. Enumeration was performed as described by Dehority [22].

pH was measured at 6, 12, 24 and 48 hrs relative to incubation using a digital pH meter (HI 8314, Hanna Instruments, Portugal). NH_3-N concentrations were determined using spectrophotometer (625 nm, UV Mini 1240, UV-VIS Spectrophotometer, Shimadzu, Japan) [23]. VFAs were determined using gas chromatography (Shimadzu, Model 15-A) with FID detector [24].

Statistical Analysis

Descriptive statistics of nutrient contents were determined using the Proc Univariate procedure [25]. Differences among feedstuffs were determined by one-way ANOVA using the Duncan Multiple Range Test option.

Two-way ANOVA with repeated measures option was employed to determine the effect of the rumen liquor form and feedstuff on pH, NH_3-N concentration, and gas production. The linear model was $y_{ijk} = \mu + RL_i + FS_j + (RL \times FS)_{ij} + T_k + (RL \times T)_{ik} + (FS \times T)_{jk} + (RL \times FS \times T)_{ijk} + e_{ijk,}$ where RL = i^{th} rumen liquor (fresh *vs*. cryopreserved), FS = j^{th} feedstuff, T = k^{th} incubation time, and e = residual error. For other variables measured at a single time point (*i.e.*, VFA, effective gas production, gas production kinetics parameters, IVME, $IVNE_L$, IVDOM), "time" parameter and its interaction terms were omitted from the linear model.

Finally, mathematical relationships between CGP and ME estimates as well as between ME and NE_L values obtained from inoculums prepared from fresh and thawed rumen liquors were established using the REG procedure. Statistical significance was declared at P<0.05.

RESULTS

Nutrient Content

Leguminous feeds (SBM > SFM = CSM) had greater CP level than gramineous feeds (BG > WG > CG) *(Table 1)*.

Protozoa Count and Viability

Rumen protozoa count in fresh rumen liquor was within normal range (1.5-5.3 x 10^5). In thawed rumen liquor the protozoa viability was 70.8% when protozoa count expressed as log basis.

Ruminal pH, NH_3-N and VFA Concentration

pH of media containing fresh ruminal liquor was lower than pH of media containing thawed ruminal liquor (6.90 *vs*. 7.01, P<0.0001; *Table 2)*. NH_3-N concentration was greater in media prepared from fresh ruminal liquor than in media prepared from thawed rumen liquor (14.4 *vs*. 12.9 mmol/l, P<0.0001; *Table 2)*. During incubation pH decreased slower and NH_3-N increased faster in media containing fresh rumen liquor than those containing thawed rumen liquor (P<0.0001 for both). As the incubation period progressed, media pH increased gradually with fermentation of protein rich-feedstuffs, whereas media pH decreased gradually with fermentation of starch rich-feedstuffs (P<0.0001). Increase in NH_3-N concentration was continuous when protein rich-feedstuffs were incubated, whereas there was a lag period in release of NH_3-N when starch rich-feedstuffs were incubated (P<0.0001), *(Table 2)*.

The acetate (51.77 vs. 54.72, P<0.0001) proportion was greater and the propionate portion was lower (20.00 vs. 24.29%, P<0.0001) when feedstuffs were incubated in fresh rumen liquor as compared to thawed rumen liquor *(Table 3)*. Cryopreservation caused 17.2 and 22.6% decreases in the Ac:Pr ratio (P<0.01) and total VFA concentration (P<0.0001), respectively *(Table 3)*.

Gas Production and Kinetics Parameters

The amount (40.32 vs. 39.10 ml, P<0.0001; *Table 2)* and rate *(Fig. 1A)* of gas production during the *in vitro* incubation with fresh rumen liquor were greater than thawed rumen liquor. Fermentation of starch-rich feedstuffs resulted in 1.73-fold greater gas production than protein rich-feedstuffs (P<0.0001; *Table 2)*. The rate of increase in gas production over time was greater for starch-rich feedstuffs than for protein-rich feedstuffs (P<0.0001; *Fig. 1B)*.

Cumulative gas production from media containing fresh and thawed rumen liquor within 24 h was highly correlated (r = 0.98) and fit to following model: *In vitro* CGP from fresh rumen liquor within 24 h (ml) = 11.74 + 0.77 x *In vitro* CGP from thawed rumen liquor within 24 h (ml) (R^2 = 0.97, Sy.x = 4.60%; P<0.0001; *Fig. 1C)*.

Cumulative gas production in media containing thawed rumen liquor fit considerably to predict ME value of feeds

Table 2. *The effect of the rumen liquor form on pH, NH_3-N concentration, in vitro gas production and kinetics parameters **

Treatments††	Response Variables†						
	pH³	NH_3-N³	CGP‡	EGP	a	b	c
Fresh rumen liquor							
BG	6.80±0.01	13.6±0.2	49.2±0.3	41.4±6.7	-10.44±1.04	86.6±10.8	0.077±0.003
WG	6.80±0.01	13.5±0.2	52.3±0.3	44.4±6.4	-9.84±1.01	93.8±10.4	0.069±0.003
CG	6.80±0.01	12.1±0.2	48.8±0.3	39.8±6.4	-19.45±1.04	100.8±10.8	0.073±0.003
SFM	7.05±0.01	15.4±0.2	26.7±0.3	23.0±5.8	-1.49±0.90	41.0±9.3	0.075±0.003
CSM	7.03±0.01	14.8±0.2	26.6±0.3	22.8±5.6	-1.71±0.88	47.6±9.1	0.055±0.003
SBM	6.94±0.01	16.8±0.2	38.3±0.3	32.8±6.4	-5.08±1.01	58.0±10.4	0.095±0.003
Group mean	6.90±0.004	14.4±0.1	40.3±0.1	34.0±0.2	-8.00±0.40	71.3±4.2	0.074±0.001
Thawed rumen liquor							
BG	6.92±0.01	12.0±0.2	48.9±0.3	40.0±6.4	-24.41±1.01	101.0±10.4	0.089±0.003
WG	6.87±0.01	11.8±0.2	55.0±0.3	43.8±6.4	-33.25±1.01	115.1±10.4	0.102±0.003
CG	6.92±0.01	10.8±0.2	47.8±0.3	38.4±6.4	-25.25±1.01	110.0±10.4	0.069±0.003
SFM	7.11±0.01	14.6±0.2	24.8±0.3	21.9±5.6	3.73±0.88	45.1±9.1	0.038±0.003
CSM	7.15±0.01	14.0±0.2	20.2±0.3	19.2±6.4	7.76±1.01	22.2±10.4	0.001±0.003
SBM	7.07±0.01	14.0±0.2	38.0±0.3	32.9±6.7	1.20±1.04	62.1±10.8	0.052±0.003
Group mean	7.01±0.004	12.9±0.1	39.1±0.1	32.7±0.2	-11.70±0.41	68.5±4.2	0.058±0.001
ANOVA (------ P > F ------)							
Rumen liquor (RL)	0.0001	0.0001	0.0001	0.0001	0.0001	0.63	0.0001
Feedstuff (FS)	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
RLxFS	0.0001	0.0001	0.0001	0.04	0.0001	0.0001	0.0001

** Data are LSM±SE,* ***n*** *= 5;* †***NH₃-N*** *= ammonia nitrogen (mmol/l);* ***CGP*** *= cumulative gas production within 24 h (ml);* ***EGP*** *= effective gas production (ml);* ***a*** *= gas production from soluble fraction (ml);* ***b*** *= gas production from insoluble but slowly fermentable fraction (ml).* ***c*** *= gas production rate from the fraction b (ml/h);* ††***BG*** *= barley grain;* ***WG*** *= wheat grain;* ***CG*** *= corn grain;* ***SFM*** *= sunflower seed meal;* ***CSM*** *= cottonseed meal;* ***SBM*** *= soybean meal*
‡P<0.0001 for the effects of incubation time (T). RLxT. FSxT. and RLxFSxT

Table 3. *The effect of the rumen liquor form on volatile fatty acid proportion **

Treatments††	Response Variables†							
	Ac (%)	Pr (%)	Isobu (%)	Bu (%)	Isova (%)	Va (%)	Ac:Pr	Total VFA (mmol/L)
Fresh rumen liquor								
BG	53.71±1.40	19.99±0.78	3.14±0.49	17.55±0.64	2.91±0.30	2.69±0.20	2.80±0.38	96.2±5.3
WG	53.59±1.40	19.44±0.78	3.11±0.49	19.26±0.64	2.35±0.30	2.25±0.20	2.88±0.38	107.6±5.3
CG	53.42±1.25	19.77±0.69	2.75±0.43	19.50±0.57	2.85±0.27	1.70±0.18	2.80±0.34	100.4±4.7
SFM	55.69±1.10	20.36±0.61	4.89±0.38	10.59±0.51	5.34±0.24	3.14±0.16	2.81±0.30	87.9±4.2
CSM	60.20±1.07	18.81±0.59	4.06±0.37	9.88±0.49	4.32±0.23	2.72±0.15	3.30±0.29	84.4±4.0
SBM	51.73±1.15	21.64±0.64	4.22±0.40	12.73±0.52	5.86±0.25	3.81±0.16	2.88±0.31	123.5±4.3
Group mean	54.72±0.50	20.00±0.28	3.97±0.18	14.92±0.24	3.94±0.11	2.72±0.07	2.91±0.14	100.0±1.9
Thawed rumen liquor								
BG	49.30±1.27	25.56±0.70	2.12±0.44	18.40±0.58	2.06±0.28	2.56±0.18	1.98±0.34	88.7±4.8
WG	49.04±1.31	23.74±0.72	2.40±0.46	20.45±0.60	1.85±0.28	2.51±0.18	2.16±0.35	92.8±4.9
CG	51.04±1.43	24.62±0.79	2.19±0.50	18.68±0.66	1.55±0.31	1.92±0.20	2.11±0.38	87.9±5.4
SFM	52.14±1.23	24.65±0.68	5.81±0.43	9.90±0.56	4.82±0.27	2.68±0.17	2.75±0.33	65.8±4.6
CSM	55.27±1.19	24.260.66	5.47±0.42	9.46±0.55	3.28±0.26	2.26±0.17	2.35±0.32	44.4±4.5
SBM	53.80±1.38	22.92±0.76	3.68±0.48	11.03±0.63	4.95±0.30	3.61±0.20	3.10±0.37	84.6±5.2
Group mean	51.77±0.54	24.29±0.30	3.61±0.18	14.65±0.24	3.08±0.12	2.59±0.08	2.41±0.14	77.4±2.0
ANOVA (----- P > F -----)								
Rumen liquor (RL)	0.0001	0.0001	0.74	0.43	0.0001	0.22	0.01	0.0001
Feedstuff (FS)	0.0001	0.40	0.0001	0.0001	0.0001	0.0001	0.44	0.0001
RLxFS	0.06	0.03	0.02	0.15	0.68	0.17	0.41	0.001

** Data are LSM±SE.* ***n*** *= 5;* †*Ac = acetate;* ***Pr*** *= propionate;* ***Isobu*** *= isobutyrate.* ***Bu*** *= butyrate;* ***Isova*** *= isovalerate;* ***Va*** *= valerate;* ***VFA*** *= volatile fatty acid*
†† ***BG*** *= barley grain;* ***WG*** *= wheat grain;* ***CG*** *= corn grain;* ***SFM*** *= sunflower seed meal;* ***CSM*** *= cottonseed meal;* ***SBM*** *= soybean meal*

estimated using fresh rumen liquor *(Fig. 1D)*. Following models were developed to predict ME and NE_L values from fresh rumen liquor using CGP measured in 24 h from thawed rumen liquor and nutrient contents: *In Vitro* ME kcal/kg (fresh rumen liquor) = 1089 + 6.98 x CGP (24 h, ml, thawed rumen liquor) + 30.79 x CP (%) + 77.11 x EE (%) + 12.14 x NFE (%) + 5.08 x aNDF (%) – 77.11 x CA (%) - 16.70 x ADF (%) (R^2 = 0.97, Sy.x = 7.07%, P<0.0001). *In Vitro* NE_L kcal/kg (fresh rumen liquor) = 644 + 9.00 x CGP (24 h, ml, thawed rumen liquor) + 2.11 x NFE (%) + 13.20 x aNDF (%) – 24.93 x ADF (%) (R^2 = 0.97, Sy.x = 3.79%, P<0.0001).

Energy Estimation and Organic Matter Digestibility

The mean ME and IVOMD values for feeds incubated with thawed rumen liquor were lower than those for feeds incubated with fresh rumen liquor (2594 *vs*. 2643 kcal/kg; P<0.0001 and 74.1 *vs*. 75.9%, P<0.003; *Table 4)*. The rumen liquor form did not affect NE_L value of feeds.

DISCUSSION

In general, nutrient contents of tested feedstuffs are in agreement with those reported in the literature [26]. Variations in nutrient contents within and among feedstuffs are natural and are mainly due to hybrid, growth condition, soil composition, and climatological factors.

Protozoa count is consistent with literature and within the normal range [22]. Controlled freezing increases viability [27], such as until -30°C, 1°C/min freezing speed for anaerobic bacteria [28]. It is suggested that frozen anaerobic bacteria should be thawed at 37°C within 1 min [28]. Nevertheless, effective freezing protocol for rumen liquor has not been defined [29]. Survival response also seems to vary by the cryopreservation protocol. It was reported that viability of *Isotricha*, *Dasytricha*, *Epidinium*, *Polyplastron*, *Eudiplodinium*, and *Entodinium* were 100, 98, 85, 79, 63, and 60%, respectively [14]. Cryoprotectant (5% DMSO) usage and controlled-2 step freezing (holding phase of -30°C for 45 min at a rate of 1.4°C/min) application to assure extracellular ice enucleation temperature (-2°C) in this experiment may explain high protozoa survival rate.

Higher pH *(Table 2)*, lower NH_3-N concentration *(Table 2)*, and lower total VFA concentration *(Table 3)* in media containing thawed rumen liquor may result from decreased survival, perhaps activity of microorganisms, and hence inefficient fermentation [9]. Energy rich grains incubated within thawed liquor reached the lowest pH at 12 h when

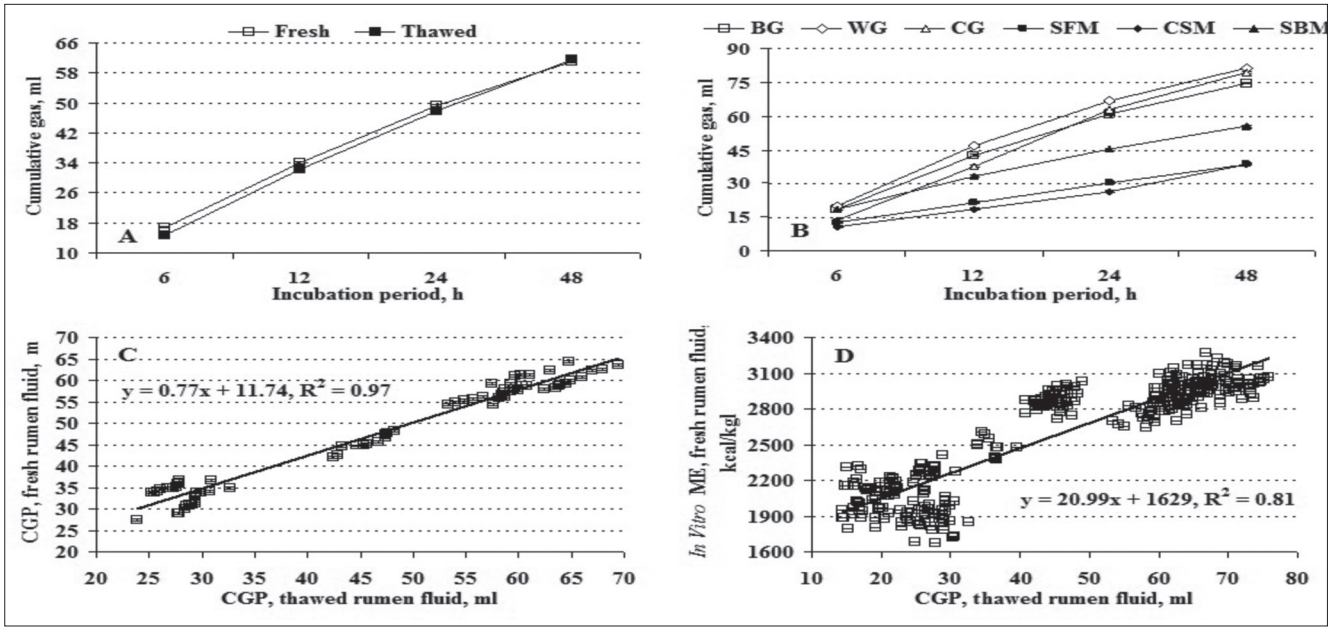

Fig 1. Comparison of fermentation products in inoculums prepared from fresh (--□--) and thawed (--■--) rumen liquor during the *in vitro* incubation Cumulative gas production in inoculums (P<0.0001, Panel A). Cumulative gas production from *in vitro* fermentation of barley grain (--□--), wheat grain (--◊--), corn grain (--Δ--), sunflower meal (--■--), cottonseed meal (--♦--), and soybean meal (--▲--) (P < 0.0001; Panel B). Regressing cumulative gas production (CGP) in inoculum prepared from thawed rumen liquor within 24-h incubation on CGP in inoculum prepared from thawed rumen liquor within 24-h incubation (Panel C) and *in vitro* ME value determined in inoculum prepared from thawed rumen liquor (Panel D)

Table 4. *Energy content and organic matter digestibility (OMD) determined in vitro gas technique employing fresh and frozen rumen liquors **

Treatments†	ME (kcal/kg)	NE_L (kcal/kg)	*In vitro* OMD (%)
Fresh rumen liquor			
BG	2882.5±23.9	1487.6±12.4	77.3±0.6
WG	3026.6±23.6	1608.4±12.3	79.6±0.6
CG	2950.3±23.6	1548.4±12.3	75.1±0.6
SFM	1989.3±21.1	827.8±11.0	65.8±0.5
CSM	2134.0±20.8	836.6±10.8	65.4±0.5
SBM	2874.5±23.6	1135.9±12.3	86.9±0.6
Group mean	2643±9.0	1241±5.0	75.9±0.2
Thawed rumen liquor			
BG	2960.4±23.6	1549.0±12.3	78.8±0.6
WG	3199.2±23.9	1759.7±12.4	83.3±0.6
CG	2971.4±23.9	1566.4±12.4	75.6±0.6
SFM	1872.7±20.8	768.9±10.8	63.5±0.5
CSM	1713.6±20.6	656.9±10.7	56.9±0.5
SBM	2849.8±24.4	1120.3±12.7	86.4±0.6
Group mean	2595±9.0	1247±5.0	74.1±0.2
ANOVA (----- P > F -----)			
Rumen liquor (RL)	0.0001	0.57	0.003
Feedstuff (FS)	0.0001	0.0001	0.0001
RLxFS	0.0001	0.0001	0.0001

** Data are LSM±SE. **n** = 5; †**BG** = barley grain; **WG** = wheat grain; **CG** = corn grain; **SFM** = sunflower seed meal; **CSM** = cottonseed meal; **SBM** = soybean meal*

gas production started to increase. Decrease in pH with SBM was more notable than CSM and SFM *(Table 2)*. High cellulose content of CSM and SFM might lead to slower decrease in pH. It was shown that freeze drying rumen liquor caused reduction in gas production, by 6-12% in concentrate feeds, 11-30% in hays, and 23-49% in straws, suggesting that freezing affects viability and/or activity of cellulolytic microorganisms [4].

Lower NH_3-N concentration in thawed rumen liquor than fresh rumen liquor is in agreement with a study by Luchini et al.[30]. Protozoa plays a role in protein degradation, as well; highly soluble protein fractions are degraded by bacteria, whereas poorly soluble protein fractions are degraded by protozoa [31]. Both decreased protozoa viability by 30% in thawed liquor and characteristics of CSM could result in its inefficient degradation.

In addition to survival and activity, freezing rumen liquor may alter bacterial cell wall, such as lipid composition [32] and porosity [13], which limits bacterial attachment, particularly to cell wall rich feedstuffs, such as CSM and SFM. This may lead to lower acetate and total VFA production *(Table 3)*. Responses of the propionate and acetate fractions [33] and the Ac:Pr ratio [30] to fermentation of starch- and protein-rich feedstuffs *(Table 3)* are in agreement with literature.

During incubation, degraded feed fraction is converted to gas, VFA, or incorporated into microbial mass [9]. Chaudhry and Mohamed [34] reported that CP and DM degradability of rapeseed meal and grass nuts were lower in thawed rumen liquor than in fresh rumen liquor. However, their degradations were highly correlated (r = 0.97), as in the

present experiment *(Fig. 1C)*. Degradation constants and effective degradabilities of feeds differed by the rumen liquor form.

Cryopreservation caused 3% reduction in gas production. In thawed rumen liquor the acetate proportion decreased by 4-fold compared to the propionate proportion. Relatively less reduced propionic acid could be due to low gas production from starch rich grains [35]. Moreover, gas loss occurs by 6-12% in *in vitro* experimentation employing thawed rumen liquor [4], which accounts for lower estimation of gas production. Rumen bacteria obtain their energy from fermentation of carbohydrate and protein. Energy from protein alone is not sufficient for bacterial growth, requiring readily available carbohydrates [36]. Indeed, the positive correlation between CGP and NFE content (r = 0.86, P<0.0001) in the present study and elsewhere [1] (r = 0.89) ascertains that bacterial growth is related to starch fermentation. Incubating starch-rich grains with thawed rumen liquor resulted in gas production at time 12 h was similar to those incubated with fresh rumen liquor, suggesting importance of starch content for bacterial activation. Pectin content of SBM stimulates growth of cellulolytic bacteria and their activities in early stage of *in vitro* incubation [37]. This could explain greater gas production from SBM than CSM and SFM in both fresh and frozen rumen liquor. Provision of soluble carbohydrates could increase efficiency of frozen rumen liquor. Moreover, even in fresh rumen liquor, gas production was shown to be negatively correlated with aNDF (r = -0.83) and ADF (r = -0.91) contents [24]. Similar relationship was determined in frozen rumen liquor (r = -0.84 for aNDF and r = -0.91 for ADF), suggesting that freezing adversely affect cellulolytic bacteria as reflected by 11-30% reduction in gas production [4].

In vitro incubation with thawed rumen liquor did not affect gas production from fraction "b", but reduced gas production per hr of incubation, fraction "c" and EGP. Hervas et al.[38] also reported these reductions in rumen liquors kept in ice for 24 hrs and those frozen in deep freezer. These were more notable in CSM and SFM, which had low fermentability and did not fit the model indicated by McDonald [39].

In this experiment, rumen liquor was subjected to condensation (centrifugation), 2-step controlled freezing, and keeping in liquid N. Freezing rumen liquor was associated with 2% underestimation of ME and NE_L values. Such a small percentage of underestimation could be due to low variability in CGP, a regression model component [40]. In a previous experiment, it was reported that ME level was 2820, 2892, and 2605 kcal/kg DM for WG, BG, and CG, respectively when IVGPT with fresh rumen liquor was employed [5]. ME values estimated from using fresh rumen liquor were lower than those reported by Getachew et al.[26], but similar to those reported by Seven et al.[41]. As compared with NRC [12], ME values were slightly lower in starch-rich grains, and markedly lower in protein-rich feedstuffs in the present experiment. Rumen liquor by feedstuff interaction revealed increases in estimate ME values for starch-rich grains and decreases in estimate ME values of protein-rich feedstuffs in thawed rumen liquor as compared to fresh rumen liquor.

In vitro OM digestibility data from fresh rumen liquors (75.1-79.6%) were lower than those reported by (78.5-87.3%) Umucalılar et al.[5], and similar to those reported by (78.2-81.3%) Şeker [2]. *In vitro* OM digestibility values had low variability in both rumen liquor forms. Limitations occurred for VFA formation and gas production in thawed rumen liquor appear to be valid for IVOMD data, as well.

Menke and Steingass [4] evaluated 700 feed samples using IVGPT and reported strong relationship between nutrient content and gas production. It was also shown that *in vivo* energy content and OMD were correlated with *in vitro* gas production measured using fresh rumen liquor [42]. However, many researchers [1,2] suggested that *in vitro* ME values were lower than their *in vivo* ME values, even in highly digestible feeds.

This experiment questioned feasibility of frozen rumen liquor usage in IVGPT through evaluating fermentation of commonly used concentrate feeds in ruminant nutrition. Despite achieving considerable viable protozoa count, usage of thawed rumen liquor increased pH and decreased NH_3-N concentration, the Ac:Pr ratio and total VFA concentration, gas production, and gas production rate as compared to usage of fresh rumen liquor. Moreover, ME and OMD values of feedstuffs were estimated to be lower in media containing thawed rumen liquor than in media containing fresh rumen liquor. However, CGP obtained using thawed rumen liquor was a good predictor of energy estimates obtained using fresh rumen liquor and digestibility. Also, response variables to fresh rumen liquor were highly correlated with response variables to thawed rumen liquor. The adverse effects of thawed rumen liquor usage were more notable on protein-rich feedstuff than starch-rich feedstuffs, particularly CSM. These may confirm detrimental effect of freezing on cellulolytic micro-organisms. It can be concluded that usage of frozen rumen liquor in IVGPT can be feasible if cryopreservation techniques are advanced to assure no change in microbial survival and activity.

REFERENCES

1. Getachew G, De Peters EJ, Robinson PH, Fadel JG: Use of an *in vitro* rumen gas production technique to evaluate microbial fermentation of ruminant feeds and its impact on fermentation products. *Anim Feed Sci Technol*, 123-124, 547-559, 2005. DOI: 10.1016/j.anifeedsci.2005.04.034

2. Şeker E: The determination of the energy values of some ruminant feeds by using digestibility trial and gas test. *Rev Med Vet*, 153, 323-328, 2002.

3. Umucalılar HD, Şeker E: Effects of sodium bicarbonate and magnesium oxide as buffers on *in vitro* digestibility of grains. *Vet Bilim Derg*, 16, 129-

135, 2000.

4. Menke KH, Steingass H: Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Anim Res Dev*, 28, 7-55, 1988.

5. Umucalılar HD, Coşkun B, Gülşen N: In situ rumen degradation and *in vitro* gas production of some selected grains from Turkey. *J Anim Physiol Anim Nutr,* 86, 288-297, 2002. DOI: 10.1046/j.1439-0396.2002.00381.x

6. Pell AN, Schofield P: Computerized monitoring of gas production to measure forage digestion *in vitro*. *J Dairy Sci*, 76, 1063-1073, 1993. DOI: 10.3168/jds.S0022-0302(93)77435-4

7. Makkar HPS: *In vitro* gas methods for evaluation of feeds containing phytochemicals. *Anim Feed Sci Technol*, 123-124, 291-302, 2005. DOI: 10.1016/j.anifeedsci.2005.06.003

8. Soliva CR, Hess HD: Measuring methane emission of ruminants by *in vitro* and in vivo techniques. **In,** Makkar HPS, Vercoe PE (Eds): Measuring Methane Production from Ruminants. 15-31, Springer Publishers, Netherlands, 2007.

9. Rymer C, Huntington JA, Williams BA, Givens DI: *İn vitro* cumulative gas production techniques: History, methodological considerations and challenges. *Anim Feed Sci Technol*, 123-124, 9-30, 2005. DOI: 10.1016/j.anifeedsci.2005.04.055

10. Thammavongs B, Poncet JM, Desmasures N, Gueguen M, Panoff JM: Resin straw as an alternative system to securely store frozen microorganisms. *J Microbiol Meth*, 57, 181- 186, 2004. DOI: 10.1016/j.mimet.2004.01.003

11. De La Fuente G, Perez-Quintana M, Cebrian JA, Fondevila M: Preliminary study on the effect of exposure to low temperature on the viability of both mixed and monocultures of rumen protozoa. *Acta Protozool*, 45, 225-230, 2006.

12. National Research Council (NRC): Nutrient Requirements of Dairy Cattle. Seventh Revised Edn, National Academy Press, Washington DC, 2001.

13. Hsu JT, Fahey GC: Effects of centrifugation speed and freezing on composition of ruminal bacterial samples collected from defaunated sheep. *J Dairy Sci*, 73, 149-152, 1990. DOI: 10.3168/jds.S0022-0302(90)78658-4

14. Nsabimina E, Kisidayova S, Macheboeuf D, Newbold CJ, Jouany JP: Two-Step freezing procedure for cryopreservation of rumen ciliates, an effective tool for creation of a frozen rumen protozoa bank. *APPl Environ Microbiol*, 69, 3826-3832, 2003. DOI: 10.1128/AEM.69.7.3826-3832.2003

15. Cone JW, Van Gelder AH: Influence of protein fermentation on gas production profiles. *Anim Feed Sci Technol*, 76, 251-264, 1999. DOI: 10.1016/S0377-8401(98)00222-3

16. Ørskov ER, Mc Donalds I: The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *J Agr Sci Cambridge,* 92, 499-503, 1979. DOI: 10.1017/S0021859600063048

17. Steingass H: Bestimmung des energetischen futterwertes von wirtschaftseigenen futtermitteln aus der gasbildung bei der pansenfermentation *in vitro*. Dissertation, Univ. Hohenheim, 1983.

18. Öğretmen T: Geviş getirenlerin beslenmesinde kullanılan önemli bazı yemlerin NEL içeriklerinin *in vivo* ve *in vitro* yöntemleri ile saptanması. *PhD Dissertation,* Ege Üniversitesi, Turkey, 1991.

19. AOAC: Official Methods of Analysis, Vol. I, 15th edn., Association of Official Analytical Chemist. Arlington, VA, USA, 1990.

20. Goering HK, Van Soest PJ: Forage Fiber Analysis (Apparatus, Reagents and some Applications). US Dep. Agric. Handb. 379, 1970.

21. Miyake Y, Karanis P, Uga S: Cryopreservation of protozoan parasites. *Cryobiol*, 48, 1-7, 2004. DOI: 10.1016/j.cryobiol.2003.10.004

22. Dehority BA: Evaluation of subsampling and fixation procedures used for counting rumen protozoa. *APPl Environ Microbiol*, 48, 182-185, 1984.

23. Weatherburn MW: Phenol-hypochlorite reaction for determination of ammonia. *Anal Chem*, 39, 971-974, 1967. DOI: 10.1021/ac60252a045

24. Supelco: Analyzing fatty acids by packed column gas chromatography. Sigma-Aldrich Corp., Bulletin 856, Bellefonte, PA, 1998.

25. SAS Institute: *SAS* User's Guide. Statistics, Version 9th. Statistical Analysis System. SAS Inst., Inc., Cary, NC, 2002.

26. Getachew G, Robinson PH, De Peters EJ, Taylor SJ: Relationships between chemical composition, dry matter degradation and *in vitro* gas production of several ruminant feeds. *Anim Feed Sci Technol*, 111, 57-71, 2004. DOI: 10.1016/S0377-8401(03)00217-7

27. Brockbank KGM, Covault JC, Taylor MJ: Cryopreservation Guide. Bro-Lecs Cryoguide-0707, Thermo Fisher Scientific Inc. USA, 2007.

28. Malik KA: Cryopreservation of bacteria with special reference to anaerobes. World Federation of Culture Collection Technical Information Sheet No: 4, Germany, 1989.

29. Luchini ND, Broderick GA, Combs DK: Preservation of ruminal microorganisms for *in vitro* determination of ruminal protein degradation. *J Anim Sci*, 74, 1134-1143, 1996. DOI: 10.2527/1996.7451134x

30. Luchini ND, Broderick GA, Combs DK: *In vitro* determination of ruminal protein degradation using freeze-stored ruminal microorganisms. *J Anim Sci*, 74, 2488-2499, 1996. DOI: 10.2527/1996.74102488x

31. Jouany JP: Effect of rumen protozoa on nitrogen utilization by ruminants. *In* Conference: Altering Ruminal Nitrogen Metabolism to Improve Protein Utilization. 1335S-1346S, 1996.

32. Beal C, Fonseca F, Corrieu G: Resistance to freezing and frozen storage of *Streptococcus thermophilus* is related to membrane fatty acid composition. *J Dairy Sci*, 84, 2347-2356, 2001. DOI: 10.3168/jds.S0022-0302(01)74683-8

33. Prates A, De Oliveira JA, Abecia L, Fondevila M: Effects of preservation procedures of rumen inoculum on *in vitro* microbial diversity and fermentation. *Anim Feed Sci Technol*, 155, 186-193, 2010. DOI: 10.1016/j.anifeedsci. 2009.12.005

34. Chaudhry AS, Mohamed RAI: Fresh or frozen rumen contents from slaughtered cattle to estimate *in vitro* degradation of two contrasting feeds. *Czech J Anim Sci*, 57, 265-273, 2012.

35. Getachew G, Blummel M, Makkar HPS, Becker K: *In vitro* gas measuring techniques for assessment of nutritional quality of feeds: A review. *Anim Feed Sci Technol*, 72, 261-281, 1998. DOI: 10.1016/S0377-8401(97)00189-2

36. Karslı MA, Russell JR: Effects of source and concentrations of nitrogen and carbohydrate on ruminal microbial protein synthesis. *Turk J Vet Anim Sci*, 26, 201-207, 2002.

37. Leedle JAZ, Hespell RB: Brief incubations of mixed ruminal bacteria: Effects of anaerobiosis and sources of nitrogen and carbon. *J Dairy Sci*, 66, 1003-1014, 1983. DOI: 10.3168/jds.S0022-0302(83)81895-5

38. Hervas G, Frutos P, Giraldez FJ, Mora MJ, Fernandez B, Mantecon AR: Effect of preservation on fermentative activity of rumen fluid inoculum for *in vitro* gas production techniques. *Anim Feed Sci Technol*, 123-124, 107-118, 2005. DOI: 10.1016/j.anifeedsci.2005.05.004

39. McDonald P: A revised model for the estimation of protein degradability in the rumen. *J Agr Sci Cambridge,* 96, 251-252, 1981. DOI: 10.1017/S0021859600032081

40. Mauricio RM, Mould FL, Dhanoa MS, Owen E, Channa KS, Thedorou MK: A semi-automated *in vitro* gas production technique for ruminant feedstuff evaluation. *Anim Feed Sci Technol*, 79, 321-330, 1999. DOI: 10.1016/S0377-8401(99)00033-4

41. Seven PT, Çerçi İH, Azman MA: The different between methods and determining of metabolisable energy levels with enzyme and gas techniques in concentrate feeds. *FU Vet J Health Sci,* 21, 159-161, 2007.

42. Getachew G, Crovetteo GM, Fondevila M, Krishnamoorthy U, Singh B, Spangero M, Steingass H, Robinson PH, Kailas MM: Laboratory variation of 24 h *in vitro* gas production and estimated metabolizable energy values of ruminant feeds. *Anim Feed Sci Technol*, 102, 169-180, 2002. DOI: 10.1016/S0377-8401(02)00212-2