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Laboratory variation of 24 h in vitro gas production and estimated metabolizable energy values of ruminant feeds

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Abstract

Intra- and inter-laboratory variation of in vitro gas production and calculated metabolizable energy (ME, MJ/kg DM) values were studied using 16 test feeds in 7 laboratories. Intra-laboratory variation was low, with six of the seven laboratories having very high relationships in gas production between runs ($R^2 \geq 0.96$) and slopes that did not differ from unity. Inter-laboratory differences were higher with highly significant ($P < 0.001$) differences among laboratories in both gas production and calculated ME values. Three of the six test laboratories generated predicted ME values that did not differ from the seventh (reference) laboratory. Combining intra-laboratory variation in gas production and inter-laboratory variation in predicted ME values, three of the six test laboratories were judged acceptable overall. ME values predicted by the gas production technique by laboratories in different parts of the world cannot be considered absolute.

Keywords: In vitro gas production; Intra- and inter-laboratory; Metabolizable energy

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1. Introduction

In vitro methods of feed evaluation have numerous advantages over in vivo methods. They are less expensive, less time-consuming and allow incubation conditions to be maintained more precisely than in vivo. In addition, in vitro techniques utilize small amounts of test feeds making them applicable to screening of feeds that are not available in sufficient quantity for in vivo experiments. The in vitro method of Tilley and Terry (1963), in sacco method of Mehrez and Ørskov (1977), and enzymatic method of Jones and Hayward (1975) have all been widely used to predict digestibility of feeds, and used as a selection tool for screening feeds for nutritional quality. Menke and Steingass (1988) reported a strong correlation between metabolizable energy (ME) values measured in vivo and predicted from 24 h in vitro gas production and chemical composition of feeds. The in vitro gas production method has also been widely used to evaluate the energy value of several classes of feeds (Getachew et al., 1998), particularly straws (Makkar et al., 1999), agro-industrial by-products (Krishna and Günther, 1987), compound feeds (Aiple et al., 1996) and various tropical feeds (Krishnamoorthy et al., 1995). The technique has also been used to assess effects of anti-nutritive factors on rumen fermentation of Mediterranean (Khazaal et al., 1994) and African (Siaw et al., 1993; Nsahlai et al., 1994; Bonsi et al., 1995) browses.

There are a number of factors that affect fermentation of feeds in vitro and could cause intra- or inter-laboratory differences. These are mainly associated with the nature of rumen fluid inoculum, although breed of animal, its physiological condition, diet, time of feeding, time of collection of rumen fluid relative to feeding time (Craig et al., 1987), method of rumen fluid collection (i.e. liquid or solid phase) (Craig et al., 1987; Cecava et al., 1990), and time elapsed between rumen fluid sampling and inoculation (Robinson et al., 1999) are all factors that have been shown to influence microbial activity in vitro. The quantitative extent of the influence of combined effects of these factors is complex and it is not possible to quantify their influence on any specific in vitro system. Attempts to completely standardize in vitro techniques to reduce or eliminate the influence of these factors is not practical due to procedural differences required by laboratories in different parts of the world to meet locally available resources. However, it is important that in vitro techniques used around the world be sufficiently robust to overcome local modifications to yield similar results.

The objective of this study was to assess intra- and inter-laboratory variability of an in vitro gas production procedure, and calculated ME values of feeds, in several laboratories in different geographical locations in the world that use the in vitro gas production technique of Menke and Steingass (1988).

2. Materials and methods

Samples of 16 conventional and by-product feeds were ground to pass a 1 mm sieve and distributed to the participating laboratories. The chemical composition of the test feeds is in Table 1. Only one sample of each feed was utilized, as feeds were selected to represent a range in fermentability, rather than to categorize the feeds.

Laboratories participating in the study were, the University of California (Davis, USA); Hohenheim University (Stuttgart, Germany); Universidad de Zaragoza (Zaragoza, Spain);

Table 1
Chemical composition[a] of the test feeds (% DM)

	CP	Fat	NDF	ADF	Ash
Forage feeds[b]					
Alfalfa hay	26.3	2.9	35.1	26.6	10.6
Almond hulls	8.0	2.9	33.6	29.6	6.8
Citrus pulp (wet)	11.3	2.5	23.8	22.2	4.7
Corn silage	6.5	2.5	51.4	31.9	5.6
Safflower meal	29.9	1.9	53.3	39.5	5.6
Soybean hull pellets	12.4	2.1	63.0	43.0	4.8
Wheat silage	7.0	3.5	53.4	37.4	14.0
Concentrate feeds[b]					
Barley grain	14.4	2.2	20.4	9.2	2.8
Beet pulp (dried)	12.5	1.0	46.4	27.2	8.3
Brewers grains (wet)	29.4	7.7	36.0	15.2	5.0
Canola meal	43.8	4.4	28.5	19.0	8.1
Corn grain	11.1	3.6	12.4	5.9	1.1
Corn hominy	12.3	4.2	23.4	9.8	1.7
Distillers dried grains	31.7	13.3	31.9	17.6	4.5
Soybean meal	54.1	1.4	9.6	7.2	8.1
Wheat mill run	19.7	4.3	36.7	13.4	5.1

[a] Assays represent duplicate assay of single sample per feedstuff. CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber.
[b] For purposes of selecting the appropriate ME calculation equation noted in the text.

University of Agricultural Sciences (Bangalore, India); Indian Veterinary Research Institute (Palampur, India); University of Udine (Udine, Italy); and Università degli Studi di Milano (Milan, Italy). Laboratories were selected on the basis that they regularly utilize the gas production procedure of Menke and Steingass (1988).

Incubations in all laboratories were completed using 30 ml of buffered rumen fluid according to Menke and Steingass (1988). Approximately 200 mg of feed was weighed and placed into a 100 ml graduated glass syringe. Pistons were lubricated with Vaseline and inserted into the syringes. Buffer and mineral solution was prepared and placed in a water bath at 39 °C under continuous flushing with CO_2. Rumen fluid (i.e. liquid and fine particles) was collected from animals into a pre-warmed thermos flask, and then filtered and flushed with CO_2. 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. Buffered rumen fluid (30 ml) was pipetted into each syringe containing feed samples and the syringes were immediately placed into an incubator with a rotating disc, as described in Menke and Steingass (1988), or into water bath at 39 °C (Blümmel and Ørskov, 1993). Three syringes with only buffered rumen fluid were incubated and considered as the blank incubation. Each incubation was completed in triplicate and each run was repeated within each laboratory. Where a waterbath was used, syringes were shaken gently every 2 h, and the incubation was terminated after recording the 24 h gas volume. Total gas values were corrected for blank incubation and hay standards with known gas values provided by Hohenheim University, except laboratories B and E, where Hohenheim standard correction is not a routine practice.

Table 2
Donor animal, diet of donor animal, sampling time, and incubation condition used by different laboratories

Laboratory	Animal(s)			Rumen fluid			Standard correction
	Number	Breed and state	Diet	Sampling time	Filtration	Incubation	
A	1-year-old calves	Jersey cross	Wheat straw ad lib plus concentrate	Before feeding	Four layers of muslin cloth	Water bath	Yes
B	Two cows	Dry Simmental	Hay plus concentrate	3 h after feeding	Two layers of gauze	Water bath	No
C	Two cows	Dry Holstein	Alfalfa hay and oat hay	After feeding	Four layers of cheese cloth	Water bath	Yes
D[a]	Two cows	Mid-lactation Holstein	Total mixed ration	Before morning feeding	Two layers of gauze	Rotor	Yes
E	Three adult sheep	Dry Rasa Aragonesa ewes	Alfalfa hay plus concentrate	Before feeding	Three layers of gauze	Water bath	No
F	One cow	Lactating Friesian crossbred	Finger millet straw ad lib plus concentrate	4 h after feeding	Cotton cloth with a pore size of approximately 420 microns	Rotor	Yes
G	Two cows	Dry	Permanent pasture hay and concentrate	2 h after feeding	Two layers of gauze	Rotor	Yes

[a] The reference laboratory (Hohenheim).

The reported 24 h gas values were expressed per 200 mg of DM. The specific incubation conditions used by each participating laboratory are summarized in Table 2.

The metabolizable energy (MJ/kg DM) content of feeds was calculated using equations of Menke and Steingass (1988) as

for forage feeds,

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

for concentrate feeds,

$$\text{ME (MJ/kg DM)} = 1.06 + 0.157\text{GP} + 0.084\text{CP} + 0.22\text{CF} - 0.081\text{CA}$$

where GP is 24 h net gas production (ml/200 mg DM); CP, CF and CA are crude protein, crude fat and crude ash (% DM), respectively. The Hohenheim gas method has been standardized and validated as a method to create these ME prediction equations using data from 400 digestibility trials (in vivo) and the corresponding in vitro gas production tests (Menke and Steingass, 1988).

3. Statistical analysis

Net gas production corrected for blanks and standards at 24 h of incubation, and ME predicted from gas production at 24 h and chemical components, were analyzed using the general linear models of SAS (SAS Institute Inc., Cary, NC, USA). The model was a 7 (laboratories) by 16 (feeds) factorial design. The relationship between in vitro gas production and ME values from each laboratory, and the values from Hohenheim (the reference laboratory), was assessed using linear regression.

4. Results

4.1. Methodological differences among laboratories

Although all laboratories utilized the method of Menke and Steingass (1988), there were some differences among laboratories in the type and physiological condition of the donor animals and rumen fluid sampling practices to adapt the original procedure to meet local facilities (Table 2). Modifications of the technique by each laboratory resulted in considerable variation in breed and diet of the donor animals as well as the time of rumen fluid collection. However, the basic procedure of Menke and Steingass (1988) was not changed in any laboratory.

4.2. Intra-laboratory variation in gas production

Laboratories A and C to F reported very high between run relationships in gas production ($R^2 \geq 0.96$) and slopes ±4% of unity (Table 3). Only laboratory B had a slope that differed from one and intercept that differed from zero.

Table 3
Relationship of in vitro gas production between run one (x) and run two (y) within each laboratory

Laboratory	Equation	R^2	P	
			Slope[a]	Intercept[b]
A	$y = 0.968x + 1.94$	0.993	0.167	0.108
B	$y = 0.830x + 5.81$	0.950	0.002	0.023
C	$y = 0.989x + 2.62$	0.978	0.773	0.225
D[c]	$y = 0.986x + 0.92$	0.997	0.379	0.293
E	$y = 1.020x - 0.00$	0.959	0.733	0.993
F	$y = 1.005x - 2.16$	0.983	0.883	0.297
G	$y = 1.061x - 0.63$	0.945	0.376	0.846

[a] That the slope differs from 1.00.
[b] That the intercept differs from zero.
[c] The reference laboratory (Hohenheim).

4.3. Inter-laboratory variation in gas production and ME values

Average gas production and ME values among laboratories ranged from 43.6 to 53.6 ml/200 mg DM and 9.92 to 11.37 MJ/kg DM, respectively (Table 4). There was a highly significant ($P < 0.001$) difference among laboratories in gas production and ME values. In vitro gas production, and estimated ME values for feeds among laboratories, are in Tables 5 and 6, respectively. Absolute differences in gas production among laboratories that reported the highest and lowest values ranged from 6.0 ml (wet brewers grains and safflower meal) to 21.4 ml (corn grain). Corresponding ME values were 0.95 and 3.40 MJ/kg DM. Labora-

Table 4
Inter-laboratory variation in 24 h in vitro gas production and estimated ME[a] values of the test feeds

Laboratory	Gas production (ml/200 mg DM)	ME (MJ/kg DM)
A	51.2	11.1
B	44.6	10.1
C	53.3	11.4
D[b]	52.7	11.3
E	46.1	10.3
F	54.7	11.6
G	47.4	10.5
S.E.M.	0.18	0.03
LSD[c]	0.72	0.11
ANOVA F-values[d]		
Laboratory	133.6	131.5
Feed	686.6	633.7
Feed × laboratory	5.2	5.5

[a] Metabolizable energy at a maintenance energy intake.
[b] The reference laboratory (Hohenheim).
[c] Least significant difference.
[d] $P < 0.001$ for the effect of laboratory, feed, and laboratory × feed interaction for gas production and estimated ME.

Table 5
In vitro gas production (ml/200 mg DM) of the test feeds among laboratory[a]

	Laboratory							S.E.M.	P[b]	LSD[c]
	A	B	C	D	E	F	G			
Alfalfa hay	43.3	37.0	46.6	46.9	39.7	47.0	44.6	0.69	0.003	2.7
Almond hulls	43.8	38.1	45.2	44.2	43.2	47.1	44.2	0.92	0.088	3.7
Barley grain	68.2	55.0	64.9	68.7	57.2	73.8	56.4	1.47	0.007	5.8
Beet pulp	65.0	59.6	69.4	64.5	61.5	69.4	57.2	1.21	0.027	4.8
Brewers grains (wet)	39.5	35.6	38.3	41.1	35.3	41.3	41.5	0.35	<0.001	1.4
Canola meal	40.5	36.4	43.5	41.3	37.8	40.7	38.0	0.67	0.035	2.6
Citrus pulp (wet)	74.3	63.5	75.8	72.5	66.3	73.2	65.8	1.03	0.009	4.0
Corn grain	75.6	64.3	74.2	74.7	60.9	82.4	60.8	0.89	<0.001	3.5
Corn hominy	63.5	57.9	69.0	68.0	54.3	74.2	55.0	0.72	<0.001	2.8
Corn silage	46.5	39.8	49.1	48.7	43.0	49.7	46.6	0.30	<0.001	1.2
Distillers dried grains	34.7	34.1	41.8	33.8	32.8	41.8	41.7	0.63	<0.001	2.5
Safflower meal	24.9	23.9	29.9	30.3	28.5	26.9	23.9	0.36	<0.001	1.4
Soybean hull pellets	62.6	49.2	64.7	70.6	54.8	66.9	56.1	0.71	<0.001	2.8
Soybean meal	49.5	43.7	49.3	46.7	45.2	49.2	44.1	0.57	0.012	2.3
Wheat mill run	53.5	44.8	53.3	53.6	45.3	54.5	48.6	0.40	<0.001	1.6
Wheat silage	34.5	30.9	38.8	38.2	32.6	37.1	33.8	0.63	0.010	2.5

[a] Mean values by laboratory are in Table 4.
[b] Significance of laboratory.
[c] Least significant difference ($P < 0.01$), if the effect of laboratory is significant ($P < 0.01$).

Table 6
Metabolizable energy[a] (MJ/kg DM) of the test feeds among laboratory[b]

	Laboratory							S.E.M.	P[c]	LSD[d]
	A	B	C	D	E	F	G			
Alfalfa hay	9.60	8.80	10.10	10.10	9.15	10.10	9.80	0.09	0.004	0.37
Almond hulls	8.65	7.85	8.85	8.70	8.55	9.05	8.70	0.12	0.078	0.48
Barley grain	13.25	11.20	12.70	13.30	11.50	14.10	11.35	0.23	0.007	0.92
Beet pulp	11.75	11.05	12.35	11.65	11.30	12.35	10.70	0.17	0.032	0.66
Brewers grains (wet)	11.05	10.40	10.85	11.25	10.35	11.30	11.30	0.06	0.001	0.24
Citrus pulp (wet)	12.95	11.50	13.20	12.70	11.85	12.85	11.80	0.15	0.011	0.58
Canola meal	11.40	10.80	11.85	11.55	10.95	11.45	11.00	0.10	0.037	0.41
Corn grain	14.55	12.75	14.30	14.40	12.25	15.65	12.25	0.14	<0.001	0.55
Corn hominy	12.85	11.95	13.70	13.55	11.40	14.50	11.45	0.12	<0.001	0.46
Corn silage	8.90	8.00	9.25	9.25	8.45	9.35	8.90	0.05	<0.001	0.21
Distillers dried grain	11.70	11.60	12.85	11.55	11.45	12.85	12.80	0.10	0.001	0.40
Safflower meal	7.25	7.20	8.00	8.05	7.80	7.55	7.15	0.05	<0.001	0.21
Soybean hull pellets	11.45	9.60	11.75	12.50	10.40	12.05	10.55	0.10	<0.001	0.39
Soybean meal	13.05	12.15	13.00	12.60	12.35	12.95	12.20	0.08	0.011	0.33
Wheat mill run	11.65	10.25	11.60	11.65	10.35	11.80	10.85	0.07	<0.001	0.26
Wheat silage	7.35	6.85	7.90	7.85	7.05	7.70	7.20	0.08	0.008	0.33

[a] Estimated for maintenance energy intake level according to Menke and Steingass (1988).
[b] Mean values by laboratory are in Table 4.
[c] Significance of laboratory.
[d] Least significant difference ($P < 0.01$), if the effect of laboratory is significant ($P < 0.01$).

Table 7
Relationship of in vitro gas production and ME between each laboratory (y) and Hohenheim (x)

Laboratory	Equation	R^2	P	
			Slope[a]	Intercept[b]
Gas production (ml/200 mg DM)				
A	$y = 1.013x - 2.188$	0.96	0.802	0.447
B	$y = 0.789x + 2.978$	0.91	0.004	0.421
C	$y = 0.938x + 3.916$	0.95	0.284	0.220
E	$y = 0.755x + 6.319$	0.93	0.053	<0.001
F	$y = 1.076x - 2.045$	0.96	0.226	0.547
G	$y = 0.683x + 11.392$	0.88	<0.001	0.004
ME (MJ/kg DM)				
A	$y = 1.072x - 1.017$	0.97	0.181	0.102
B	$y = 0.879x + 0.194$	0.88	0.179	0.842
C	$y = 0.990x + 0.213$	0.94	0.882	0.783
E	$y = 0.776x + 1.566$	0.88	0.007	0.087
F	$y = 1.159x - 1.491$	0.96	0.014	0.041
G	$y = 0.767x + 1.836$	0.79	0.037	0.014

[a] That the slope differs from 1.00.
[b] That the intercept differs from zero.

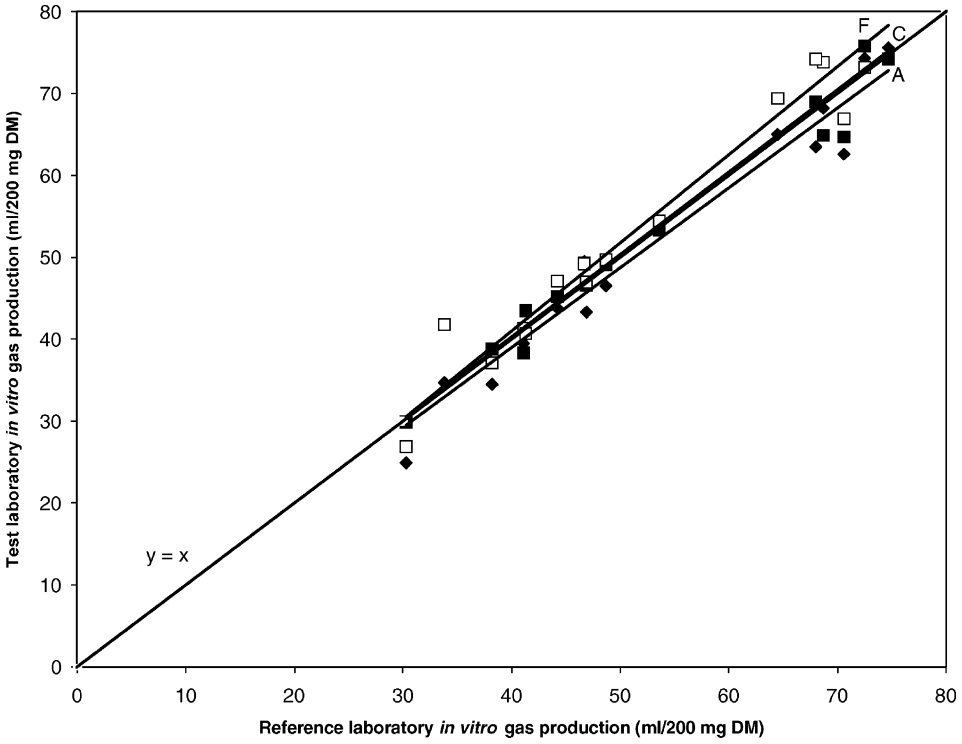

Fig. 1. Relationship of gas production between the reference laboratory (Hohenheim) and test laboratories A, C and F where the relationship did not differ from unity (A (◆), C (■) and F (□)).

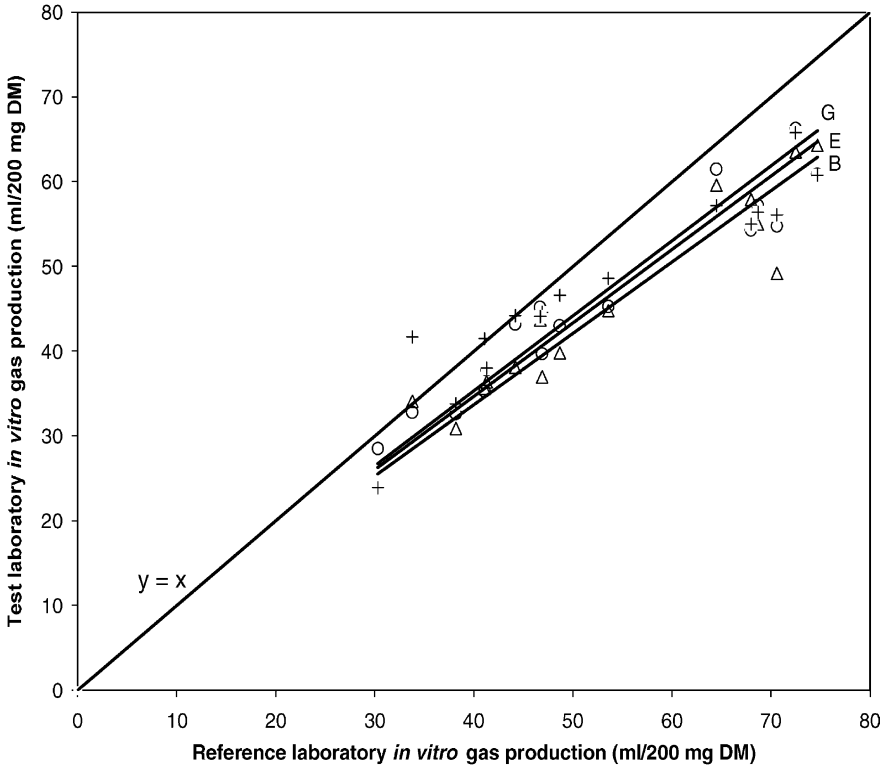

Fig. 2. Relationship in gas production between the reference laboratory (Hohenheim) and test laboratories B, E and G where the relationship differed from unity (B (△), E (○), and G (+)).

tories B and E reported lower gas production and ME values for more than half the feeds and laboratory G tended to have higher values for some feeds.

The relationship of in vitro gas production and ME for each of the six laboratories and the values from Hohenheim, the reference laboratory, are in Table 7 and in Figs. 1 and 2. For gas production, the slope of the relationship differed significantly from unity for laboratories B and G, and the intercept differed significantly from zero for laboratories E and G. For ME values, the slope of the relationship was significantly different from unity for laboratories E to G, and the slope was always less than the reference laboratory (Fig. 2). The intercept significantly differed from zero for only laboratories F and G.

5. Discussion

5.1. Intra-laboratory variation of gas production

Sources of among day variation in gas production may be due to changes in the microbial population in the donor animals and/or inconsistent inoculation procedures. It is not possible

to differentiate the extent of the contribution of each of these factors within laboratories. However, it is clear that these factors had a minimal impact overall as six of the seven laboratories had inter-run variation that did not differ from unity.

5.2. *Inter-laboratory variation of gas production*

There was a significant interaction between 'laboratory' and 'feed' in both gas production and ME values. However, since the main effects (i.e. feed and laboratory) are relatively much larger than the interaction (i.e. 25.7 and 23.9 times larger than the interaction for gas production and ME for 'laboratory', and 132.0 and 115.2 times larger than the interaction for gas production and ME for 'feed'), the interaction significance probably implies a minor variation in the effect of feed within laboratory (Snedecor and Cochran, 1980), rather than a substantive impact, and as such can be discounted.

Despite correction of gas production values by a hay standard supplied by Hohenheim, the gas production values from laboratories B, G, and E differed significantly from Hohenheim values (i.e. laboratory D). Variation of in vitro gas production among laboratories could partly be due to differences in sources of rumen fluid (i.e. animal and (or) physiological state of the animal). For example, Grant et al. (1974) reported that inoculum from buffalo resulted in consistently higher DM digestibility compared to cattle when both were fed a similar diet, and inoculum from cattle at Cornell University (USA) resulted in lower digestibility in test feeds when compared to results obtained from cattle in the Philippines using the same test feeds. The difference in inoculum activity between cattle in the Philippines and Cornell was attributed to differences in the diet of the donor animals (Grant et al., 1974). Bonsi et al. (1995) studied the influence of donor animal diet on in vitro gas production and reported that rumen fluid from animals on different diets resulted in different gas values at different times of incubation. For example, rumen fluid from animals fed teff straw resulted in lower gas values compared to those fed either *Sesbania* or *Leucaena*. This could be due to the low N content of teff straw resulting in low rumen ammonia N concentrations (Bonsi et al., 1995), which reduced microbial growth. These authors also reported an interaction between the diet of the donor animal and the type of feed incubated, and Trei et al. (1970) also reported differences in gas production between rumen fluid from two animals fed the same diet.

The relative proportion of concentrate and forage in the diet will have a considerable influence on in vitro gas production. Nagadi et al. (2000) reported that differences in the diet of the donor animal influenced gas production from different substrates differently, thereby indicating that there is an interaction of diet of the donor animal and type of feed incubated. The diet of the donor animal exerted considerable influence on bacterial concentrations and so influenced in vitro gas production. Since different feeds can affect the relative proportion of microbes in the rumen, this may influence the extent of fermentation of feeds. The magnitude of the diet effect can vary with the type of feed incubated (Bonsi et al., 1995; Nagadi et al., 2000; Ngwa et al., 2001). The major microbial species involved in cellulose degradation adhere closely to plant cell wall surfaces to digest cell wall (Cheng et al., 1983), and use of hay-based diets to donor animals may promote growth of such bacteria thereby increasing rate and extent of fiber digestion.

Thus, it seems clear that in vitro biological assays can only be standardized to a limited extent. Even where all laboratories are using the same base procedure, as was the case here,

only three of the six test laboratories were able to reproduce the values of the reference laboratory. That the gas production of the three laboratories which failed to reproduce the reference laboratory was less than the reference laboratory in all cases, suggests that procedural modifications are most likely to suppress, rather than enhance gas production.

5.3. *Inter-laboratory variation of calculated ME values*

While the in vitro gas production procedure measures gas production, its purpose, at least in the context of this study, was to allow estimation of the ME values of feeds. These ME values are valuable for purposes of ration formulation and to set the economic value of feeds for trading purposes. It seems clear that variation of ME values as high as 3 MJ/kg DM for corn grain and corn hominy, as found here, will be deemed unacceptable by feed traders (i.e. feed companies, forage brokers, nutritionists, farmers and forage producers) where commodities are valued, at least partially, on the basis of their energetic values. In addition, our finding that only three of the six test laboratories could reproduce the ME values of the reference laboratory indicates that the impact of the methodological changes to the base procedure are different enough, at least in these three laboratories, to result in inaccurate calculated ME values.

6. Conclusions

In this study to examine intra- and inter-laboratory variation in gas production by laboratories using the gas production technique of Menke and Steingass (1988), six of the seven laboratories had an acceptably low level of intra-laboratory variation, and three of the six test laboratories generated predicted ME values that did not differ from the reference laboratory. Combining the intra-laboratory variation in gas production and the inter-laboratory variation in predicted ME values, only three of the six test laboratories were judged acceptable overall. ME values predicted by the gas production method of Menke and Steingass (1988) by laboratories in different parts of the world cannot be considered absolute.

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