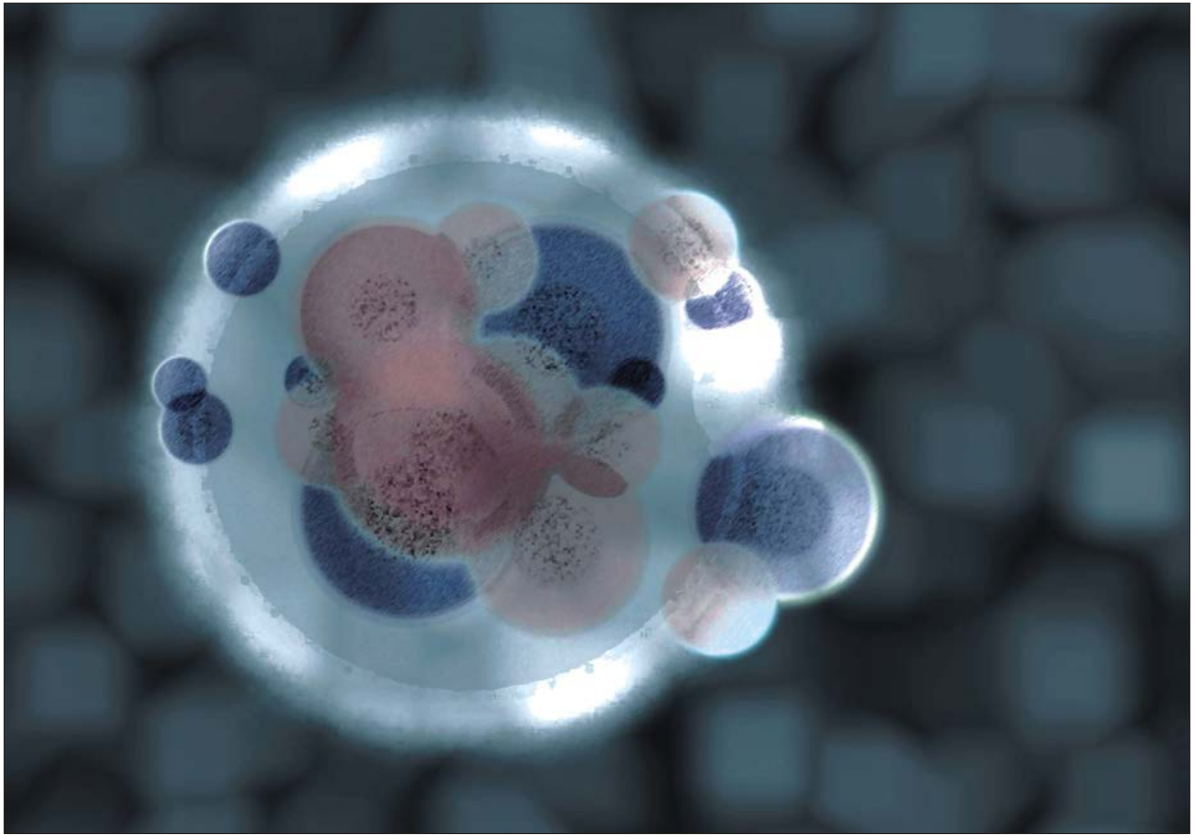




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Gas Production: A Proposed *in vitro* Method to Estimate the Extent of Digestion of a Feedstuff in the Rumen

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Abstract: This study highlights the potential of a novel approach using an *in vitro* rumen fermentation technique for evaluation of nutritional quality of feed resources. *In vitro* techniques have been used extensively in feed evaluation and in studies of ruminal fermentation. This technique enables selection of feed or feed constituents for high efficiency of microbial protein synthesis in the rumen along with high dry matter digestibility and provides a basis for development of feeding strategies to maximize substrate fixation into microbial cells. This could lead to increase in the supply of protein to intestine and reduce methane production from ruminants. Gas production method is widely used to evaluate the nutritive value of different classes of feeds; this technique is more efficient than other *in vitro* techniques in determining the nutritive value of feeds containing tannins.

Key words: Gas production, rumen, *in vitro*, feed fermentation

INTRODUCTION

Yield of ruminants is largely limited by forage quality which is mainly reflected in low voluntary intake and digestibility. The importance of these parameters in animal nutrition has been recognized. The determination of intake and digestibility of feedstuffs *in vivo* is time-consuming, laborious and expensive, requires large quantities of feed and is unsuitable for large-scale feed evaluation. Therefore, many attempts have been made to predict intake and digestibility using laboratory techniques. Much effort has been directed towards the development of regression equations to predict digestibility from forage chemical composition, but a regression equation that satisfactorily predicts a wide range of forages has not yet been derived. *In vitro* methods for laboratory estimations of degraded feeds are important for ruminant nutritionists. An efficient laboratory method should be reproducible and should correlate well with actually measured *in vivo* parameters. *In vitro* methods have the advantage not only of being less expensive and less time-consuming, but also they allow maintaining experimental conditions more precisely than do *in vivo* trials. Three major biological digestion techniques are currently available to determine the nutritive value of ruminant feeds: 1) digestion with rumen micro organisms (Tilley and Terry, 1963) or using a gas method (Menke *et al.*, 1979) *in situ* incubation of samples in nylon bags in the rumen (Mehrez and

Orskov, 1977) cell-free fungal cellulose (De Boever *et al.*, 1986). These biological methods are more meaningful since microorganisms and enzymes are more sensitive to factors influencing the rate and extent of digestion than are chemical methods (Van Soest, 1994). Tilley and Terry (1963) reported nylon bag methods are based on residue determinations and may result in overestimation of dry matter digestibility for tannin-rich feeds, since tannins are solubilised in both these systems but might be indigestible and do not contribute nutrient supply to animal (Makkar *et al.*, 1993). Aiple *et al.* (1996) compared three laboratory methods (enzymatic, crude nutrient and gas measuring technique) as predictor of net energy (as estimated by equations based on *in vivo* digestibility) content of feeds and found that for predicting net energy content of individual feeds, the gas method was superior to the other two methods. The variation in the rate and extent of disappearance for both fiber and starch are large enough to cause major problems when formulating dairy rations. To put in it perspective, Oba and Allen, (1999) estimated that a 1% increase in ruminal NDF digestibility *in vitro* or *in situ* will lead to a 0.25 kg increase in 4.0% fat-corrected milk production. Looking at the problem from another angle, St-Pierre and Harvey, (1986) estimated that the economic benefit of testing feeds equates to \$0.27 per cow per day. There is no question that information on ruminal digestion and nutritive value of feedstuffs is valuable for practicing nutritionists.

However, the problem becomes one of capturing the various characteristics of digestion in a manner that is practical in the field (Johnston and Tricarico, 2006). Gas production technology allows for a more useable collection of digestion kinetics data and has allowed for a growing body of knowledge that is directly applicable to the feeding programs that are in daily practical field use (Pitt *et al.*, 1999). This review highlights the potential of a main approach using an *in vitro* rumen fermentation technique for evaluation of the nutritional quality of conventional and unconventional feed resources.

Gas production method: Since the late 1970s, measurement of *in vitro* gas production has become increasingly popular for determining forage digestion characteristics and the kinetics of fermentation (Theodorou *et al.*, 1998). Gas production is measure from the *in vitro* digestion of forage with bicarbonate-buffered rumen fluid. Rumen microorganisms ferment substrate to end-products includes gases, carbon dioxide and methane and Volatile Fatty Acids (VFA), including acetate, propionate and butyrate. The amount of gas produced depend on the amount of substrate fermented and the amount and molar proportions of the VFA produced (Davies *et al.*, 2000). The gas measuring technique has been widely used for evaluation of nutritive value of feeds. More recently, the increased interest in the efficient utilization of roughage diets has led to an increase in the use of this technique due to the advantage in studying fermentation kinetics. Gas measurement provides a useful data on digestion kinetics of both soluble and insoluble fractions of feedstuffs. Several gas measuring techniques and *in vitro* gas methods are in use by several groups. Advantages and disadvantages of these methods are discussed by Getachew *et al.* (1998). The *in vitro* gas method based on syringes (Blummel *et al.*, 1997) appears to be the most suitable for use in developing countries. Other *in vitro* methods of Mehrez and Orskov (1977) such as Tilley and Terry (1963) and nylon bag methods are based on gravimetric measurements which follow disappearance of the substrate, whereas gas measurement focuses on the appearances of fermentation products. In the gas method, kinetics of fermentation can be studied on a single sample and therefore a relatively small amount of sample is required or a larger number of samples can be evaluated at a time. The *in vitro* gas method is more efficient than the *in sacco* method in evaluating the effects of tannins or other anti-nutritive factors. In the *in sacco* method these factors are diluted in the rumen after getting released from the nylon bag and therefore do not affect rumen fermentation appreciably. In addition, the *in vitro* gas method can better monitor nutrient-anti

nutrient and anti nutrient-anti nutrient interactions (Makkar *et al.*, 1995). Gas production method is widely used to evaluate the nutritive value of different classes of feeds (Getachew *et al.*, 1998) particularly to estimate energy value of straws (Makkar *et al.*, 1999), agro-industrial by-products (Krishna and Gunther, 1987), compound feeds (Aiple *et al.*, 1996) and various types of tropical feeds (Krishnamoorthy *et al.*, 1995). The gas production technique is more efficient than other *in vitro* techniques in determining the nutritive value of feeds containing tannins. The binding effect of tannins to macromolecules such as protein and carbohydrates creates problems (Makkar *et al.*, 1995) in the use of the conventional *in vitro* method of Tilley and Terry (1963) and nylon bag method of Mehrez and Orskov (1977). Furthermore, the latter techniques are based on gravimetric determination of residues, leading to solubilization of tannin which, although making no contributions to energy production in the system, is measured as dry matter digestibility. In the *in vitro* gas production method, the effects of tannins on rumen fermentation are reflected in the gas production. The technique has been used to assess actions of anti nutritive factors on rumen fermentation of Mediterranean (Khazaal *et al.*, 1994) and African browses (Bonsi *et al.*, 1995). The gas production on incubation of cereal straws (Blummel and Orskov, 1993), cereal grains (Opat-Patanakit *et al.*, 1994) and different classes of feed (Blummel *et al.*, 1999) in buffered rumen fluid was closely related to the production of Short Chain Fatty Acids (SCFA) calculated using the stoichiometry outlined by (Wolin, 1960), which was based on carbohydrate fermentation. Little work has been done to investigate the effect of proteins and fats on stoichiometry of gas production. Cone and van Gelder (1999) reported a poor correlation between measured and calculated gas volume on incubation of starch and glucose with increasing levels of casein (Getachew *et al.*, 2002). Huhtanen *et al.* (2008) with use of an automatic *in vitro* gas production technique, evaluated the predicting *in vivo* fiber (NDF) digestibility and effective first-order digestion rate of potentially digestible NDF (pdNDF) of 15 grass silages. They concluded that a simple first-order digestion rate can be estimated from a complicated gas production kinetic model. This rate constant can be used in continuous steady-state dynamic mechanistic rumen models predicting the nutrient supply to the host animal. More recently, Razmazar (2010) determined the nutritive value of grain and forage three species of legumes using gas production technique.

Different systems of gas production: A number of different systems have been used to measure gas

production. Menke *et al.* (1979) described a method in which fermentations were conducted in 100 mL gas-tight, ground-glass syringe barrels and gas evaluation was measured after 48 h of incubation. The technique was primarily used for end-point digestion studies, but by measuring the rate of ascent of the plunger in the syringe barrel, information on the kinetics of digestion of the feedstuff was also obtained. More recently, Theodorou *et al.* (1994) described a simple gas production method using an electronic measuring procedure employing a pressure transducer to measure gas from incubations in 160 mL gas-tight culture bottles. Gas accumulated in the head-space of the bottle as the fermentation proceeded and was measured at regular intervals by a pressure transducer connected to a digital readout voltmeter, gas-tight syringe and needle. This method, although technically straight forward was labor intensive since frequent readings were needed, especially over the initial stages of fermentation. Several automated systems have been developed to measure gas production. Beuvink *et al.* (1992) used a liquid displacement system. In this system, a 100 mL serum bottle was connected to a water displacement bottle and collection vessel placed on a balance. Readings were taken every 25 min when the weight of liquid displaced by the gas was recorded and stored in a data-logger. The bottles were held in a shaking water bath throughout the fermentation. Pell and Schofield (1993) described a gas production system using a series of closed 50 mL serum bottles, each with its own stirrer. Each bottle had its own individual pressure sensor that remained in place throughout the entire incubation. These pressure sensors were linked to an IMB-compatible computer. In the system of Cone *et al.* (1994), each bottle was fitted with its own pressure transducer and electric micro-valve. The pressure transducer measured the pressure build up in each bottle until a preset upper value was reached (ca. 0.65 kPa). Every valve opening represented a known amount of gas, so the number of valve openings was proportional to gas production. Each valve opened for just a fraction of a second (50 ms) (Cone, 1998). These various systems have been used to monitor the fermentation characteristics of a range of different feedstuffs including silages (Beuvink and Spoelstra, 1994; Doane *et al.*, 1997), tropical forages (Longland *et al.*, 1995; Sileshi *et al.*, 1996) straw (Prasad *et al.*, 1994; Williams *et al.*, 1996) and cereal grains (Opat-Patanakit *et al.*, 1994). Gas production has also been used to determine growth rates of anaerobic fungi on soluble and cellulosic substrates (Theodorou *et al.*, 1995) and to elucidate the relative role of fungi and bacteria in the digestion of fibrous substrates (Davies, 1991; France *et al.*, 1993; Davies *et al.*, 2000).

Protein and fat affect *in vitro* gas production estimates:

Consideration of the fermentative characteristics of protein fractions must be considered when reviewing total gas production. Fermentation of casein, for example produces only 32% of the gas amount produced by carbohydrates. In addition, Cone and van Gelder (1999) estimated that an increase in Crude Protein (CP) of one percent will reduce gas production by 2.48 mL g⁻¹ of Organic Matter (OM). Therefore, it is important to consider and correct for the CP content when comparing gas production from different feedstuffs. Fermentation of protein results in both amino acids and short chain peptides which can end up either in microbial biomass or in fermentation end products such as VFA, CO₂, or NH₃. As the breakdown of proteins takes place in the first few hours and is not linear, it is perhaps incorrect to draw inferences from gas production measurements relative to protein degradation rates or extents. One possible suggestion to get around this difficulty is to suppress amino acid incorporation in microbial protein through the use of hydrazine and chloramphenicol but this technique is beyond the practical application of gas production technology. Fats have long been added to ruminant diets as a method of increasing the energy density of the diet. The addition of palmitate, stearate, or oleate triglycerides to *in vitro* incubations do not affect total VFA production, acetate, propionate and the acetate to propionate ratio. More recent experiments have reviewed the addition of fat on VFA, IVTD and ammonia-N concentrations using an *in vitro* gas production system. The fat sources utilized were corn oil, tallow, or yellow grease provided as triglycerides or potassium soaps. Triglycerides had no major effects on gas production, digestion or VFA production, however all potassium soaps reduce gas production digestion and VFA production. The suspected negative effects of fat on ruminal fermentation and digestion cannot be generalized and are dependent on the form supplied, with triglycerides having smaller effects than the corresponding free fatty acids (Johnston and Tricarico, 2006).

Liquor and feces as inoculums in gas method: A sample of feed is incubated in batch cultures, measuring substrate disappearance (*in vitro* digestibility) and/or end-product accumulation (fermentation gas) either at an endpoint or at a sequence of different time points. Mixed ruminal microorganisms are the inoculum of choice to recreate ruminal conditions. Ruminal liquor, even when diluted, provides sufficient microbes to achieve rapid degradation of feedstuffs *in vitro*. An alternative to ruminal liquor as the inoculum, not requiring surgical intervention while maintaining the link to the animal, is fecal matter. The fermentation process seems to be similar

in cultures of ruminal or fecal microorganisms, as the pattern of end-product yield is similar when the same substrate is incubated (El-Meadaway *et al.*, 1998). However, low levels of surviving microbes mean that inocula derived from fecal matter will exhibit reduced degrading potency. This will be expressed as a longer lag phase and slower degradation rate at the outset (Mauricio *et al.*, 2001). Eventual cumulative gas production will be a little less than what is expected from ruminal liquor because not all the microbial species survive post-ruminal processes (El-Meadaway *et al.*, 1998). Thus, mathematical adjustments will be required to convert or translate the degradation profiles produced from fecal inoculum to those obtained when the inoculum originates from ruminal liquor. *In vitro* gas production with inocula derived from fecal matter suggests that it might be possible to move away from ruminal liquor sampling (Mauricio *et al.*, 2001). *In vitro* gas production based on ruminal liquor is limited by the need for donor animals and facilities for surgical intervention, whereas gas production based on fecal matter has no such limitations. Under this system, research is not restricted to housed herbivores and animals can be studied in their natural environment behaving as their biology dictates. Furthermore, it creates opportunities to study exotic species of herbivores in the wild. However, more fermentation gas is produced with a ruminal than with a fecal inoculum and, although the ranking of feeds using one or the other is similar, within each profile, there is a biphasic relationship in gas volume recorded from cultures of ruminal or fecal microorganisms. The difference between ruminal and fecal gas volumes increases during early incubation and is maintained or even reduced slightly during prolonged incubation. This confirms that fecal matter has reduced fermentation activity compared to ruminal liquor, resulting in lower gas volume, longer lag time, slower fermentation rate and lower OM disappearance (Mauricio *et al.*, 2001). Fecal microorganisms originate from the hindgut where fermentation activity is lower than in the rumen for a number of reasons. Firstly, total and cellulolytic bacterial populations and the diversity of bacterial species are higher in the rumen than in the hindgut or in feces (El-Meadaway *et al.*, 1998; Omed *et al.*, 2000). Secondly, fecal suspensions may contain some anaerobic fungi (Davies *et al.*, 1993) but lack protozoa. Thirdly, the microorganisms in feces are perhaps in a state of low metabolic activity in contrast with the rumen where they show an actively growing state (Mauricio *et al.*, 2001). Finally, ruminal liquor supplies some trace elements, micronutrients and unknown factors deemed essential for microbial growth that may not be present in fecal

suspensions (Omed *et al.*, 2000). Therefore, inoculum type, size and activity have a profound effect on microbial growth and substrate degradation in batch cultures and thus on gas production. Cultures inoculated with fecal matter need longer to achieve their degradation potential than with ruminal liquor, but once the microbial numbers reach a given threshold, the rate of gas production is similar in both cases. Hidayat *et al.* (1993) suggested that once the maximal degradation rate is achieved, the addition of more microorganisms fails to stimulate further digestion (Dhanoa *et al.*, 2004).

Estimation of ruminal microbial protein synthesis:

in vitro gas tests are attractive for ruminant nutritionist since it is very easy to measure the volume of gas production with time, but the measurement of gas only implies the measurement of nutritionally wasteful and environmentally hazardous products. In most studies the rate and extent of gas production has been wrongly considered to be equivalent to the rate and extent of substrate degradation. Current nutritional concepts aim at high microbial efficiency, which cannot be achieved by measurement of gas only. *In vitro* gas measurements reflect only SCFA production. The relationship between SCFA and microbial mass production is not constant and the explanation for this resides in the variation of biomass production per unit Adenosin-Three Phosphate (ATP) generated. This can impose an inverse relationship between gas volume and microbial mass production particularly when both are expressed per unit of substrate truly degraded. This implies that selecting roughages by measuring only gas using *in vitro* gas methods might result in a selection against the maximum microbial mass yield (Makkar, 2000).

Therefore, gas method estimates microbial protein synthesis from the stoichiometric partitioning of degraded substrate between gas production, VFA and microbial biomass. A simplified approach for forage evaluation has recently been proposed by Grings *et al.* (2005) where microbial biomass production is predicted with the equation:

$$\text{MBP} = \text{TSD} - (\text{gas volume} \times \text{SF})$$

In this equation, MBP represents microbial biomass production, TSD represents true substrate degradability as defined by Goering and van Soest (1970) and SF represents a stoichiometric factor. Once the MBP figure is known, the efficiency of microbial protein synthesis (EMP) can be calculated according to the equation (Johnston and Tricarico, 2006):

$$\text{EMP} = \frac{\text{TSD-Gas volume} \times \text{SF}}{\text{TSD}}$$

Interaction between dietary constituents in gas method:

Gas measurement was also employed for evaluation of the interaction between basal and supplementary diets by incubating basal diet and supplementary diet separately as well as in combination and monitoring gas production at different hours of incubation using the pressure transducer system (Liu *et al.*, 2000). This will indicate the availability of readily fermentable material as a ready energy source, which will stimulate the activity of the rumen micro organisms which in turn would accelerate the digestion of roughages. These workers, by incubating the basal diet and the supplement, observed a positive interaction in gas production in the early hours of incubation, which according to the authors can be an approach to study the synergetic effects of supplementation. However, it must be pointed out that measurement of gas only, could lead to misleading results. It is suggested to determine microbial mass production in addition to the gas measurement for such studies.

Voluntary intake prediction in gas test: The main constraint to the utilization of roughages by ruminants is voluntary feed intake so prediction of feed intake, particularly of fibrous roughage, is one of the important aspects of ruminant nutrition. *In vitro* gas production has been used to predict dry matter intake. Various workers have reported significant correlation between *in vitro* gas production and dry matter intake. Forage cell walls have considerable influence on voluntary feed intake through rumen fill mechanism (Van Soest, 1994). Gas production from extracted neutral detergent fiber was shown to be better correlated to voluntary feed intake than the values obtained from the incubation of whole roughage. The use of various models for intake prediction was investigated and it currently appears that combination of gas volume measurements (4-8 h) with concomitant determination of the amount of substrate degraded (>24 h) is superior to the models based on kinetics of gas production only. The *in vitro* gas production from NDF explained more (82% vs. 75%) of the variation in dry matter intake than gas production from whole roughage (Getachew *et al.*, 1998).

CONCLUSIONS

The *in vitro* rumen fermentation method in which gas production and microbial mass production are concomitantly measured has several major advantages: (1) it has the potential for screening a large number of feed resources, for example in breeding programs for the

development of varieties and cultivars of good nutritional value, (2) it could also be of great value in the development of supplementation strategies using locally available conventional and unconventional feed constituents to achieving maximum microbial efficiency in the rumen, (3) it has an important role to play in the study of rumen modulators emission of methane, an environmental polluting gas, (4) it provides a better insight into nutrient-antinutrient and antinutrient-antinutrient interactions and into the role of various nutrients with respect to production of fermentative gases, SCFA and microbial mass. The method is also being used increasingly to screen plant-derived rumen modulators. These products have a lower toxicity to animals and humans and are environmentally friendly. Consequently, they are becoming increasingly popular with consumers.

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