Recent advances in the *in vitro* gas method for evaluation of nutritional quality of feed resources

Harinder P.S. Makkar
Animal Production and Health Section
Joint FAO/IAEA Division
International Atomic Energy Agency
Vienna, Austria

This paper highlights the potential of a novel approach using an *in vitro* rumen fermentation technique for evaluation of nutritional quality of feed resources. 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 maximise substrate fixation into microbial cells. This could lead to increase in the supply of protein to intestine and reduce methane production from ruminants. In addition, this technique provides an easy tool to study the effects of various plant active moieties or synthetic compounds for their adverse or beneficial effects on partitioning of nutrients to fermentative gases, short chain fatty acids, and microbial mass.

1. INTRODUCTION
A major constraint to livestock production in developing countries is the scarcity and fluctuating quantity and quality of the year-round feed supply. Providing adequate good-quality feed to livestock to raise and maintain their productivity is and will be a major challenge to agricultural scientists and policy makers all over the world. Increase in population and rapid growth in world economies will lead to increase in demand for animal products; an increase
of approximately 30 percent in both meat and milk production is expected in the coming 20 years. At the same time, the demand for food crops will also increase. Future hopes of feeding the millions and safeguarding their food security will depend on the enhanced and efficient utilisation of unconventional resources, which can not be used as food for humans, as feed for livestock. In addition, a large area of land in the world is degraded, barren or marginal and the amount is increasing every year. This also calls not only for identification and introduction of new and lesser-known plants capable of growing in poor soils, which can play a vital role in the control of soil erosion in addition to providing food and feed. In developing countries, livestock are fed mainly on agro-industrial by-products containing a larger proportion of ligno-cellulosic feeds like cereal straws, stovers, sugarcane by-products and similar other feeds. These feeds are poor in protein, energy, minerals and vitamins. Addition of foliage from tree leaves or supplementation with seed meals or even urea can improve the utilization of low quality roughages mainly through the supply of nitrogen to rumen microbes. The use of simple but robust techniques for evaluation of the nutritional quality of these feed resources will contribute to their efficient utilization.

Both growth and milk yield of ruminants are largely limited by forage quality which is mainly reflected in low voluntary intake and digestibility. The importance of these parameters in animal nutrition has long been recognised. 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.

This paper highlights the potential of a novel approach using an in vitro rumen fermentation technique for evaluation of the nutritional quality of conventional and unconventional feed resources.
2. EVALUATION OF FEED RESOURCES

Recent advances in ration balancing include manipulation of feed to increase the quantity and quality of protein and energy delivered to the small intestine. Selection of fibrous feeds based on high efficiency of microbial protein synthesis in the rumen along with high dry matter digestibility, and development of feeding strategies based on high efficiency as well as high microbial protein synthesis in the rumen will lead to higher supply of protein post-ruminally. This concept of feed evaluation has an extra element of the efficiency of microbial protein synthesis in addition to the more conventional one of the dry matter digestibility. The limited supply of protein post-ruminally under most feeding systems in developing countries is an important limiting factor which prevents an increase in animal productivity.

There are a number of methods used to determine net microbial protein synthesis in the rumen based on the use of microbial markers. They require the use of post-ruminally cannulated animals to determine flow of digesta. The cannulation approach is tedious and has several limitations (Chen et al., 1990) to its applicability under most research conditions in developing countries. A simpler technique for determination of microbial protein supply to the intestine is based on the determination of total urinary purine derivatives (IAEA, 1997). This approach is being thoroughly investigated under a Joint FAO/IAEA Coordinated Research Project (IAEA, 1998). Although the method is based on the collection of urine for determination of purine derivatives (allantoin and uric acid for cattle, and allantoin, uric acid, xanthine and hypoxanthine for sheep), the approach is being further simplified using spot urine samples. This technique does not require cannulated animals, but it involves feeding the diets under investigation to animals and therefore is not suitable for screening large numbers of samples or for developing feed supplementation strategies using various feed constituents.

In vitro methods

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 \textit{in vivo} parameters. \textit{In vitro} methods have the advantage not only of being less expensive and less time-consuming, but they allow maintaining experimental conditions more precisely than do \textit{in vivo} trials. Three major biological digestion techniques are currently available to determine the nutritive value of ruminant feeds: 1) digestion with rumen microorganisms as in Tilley and Terry (1963) or using a gas method (Menke \textit{et al.}, 1979), 2) \textit{in situ} incubation of samples in nylon bags in the rumen (Mehrez and Orskov, 1977), and 3) cell-free fungal cellulase De Boever \textit{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). The nylon bag technique has been used for many years to provide estimates of both the rate and extent of disappearance of feed constituents. This technique provides a useful means to estimate rates of disappearance and potential ruminal degradability of feedstuffs and feed constituents whilst incorporating effects of particulate passage rate from the rumen. The disadvantage of the method is that only a small number of forage samples can be assessed at any one time, and it also requires at least three fistulated animals to account for variations due to animal. It is therefore of limited value in laboratories undertaking routine screening of a large numbers of samples. It is also laborious, and requires large amounts of samples. Substantial error could result in values obtained at early stages of digestion due to a low weight loss; and for poor quality roughages, adherence of microbes at early stages can even lead to higher weights and thus distortion of results if kinetic modelling does not incorporate the lag-phase (McDonald, 1981; Dhanoa, 1988).

The Tilley and Terry (1963) technique is used widely because of its convenience, particularly when large-scale testing of feedstuffs is required. This method is employed in many forage evaluation laboratories and involves two stages in which forages are subjected to 48 h fermentation in a buffer solution containing rumen fluid, followed by 48 h of digestion with pepsin in an acid solution. The
method was modified by Goering and Van Soest (1970), in that the residue after 48 h incubation was treated with neutral detergent solution to estimate true dry matter digestibility. Although the method of Tilley and Terry (1963) has been extensively validated with *in vivo* values (Van Soest, 1994), the method appears to have several disadvantages. The method is an end-point measurement (gives only one observation) thus, unless lengthy and labour-intensive time-course studies are made, the technique does not provide information on the kinetics of forage digestion; the residue determination destroys the sample and therefore a large number of replicates are needed. The method is therefore difficult to apply to materials such as tissue culture samples or cell-wall fractions.

Both the Tilley and Terry and 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 to nutrient supply to animals (Makkar *et al.*, 1993).

**In vitro gas method**
The gas measuring technique has been widely used for evaluation of nutritive value of feeds. More recently, the increased interest in the efficient utilisation 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.* (1998a). The *in vitro* gas method based on syringes (Menke *et al.*, 1979; Blümmel *et al.*, 1997) appears to be the most suitable for use in developing countries. Other *in vitro* methods such as Tilley and Terry and nylon bag methods are based on gravimetric measurements which follow disappearance of the substrate (the components which may or may not necessarily contribute to fermentation), whereas gas measurement focuses on the appearances of fermentation products (soluble but not fermentable products
do not contribute to gas production). 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-antinutrient and antinutrient-antinutrient interactions (Makkar et al., 1995a).

A simple in vitro approach is described below which is convenient and fast, and allows a large number of samples to be handled at a time. It is based on the quantification of substrate degraded or microbial protein produced using internal or external markers and of gas or short chain fatty acid (SCFA) production in an in vitro rumen fermentation system based on syringes (Menke et al., 1979). This method does not require sophisticated equipment or the use of a large number of animals (but one or preferably two fistulated animals are required) and helps selection of feeds or feed constituents based not only on the dry matter digestibility but also on the efficiency of microbial protein synthesis.

In the method of Menke et al. (1979), fermentations are conducted in 100 ml capacity calibrated glass syringes containing feedstuff and a buffered rumen fluid. The gas produced on incubation of 200 mg feed dry matter after 24 h of incubation together with the levels of other chemical constituents are used to predict digestibility of organic matter determined in vivo and metabolizable energy.

For roughages, the relationships are:

\[
\begin{align*}
\text{ME (MJ / Kg DM)} &= 2.20 + 0.136 \text{Gp} + 0.057 \text{CP}, \quad R^2 = 0.94 \\
\text{OMD ( percent)} &= 14.88 + 0.889 \text{Gp} + 0.45 \text{CP} + 0.0651 \text{XA}, \quad R^2 = 0.92
\end{align*}
\]

Where ME is the metabolizable energy; DM dry matter, OMD organic matter digestibility; CP, crude protein in percent; XA, ash in percent; and Gp, the net gas production in ml from 200 mg dry sample after 24 h of incubation and after correction for the day-to-
day variation in the activity of rumen liquor using the Hohenheim standard.

Aiple et al. (1996) compared three laboratory methods (enzymatic, crude nutrient and gas measuring technique) as predictors 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 method of Menke et al. (1979) was modified by Blümmel and Ørskov (1993) in that feeds were incubated in a thermostatically controlled water bath instead of a rotor in an incubator. Makkar et al. (1995b) and Blümmel et al. (1997) modified the method further by increasing the amount of sample from 200 to 500 mg and increasing the amount of buffer two-fold as a result the incubation volume increase from 30 ml in the method of Menke et al. (1979) to 40 ml in the modified method. In the 30 ml system, the linearity between the amount of substrate incubated and the amount of gas produced is lost when the gas volume exceeds 90 ml because of the exhaustion of buffer of the medium; and in 40 ml system, the linearity is lost when the gas volume exceeds 130 ml (Getachew et al., 1998b). The exhaustion of the buffer decreases pH of the incubation medium; consequently the fermentation is inhibited. If the amount of gas production exceeds 90 ml using the 30 ml system and 130 ml using the 40 ml system, the amount of feed being incubated should be reduced.

The main advantages of the modified method (the 40 ml system and incubation in a water bath) are: i) there is only a minimum drop in temperature of the medium during the period of recording gas readings on incubation of syringes in a water bath. This is useful for studying the kinetics of fermentation where gas volume must be recorded at various time intervals, ii) because of large volume of water in the water bath and also its higher temperature holding capacity, drastic drop in the temperature of the incubation is prevented in case of power breakdown for a short duration, and iii) an increase in amount of sample from 200 to 500 mg reduces the inherent error.
associated with gravimetric determination needed to determine concomitant in vitro apparent and true digestibility (see below).

When a feedstuff is incubated with buffered rumen fluid in vitro, the carbohydrates are fermented to produce short chain fatty acids, gases and microbial cells. Gas production is basically the result of fermentation of carbohydrates to acetate, propionate and butyrate. Gas production from protein fermentation is relatively small as compared to carbohydrate fermentation. The contribution of fat to gas production is negligible. When 200 mg of coconut oil, palm kernel oil and/or soybean oil were incubated, only 2.0 to 2.8 ml of gas were produced while a similar amount of casein and cellulose produced about 23.4 ml and 80 ml gas.

The gas produced in the gas technique is the direct gas produced as a result of fermentation and the indirect gas produced from the buffering of SCFA. For roughages, when bicarbonate buffer is used, about 50 percent of the total gas is generated from buffering of the SCFA and the rest is evolved directly from fermentation. At very high molar propionate the amount of CO2 generated from buffering of SCFA is about 60 percent of total gas production. Each mmol of SCFA produced from fermentation releases 0.8–1.0 mmol of CO2 from the buffered rumen fluid solution, depending on the amount of phosphate buffer present. Highly significant correlation has been observed between SCFA and gas production (see below).

Gas is produced mainly when substrate is fermented to acetate and butyrate. Substrate fermentation to propionate yields gas only from buffering of the acid and, therefore, relatively lower gas production is associated with propionate production. The gas that is released with the generation of propionate is only the indirect gas produced from buffering. The molar proportion of different SCFA produced is dependent on the type of substrate. Therefore, the molar ratio of acetate of propionate has been used to evaluate substrate related differences. Rapidly fermentable carbohydrates yield relatively higher propionate as compared to acetate, and the reverse takes place when slowly fermentable carbohydrates are incubated. Many workers
reported more propionate and thus lower acetate to propionate ratio in the ruminal fluid of cows fed a high grain diet. If fermentation of feeds leads to a higher proportion of acetate, there will be a concomitant increase in gas production compared with a feed with a higher proportion of propionate. In other words, a shift in the proportion of SCFA will be reflected by changes in gas production.

The gas produced on incubation of cereal straws (Blümmel and Ørskov, 1993), a wide range of feeds including many dairy compound feeds and their individual feed components whose protein and fat contents vary greatly (Blümmel et al., 1999a), and tannin containing browses (Getachew et al., 2000a) in absence or presence of polyethylene glycol (a tannin complexing agent) in the buffered rumen fluid was closely related to the production of short chain fatty acids as per Wolin (1960) stoichiometry, which is as follows:

\[
\text{Fermentative CO}_2 = \frac{A}{2} + \frac{P}{4} + 1.5B
\]

where A, P and B are moles of acetate, propionate, and butyrate respectively.

\[
\text{Fermentative CH}_4 = (A+2B) - \text{CO}_2
\]

where A and B are moles of acetate and butyrate respectively and \( \text{CO}_2 \) is moles of \( \text{CO}_2 \) calculated from previous equation.

Assumption: one mole of SCFA releases one mole of \( \text{CO}_2 \) from bicarbonate-based buffer described as buffering \( \text{CO}_2 \) and therefore, mmol of buffering \( \text{CO}_2 \) is equal to mmol of total SCFA generated during incubation.

\[
\text{Gas volume} = \text{mmol of gas} \times \text{gas constant (R)} \times T
\]

where:

R = the ratio between molar volume of gas to temperature (Kelvin zero; K) i.e. (22.41l/273 = 0.082),
T = incubation temperature (Kelvin); 273 + 39°C = 312 K
Total volume of gas (ml) calculated from SCFA production = (BG + FG) x CF
BG = gas volume (ml) from buffering of SCFA,
FG = fermentative gas (ml) (CO2 + CH4),
CF = correction factor for altitude and pressure which is 0.953 for Hohenheim at altitude 400m above sea level (Blümmel et al., 1999).
(The volume of 1 mmol of gas at 39°C in Hohenheim would be; 1 x 0.082 x 312 x 0.953 = 24.4 ml).

The origin and stoichiometry of gas production have been described in details in Blümmel et al. (1997) and Getachew et al. (1998a).

The in vitro gas production measured after 24 h incubation of tannin containing browses in the presence or absence of polyethylene glycol (PEG) was strongly correlated with gas volume stoichiometrically calculated from SCFA. The relationship between SCFA production (mmol) and gas volume (ml) after 24 h of incubation of browse species with a wide range of crude protein (5.4-27 percent) and phenolic compounds (1.8-25.3 percent and 0.2-21.4 percent total phenols and total tannins as tannic acid equivalent respectively) was (Getachew et al., 2000a):

In the absence of PEG: SCFA = 0.0239.Gas -0.0601; R² = 0.953; N = 39; P<0.001 (I)
In the presence of PEG: SCFA = 0.0207.Gas + 0.0207; R² = 0.925; N = 37; P<0.001 (II)

These relationships are similar to that obtained for wheat straw (Blümmel et al., 1993).

The SCFA production could be predicted from gas values using the above relationship. The level of SCFA is an indicator of energy availability to the animal. Since SCFA measurement is important for relating feed composition to production parameters and to net energy values of diets, prediction of SCFA from in vitro gas measurement will be increasingly important in developing countries.
where laboratories are seldom equipped with modern equipments to measure SCFA.

The stoichiometric balance also allows calculation of the CH4 and CO2 expected from the rumen fermentation if the molar proportions and amount of SCFA are known.

Kinetics of fermentation feedstuffs can be determined from fermentative gas and the gas released from buffering of SCFA. Kinetics of gas production is dependent on the relative proportion of soluble, insoluble but degradable, and undegradable particles of the feed. Mathematical descriptions of gas production profiles allows analysis of data, evaluation of substrate- and media-related differences, and fermentability of soluble and slowly fermentable components of feeds. Various models have been used to describe gas production profiles. France et al. (1993; 2000) combine modelling of the gas profiles with estimates of substrate loss and ruminal rate of passage and derive estimates of ruminal extent of degradation thus linking gas production to events in the rumen proper.

**In vitro gas production with concomitant microbial mass measurement**

Determination of microbial mass. *In vitro* gas tests are attractive for ruminant nutritionists 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 (feed) 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 (or SCFA production) and microbial mass
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Assessing quality and safety of animal feeds 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. Blümmer et al. (1997) have demonstrated how a combination of in vitro gas production measurements with a concomitant quantification of the truly degraded substrate provides important information about partitioning of fermentation products, and the in vitro microbial mass production can be calculated as:

\[ \text{Microbial mass (mg)} = \text{mg substrate truly degraded} - \text{(ml gas volume x stoichiometrical factor)} \]

For roughages, the stoichiometrical factor was 2.20.

Partitioning factor. The parameters in the above equation also allow the calculation of a partitioning factor (PF). The PF is defined as the ratio of substrate truly degraded in vitro (mg) to the volume of gas (ml) produced by it (equivalent to the reciprocal of parameter Y in France et al., 1993). The above equation becomes

\[ \text{Microbial mass (units)} = \text{gas volume (PF - stoichiometrical factor).} \]

A feed with higher PF means that proportionally more of the degraded matter is incorporated into microbial mass, i.e., the efficiency of microbial protein synthesis is higher. Roughages with higher PF have been shown to have higher dry matter intake. Different in vitro PF values are also reflected by in vivo microbial protein synthesis as estimated by purine derivatives (the higher the PF, the higher the excretion of urinary purine derivatives; Blümmer et al., 1999b) and in methane production by ruminants (the higher the PF, the lower the methane output; Blümmer et al., 1999b). These results show that the PF calculated in vitro provides meaningful information for predicting the dry matter intake, the microbial mass production in the rumen, and the methane emission of the whole ruminant animal.

The procedures for the determination of truly degraded substrate and the calculation of the stoichiometrical factor; stoichiometrical
relationship between SCFAs and gas volume; and relationship between SCFA production, ATP production and microbial mass yield can be obtained from Blümmer et al. (1997) and Getachew et al. (1998a). It may be noted that these procedures and relationships are valid for substrates consisting predominantly of structural carbohydrates, and the findings might not extend to substrates such as those high in soluble carbohydrate, protein or fat. Rymer and Givens (1999) have shown that, as observed by Blümmer et al. (1997), good quality feeds (grass silage, wheat, maize, molasses sugar beet feed and fish meal) which produce large amounts of gas and SCFA yield small amounts of microbial mass per unit of feed truly degraded.

It seems therefore justified to suggest that feeds or feed ingredients should be selected that have a high in vitro true degradability but low gas production per unit of truly degraded substrate. Dijkstra et al. (2000) have described modelling of microbial protein synthesis in vivo from the in vitro gas parameters.

**Incubation time and PF.** Another study by Blümmer et al. (1998a), in addition to once again describing the importance of measuring microbial mass, has highlighted the importance of the fermentation time at which the microbial mass should be measured. In this study, substrate degradation and kinetics of in vitro partitioning of three hays, with similar in vivo digestibilities, into SCFA, microbial mass yield, and ammonia, carbon dioxide and methane production was examined at 8, 12, 18 and 24 h of incubation in the gas method under both low and adequate nitrogen levels. Microbial synthesis was quantified gravimetrically (Blümmer et al., 1997), by nitrogen balance (Getachew et al., 2000b) and by purine analysis (Makkar and Becker, 1999). SCFA and gas production were positively correlated (P < 0.0001) and cumulative at all times of incubation under both low and adequate nitrogen levels. On the other hand, microbial mass, microbial nitrogen and microbial purine yields declined after 12 h of incubation while ammonia production increased. Per unit of substrate degraded, gas and SCFA production were always inversely (P < 0.05) related to microbial mass yield regardless of incubation
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At later incubation times, continuously more SCFA or gas and less microbial mass were produced reflecting microbial lysis and probably increasing microbial energy spilling. All three hays differed (P < 0.05) consistently in how the degraded substrate was partitioned into SCFA and gas and into microbial mass in both the low and adequate nitrogen medium. Purine analysis indicated substantial differences in microbial composition across treatments, which might be one explanation for these different microbial efficiencies (Blümmel et al., 1998a).

The efficiency of microbial growth was higher for 16 h incubation than 24 h for tannin-rich feeds when these were incubated in presence or absence of PEG, a tannin-inactivating agent. Additional nitrogen in the medium also affected the efficiency of microbial protein synthesis from tannin-tannins feeds both at 16 h and 24 h (Getachew et al., 2000c). For proper characterisation of feed and feed ingredients, approaches need to be developed for measuring the PF at the incubation time at which the lysis of microbes is minimal. Some possible simple approaches worth investigating to identify this incubation time are: i) the time at which half of the maximum gas is produced, and ii) the inflection point at which the rate of gas production is maximum (the rate increases up to a certain incubation time and thereafter decreases as the incubation progresses), both these parameters can be mathematically calculated from the gas profiles. The effect of the nitrogen level in the medium on the PF and the significance in vivo of these PFs also need to be investigated.

Digestion kinetics of neutral detergent-soluble fraction. The gas measurement method has also been used to study digestion kinetics of the neutral detergent soluble fraction of forages, starch-rich feeds and other highly digestible carbohydrate components, which was obtained by subtracting the average gas production curve for the digestible neutral detergent fibre (NDF) from that of the unfractionated whole feed (Chen et al., 1999). The subtraction procedure might give some useful information relevant to low-NDF fibre feeds, e.g., corn grain Chen et al., 1999), but it is not suitable for
Recent advances in the in vitro gas method for evaluation of nutritional quality of feed resources (Blümmel et al., 1998b). Blümmel et al. (1998b) examined the rate and extent of fermentation of whole roughage and extracted NDF, dry matter degradability of extracted NDF and the PF for whole roughage and the extracted NDF of 54 roughages. The 24-h degradabilities of extracted NDF were higher than NDF degradabilities in whole roughages, and the PF values were lower for extracted NDF than for whole roughages (2.5 vs 3.1; i.e. the efficiency of microbial protein synthesis with extracted NDF was lower). Both the higher degradability and lower PF contributed to higher gas volumes obtained from extracted NDF compared with whole roughage. Supplementation of amino acids and sugars, which essentially constitute the solubles, may increase the efficiency of microbial synthesis from cell walls during fermentation (a situation similar to that in unfractionated forages) and the removal of solubles may result in lower microbial efficiencies. A considerable effect of cell solubles on partitioning of nutrients from the NDF raises doubts as to the significance in vivo of the kinetic parameters calculated using the subtraction procedure (Blümmel et al., 1998b).

Voluntary intake prediction. The main constraint to the utilisation 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 fibre 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
percent vs. 75 percent) of the variation in dry matter intake than gas production from whole roughage (Getachew et al., 1998a).

Interaction between dietary constituents. 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 (Sampath et al., 1995). 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 (see above) It is suggested to determine microbial mass production in addition to the gas measurement for such studies.

Evaluation of tannin-containing by-products and forage

The need to determine microbial mass using internal or external markers. Unfortunately, the method based on the gas method and the detergent system of fibre analysis to calculate the microbial mass produced for fibrous feeds (the method outlined above) did not work for tannin-rich feeds. The PF for tannin-rich feeds (calculated as mg truly degraded substrate needed to produce one-ml gas) ranged from 3.1 to 16.1 (Getachew et al., 2000c), which is well above the theoretical range of PF (2.75 to 4.41) (Blümmel et al., 1997). The high PF could be due to: a) solubilization of tannins from the feed. These tannins would make no contribution to gas or energy in the system but would contribute to dry matter loss, b) the cell solubles contributing to dry matter loss but not to gas production because the gas production is inhibited by tannins or c) a combination of a) and b). In addition, the appearance of tannin-protein complexes as artefacts in the true residue also makes the gravimetric approach
(Blümmel et al., 1997) of quantification of microbial mass redundant (Makkar et al., 1997a). The presence of tannin-protein complexes in faecal samples (the origin of proteins could be microbes, feed or endogenous secretion from the gastro-intestinal tract) from animals fed tannin-rich forage and their non-removal by the detergent system of fibre analysis (Van Soest et al., 1991) leads to misleading values of fibre and also causes problems in the in vivo evaluation of tannin-rich feeds (Degen et al., 1995; Makkar et al., 1995c). Therefore, caution is required in interpreting results obtained from in vivo or in vitro experiments on the evaluation of tannin-containing feeds using the detergent system of fibre analysis.

For the in vitro evaluation of tannin-rich feeds, the microbial mass should be quantified using diaminopimelic acid (DAPA) or purines as markers, or by 15N incorporation into the microbes (Makkar et al., 1998a), and the PF for tannin-rich feeds can be expressed as the microbial mass determined by these markers per ml of gas produced (or per mmol SCFA produced). The system developed for evaluation of tannin-containing feeds depends on incubation of the feed in the presence and absence of polyethylene glycol (PEG MW 4000 or 6000 preferably of 6000; (Makkar et al., 1995b)) and measurement of gas (or SCFA) and microbial mass using the above mentioned markers. PEG has a high affinity for tannins. Addition of PEG results in the formation of PEG-tannin complexes which inactivates tannins. The changes in gas (or SCFA) and microbial mass as a result of PEG addition represents ‘in totality’ the tannin effects (biological) as a function of the rumen fermentation parameters. This bioassay based on an in vitro rumen fermentation system coupled with the use of a tannin-complexing agent, PEG could be complementary to other tannin assays (Makkar et al., 1995c; Makkar, 1989; Makkar, 2000) in evaluating the nutritional quality of tanniniferous feeds.

**Significance of bound tannins and the efficiency of microbial protein synthesis.** The above approach of incubating tannin-containing feeds in the presence and absence of PEG also enables studies to be made on the nutritional significance of both extractable and unextractable (bound) tannins. Addition of PEG during the
incubation of tannin-rich NDF led to an increase in gas production, suggesting that tannins released as a result of NDF degradation by rumen microbes are biologically active and have the potential to influence rumen fermentation (Makkar et al., 1997b). Similar results were obtained on incubation of tannin-rich browse made free of extractable tannins by repeated use of 70 percent aqueous acetone.

Another application of this method is to study the effect of tannins on the partitioning of nutrients between microbial protein and SCFAs or gases or to study the efficiency of rumen microbial protein synthesis. Using DAPA, purines and 15N approaches for measuring microbial mass it has been shown that in the presence of PEG, the degradabilities of substrate and microbial mass production were higher, the efficiency of microbial protein synthesis was lower (Makkar et al., 1995a, 1998a; Getachew et al., 2000b). Similar results have also been obtained by other approaches based on the gas method in which the rate of ammonia uptake is taken as the efficiency of microbial protein synthesis (Makkar et al., 1998a), and using nitrogen balance approach (Getachew et al., 2000b). Conversely, efficiency of microbial protein synthesis is expected to be higher in the presence of tannins. The net microbial mass production would depend on the balance between decreased degradable dry matter and higher microbial mass production per unit of dry matter digested in presence of tannins.

This method has also been used to develop strategies to enhance the microbial mass production through enhancing efficiency of microbial protein synthesis for tannin rich feeds. Incorporation of PEG in small amounts and scattered over the incubation period increased the efficiency of microbial mass production (Getachew et al., 2000d) which translated into the inclusion of PEG in fed blocks. The licking of these blocks by animals resulted in slow release of PEG in the rumen which produced higher animal productivity compared to when the same amount of PEG was given to animals in one portion (Ben Salem et al., 1999).

**Protein degradability.** Although the 24-h *in vitro* degradable nitrogen values obtained from tannin-rich browse and herbaceous
legumes were lower than those reported for low quality roughages (Getachew et al., 1998b; 2000b), the relatively high crude protein content of these browse and herbaceous legumes could play a significant role in supplying rumen degradable nitrogen. In the presence of a tannin-inactivating agent, PEG, their in vitro degradable nitrogen values were raised. The difference between these values observed in the presence and absence of PEG indicates the amount of protein protected by tannins from degradation in the rumen (Getachew et al., 2000b). Whether the protein protected by tannins from microbial degradation is fully available to animal’s post-ruminally requires further research. Raab et al. (1983) reported a close relationship between in vivo and in vitro values when incubation was terminated after 17 h. When normal protein feed was tested about 80 percent of the 24 h value was degraded in the first 8 h incubation whereas in protected protein feed only 60 percent of the 24 h value was degraded in this time (Raab et al., 1983). The appropriate incubation time for in vitro degradability studies in the presence and absence of PEG for tannin-rich feeds may depend on the nature of protein and tannins, which should be identified. Also, this method for quantifying feed proteins that have been protected by tannins from degradation in the rumen needs to be validated in vivo.

Measurement of feed proteins during fermentation using polyacrylamide gel electrophoresis coupled with the use of an image analyser could be another attractive approach for measuring the rumen degradability of nitrogen, and for studying the influence of various natural plant products (e.g., tannins, saponins, alkaloids; (Makkar et al., 1997a, 1998a,b).

3. SOME PRACTICAL CONSIDERATIONS FOR FEED EVALUATION USING THE GAS METHOD

After 16 h (for concentrate feeds) and 24 h (for roughage samples) of incubation (The termination of the incubation should be at the time at which the efficiency of microbial production is maximum, which is difficult to ascertain under normal feed evaluation experiments for a large number of feeds. An approach that could be applied is running of
200 mg sample for the kinetic experiment and calculating the time (t/2) at which the gas production is half of the potential gas production. The time ‘t/2’ is near to the time at which the efficiency of microbial production is the maximum. This means that one has to run a kinetic experiment before determination of truly degraded organic matter and microbial protein using purines or 15N approaches. The time 16 h for concentrate feeds and 24 h for roughages might be a good compromise for routine feed evaluation), measure:

a) True organic matter degradability and gas production (weigh air dried 375 + 5 mg sample in 30 ml medium containing double the level of bicarbonate ions than the conventional medium of Menke et al. (1979) used for estimation of metabolizable energy and degradability of organic matter (DOM)). Record net gas production (ml gas production in syringe containing feed minus ml gas production in blank). After termination of the incubation, transfer the contents of the syringe quantitatively in a beaker and digest it with the neutral digestion solution for 1 h (the purpose of this treatment is to solubilize the microbes and obtain only the under graded feed). Filter the contents through crucible no. 2, wash the residue on the crucible with hot water till the residue is free of the detergent. Dry the crucibles at 130 ºC for 2 h or at 100 ºC for 10 h (overnight). Record weight of the crucibles after transferring them to a desiccator. This weight minus weight of empty crucible gives the weight of under graded feed in that particular syringe. [Note: this method of measurement of under graded residue does not work satisfactorily with tannin-containing feeds/samples (presence of tannin-protein complexes as artefacts in the residue) and starch-rich feeds (some starch might not be degraded by microbes up to 16 h of incubation but it would get solubilized in the neutral detergent solution leading to underestimation of truly under graded residue). It may be noted that this method of measurement of under graded residue should not be applied at the initial hours of incubation (before 16 h of incubation) since
during this period a portion of the feed, which is under graded by microbes could be solubilised in the neutral detergent solution. Let the weight of this residue (under graded feed) be ‘a’. Now transfer the crucibles containing this residue to Muffle furnace and ash the sample. The organic matter will disappear leaving the ash. After transferring the crucibles to a desiccator, weigh them and subtract from this weight the weight of the empty crucible to obtain the weight of ash (called as ‘b’), i.e. subtract ‘b’ from ‘a’ to obtain truly under graded organic matter (a-b). Calculate the organic matter degradability in percentage unit. Please note that for calculation of organic matter degradability, one needs to have dry matter and ash contents of the feed sample. This will enable to calculate organic matter in 375 + 5 mg of the air-dried matter weighed in the syringe for the incubation.

Organic matter weighed into the syringe
= (375 x DM in percentage/100)) minus (375 x (DM in percentage/100) x (Ash in percentage/100)).
Or (375 x DM in percentage/100) (1 – Ash in percentage/100)
Let this value be ‘c’.
Percent organic matter degradability is (a-b)100/c.

b) For most conventional feed resources (not the tannin-containing feeds and starch-rich feed ingredients), microbial mass production can be estimated at the time of termination of the incubation. Please note that this approach should not be applied for the initial hours of incubation (earlier than 16 h).

mg microbial mass production = ((a-b) – 2.2 x net gas in ml)); (a-b) is in mg and 2.2 is the stoichiometric factor.

Efficiency of microbial mass production = ((a-b) – 2.2 x net gas in ml)/(a-b)

c) Calculation of PF. PF is a measure of efficiency of microbial mass production or efficiency of microbial protein production. Higher the factor, higher is the efficiency.
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PF = (mg truly degraded organic matter)/ml gas

Or

PF = c – (a-b) in mg truly degraded organic matter divided by ml gas produced in that particular syringe for which ‘c – (a-b)’ has been calculated. This value is the PF.

The theoretical range for PF is 2.74 to 4.41. Any value above or below this range should be critically evaluated. For tannin-rich samples, the PF is normally above 4.41. The higher PF (for example 7.2) would mean that 7.2 mg of the truly degraded organic matter produce one ml of the gas. The high values for tannin-rich feeds appear to be due to the solubilization of tannins from the feeds during fermentation, contributing to the dry matter loss but without contributing to the gas, and inhibition of gas production from cell solubles by tannins through inhibition of rumen fermentation (cell solubles will also contribute to dry matter loss). The presence of tannin-protein complexes in the truly under graded residue and hence underestimation of the truly degraded organic matter also contribute to the errors in the PF, but the presence of these complexes in the truly unde graded will lower the PF. The PF, which is obtained for the tannin-containing sample is the net effect of solubilization of the feed components during fermentation but not contributing to the gas production and the presence of tannin-protein complexes in the truly undegraded residue.

d) For tannin-containing feeds, the syringe contents are not digested after incubation with the neutral detergent solution (see above) to determine truly undegraded organic matter. Instead, the syringe contents are taken to the determination of purines and/or 15N incorporation studies.

Efficiency of microbial mass production can be expressed as: mg net purines in syringe divided by ml of net gas production (both these values need to be corrected for the blanks).

Or as:
mg (or microgram) of 15N in apparently under graded residue/ml of net gas production (both these values need to be corrected for the blanks).

If need be, purine amount can be converted to microbial-N by taking rumen fluid sample, centrifuging it at approx. 17,000 g to obtain microbial pellet, washing it once with distilled water and recentrifuging, and lyophilising it. In a weighed lyophilised pellet, purines can be determined by high performance chromatography (HPLC) or using a spectrophotometric method after precipitation of purine with silver nitrate. Using this purine to microbial mass ratio, the purine can be converted into microbial mass. If there is a need to convert purine to microbial-N, a portion of the same lyophilised pellet can be subjected to the determination of N using micro-Kjeldahl method or by converting N in the pellet to ammonia and determination of ammonia using sodium nitroprusside and hypochlorite reaction (Makkar and Becker, 1999).

Note: Purines are only in the microbes and not in the supernatant, but 15N is present both in the apparently undegraded residue as well as in the supernatant after the incubation. Therefore, there is a need to take apparently undegraded residue for 15N incorporation studies. The apparently undegraded residue is prepared by centrifuging the syringe content using the high speed centrifuge (17,000 g for 20 min at 4o C), discarding supernatant, washing the residue with distilled water followed by again the high speed centrifugation and discarding the supernatant. The residue should be lyophilized or can be dried in a vacuum oven at approx. 50o C. The weight of this residue should be determined (weight of centrifuge tube plus the residue) minus weight of empty centrifuge tube. This weight needs to be taken into account in the calculations at a later stage. The residue should be quantitatively collected and ground to fine powder preferably using ball mill. In addition to determination of 15N, purines could also be
determined in this residue. This section addresses the use of this approach for tannin-containing feeds but this could be used for any feed samples.

e) The gas production can be converted to mmol SCFA production using the equations (I) and (II).

f) Estimation of in vivo metabolizable energy and digestibility of organic matter (DOM) using the net gas value from 375 mg sample and converting the gas value from 375 mg to 200 mg dry sample or directly incubating 200 mg sample for 24 h.

g) Determination of tannin activity by incubating 375 mg of sample in presence and absence of PEG 6000 in 30 ml of the medium containing double the level of bicarbonate ions (see section ‘a’ above); termination of the incubation at 16 and 24 h; and expressing the results at both 16 and 24 h of the incubation, as:
   • Difference in the gas production as percent increase on addition of the PEG compared to the syringe without the PEG,
   • Difference in purine as percent increase or decrease on addition of the PEG compared to the syringe without the PEG,
   • ME and DOM values with and without PEG (after converting gas value for 200 mg sample), and percent change in ME and DOM values on addition of the PEG (taking the value without PEG as 100 percent), and
   • Change in the efficiency of microbial protein production when expressed as mg purine (or mg 15N incorporation)/ml gas, on addition of the PEG.

h) Determination of the kinetics of gas production (France et al., 2000) by incubating 200 mg of the sample for 96 h for roughages and 72 h for concentrate samples.

i) Microbial N (MN) could also be measured after incubation by
following two nitrogen balance approaches (Getachew et al., 2000b). The first approach is:

\[ MN = TN - (NDF-N + Ammonia-N), \]

where TN is total N i.e., feed N + N in buffered rumen fluid in the syringe before incubation (at 0 time), NDF-N is the N bound to NDF fraction following incubation and Ammonia-N is the ammonia-N in the supernatant following the incubation. In a closed system, the total N present at the start of the incubation can be in microbial mass, NDF-N, ammonia-N and amino acids during any time of the incubation. Negligible amounts of amino acids and peptides are present in the supernatant during fermentation and therefore these can be ignored in calculation of microbial N.

For determination of NDF fraction for N bound to NDF after incubation, the syringe contents are transferred into a 600 ml beaker and the syringes are washed twice with a total of 50 ml neutral detergent solution (NDS) and emptied into the beaker. The contents are refluxed for 1 h, and then filtered through pre-tarred filter crucibles (no. 2). The crucibles are dried overnight at 100 ºC and weighed. True degradability can be calculated as the weight of substrate incubated minus the weight of the residue after NDS treatment. The residue after NDS treatment (neutral detergent residue, NDF) is subjected to micro-Kjeldahl digestion for determination of NDF-N.

The second approach is:

\[ MN = APUR-N - NDF-N, \]

where APUR-N is N bound to apparent under graded residue after incubation. The preparation of apparent under graded residue is given above (section ‘d’). N bound to this fraction is determined in a manner similar to NDF-N determination.

From the weight of apparent under graded residue, apparent digestibility can also be calculated.
Do’s and don’ts for the gas method

- Soon after getting the syringes from the firm, mark the plunger and the corresponding graduated outer portion (barrel) with a diamond pencil (just after opening the case containing the syringe comprising of the plunger and the barrel). Give the same number to both the parts of the syringe.
- The plunger should be properly lubricated using white Vaseline (apply less amount of Vaseline for incubations up to 24 h and more for incubations up to 96 h).
- Collect rumen liquor from both the liquid and the solid phase and handle it properly (use of warm containers, flushing the containers with carbon dioxide, always keeping the rumen liquor under carbon dioxide).
- Reducing solution should be prepared fresh on the same day of conducting the experiment.
- Start flushing the medium with carbon dioxide well before (approx. 10 min) adding the reducing solution. Also flush the medium for at least 10 min after adding the rumen liquor and before starting filling the syringes. Keep flushing the medium with carbon dioxide while filling the syringes (the flow could be reduced at this stage).
- While filling the syringes with the medium, keep an eye on the medium (carbon dioxide gas should be flushing into the medium and the medium should be stirring).
- After dispensing 30 ml of the medium into the syringe, create a light vacuum by pushing back the plunger and then open the clip. This procedure will bring the medium lying in the nozzle back into the syringe. Otherwise there could be a loss of the medium and/or sample.
- After filling of the syringes has been completed (might take 30-40 min), shake the syringes. Shake them again after every 30 min till first 2 h of the incubation, and then after every two hours till the first 10 or 12 h of the incubation. Thereafter, shake the syringes after taking the gas volume readings (24, 30, 36, 48, 60, 72, 96 h, as the case may be). Make sure that all feed particles are
taken into the medium while stirring (swirling shaking action might help).

- Wash the dispenser with distilled water immediately after finishing filling the syringes, otherwise the dispenser could get stuck up and might not then be usable.
- Check temperature and level of water in the water bath at least twice a day.
- In the evening before going home, if the plunger is above 80 ml level, push it back, record the readings (before and after) pushing back the plunger.
- When you push back the syringe in the evening, give a shake after approximately 30 min in order to prevent taking up the sample along with the bottom portion of the plunger and out from the incubation medium.
- Use the carbon dioxide gas cylinder with caution. Ask someone if you do not know its operation. Misuse could cause an accident.
- While taking the gas volume readings, use the brown ring marked on the plunger and not the bottom end of the plunger. Keep the syringe in inverted position and in parallel with eye while recording the gas reading. Immediately transfer the syringe into the water bath after taking the reading.
- For cleaning the syringes, the syringe should be emptied (preferably pulling back the plunger and removing contents from the back and not from the nozzle). Clips should be removed. The plunger and the outer graduated part of the syringe (barrel) should be separated. Excess Vaseline on the plunger should be cleared with a tissue paper or a piece of soft cloth, and then transfer both the parts in hot water containing detergent (soap) solution. Rub the plunger with hand and inside of the barrel with a soft brush to clean these. Wash thoroughly both the portions with hot water and finally rinse them with distilled water. Dry them well before weighing sample into the syringe.
• Fix the clip in such a manner (by keeping the portion, where pressure is applied to open or close it, facing the syringe) that it does not open by striking on the edges of the lid of the water bath while taking out the syringe for taking reading.

• Mark the crucibles well (preferably with a diamond pencil). Keep them in increasing or decreasing order; this might help you in identifying the crucibles, which have not been marked well, especially after these have been placed in the Muffle furnace.

• For tannin bioassay use only PEG-4000 or PEG-6000 (preferably the latter).

• For a slide show on the gas method, refer to: http://www.iaea.org/programmes/nafa/d3/mtc/invitro-slideshowapr01.pdf

4. CONCLUSIONS AND FUTURE RESEARCH
The in vitro rumen fermentation method in which gas production and microbial mass production are concomitantly measured has several major advantages: i) it has the potential for screening a large number of feed resources, for example in breeding programmes for the development of varieties and cultivars of good nutritional value, ii) 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; iii) it has an important role to play in the study of rumen modulators for increasing efficiency of microbial protein synthesis and decreasing emission of methane, an environmental polluting gas, and iv) it provides a better insight into nutrient-antinutrient and antinutrient-antinutrient interactions, and into the roles of various nutrients (by changing the composition of the incubation medium) 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.

Further studies are required on: i) the development of simple approaches for identifying the incubation time in the in vitro gas system at which the PF (a measure of the proportion of fermented substrate which leads to microbial mass production) is maximum, ii) the effect of nitrogen in the incubation medium on the PF, and iii) the in vivo significance of the PF so obtained. The results of the limited experiments conducted so far have shown that simple models employing gas kinetic parameters and the PF are capable of predicting the dry matter intake of roughages and level of emission of methane by ruminants. Experiments also need to be done to test whether, for any given feed, the microbial protein synthesis as derived from digestion kinetic parameters (including PF) in vitro is sufficient to explain the observed microbial protein supply to the small intestine in vivo. At present, the simplest way of determining the latter parameter is to calculate it from the level of urinary purine derivatives. This validation exercise should be conducted for a wide range of feed constituents and diets which should enable the above mentioned simple technique of measuring gas and microbial mass to be a routine and powerful tool for feed evaluation thus avoiding the need for time-consuming, laborious and expensive feeding studies. Lately, much emphasis has been given to the development of statistical or mathematical models that best fit the gas production profiles and describe the gas evolution with high accuracy. Experiments must be designed to understand the biological significance of the various statistical and functional parameters being calculated using these models, and also to incorporate a measure of microbial mass into these mathematical descriptions.

Research and development efforts are required to establish a feed library for unconventional feedstuffs that includes information on nutritive values in addition to routine composition analysis. In the case of tannin-containing feedstuffs, there is a need to incorporate
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approach(s) measuring the biological activities of tannins as well as measuring tannin levels by chemical methods.

REFERENCES


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