Chapter 7
In Vitro Screening of Feed Resources for Efficiency of Microbial Protein Synthesis

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Introduction

Recent advances in ration balancing include manipulation of feed to increase the quantity and quality of protein and energy delivered to the small intestine. Proportionally high conversion of feed degraded in the rumen into microbial mass, i.e. a high efficiency of microbial mass production, will lead to efficient utilization of feed nitrogen and carbon. Selection of fibrous feeds based on high efficiency of microbial protein synthesis in the rumen along with high dry matter and fibre 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 will decrease both the need for supplementing rumen undegradable feed protein and the flow of feed carbon flow to fermentative carbon dioxide and methane (environment pollutants). The prediction of feed-dependent differences in efficiency of microbial protein synthesis is, therefore, of considerable interest in feed analysis. In addition, as a result of strong pressure from consumers to phase out antibiotics and other chemicals from feeds, because of the risk to human of chemical residues in food and of antibiotics resistance being transferred to human pathogens, intensive efforts are being made to identify plants, plant extracts or plant compounds which could substitute antibiotics and growth promoters. In quest for this, plants are being screened for properties that could lead to the use of plants or plant products for enhancing efficiency of rumen fermentation. In this context, how these plants or plant extracts when used as additives to diets affect the efficiency of microbial protein synthesis becomes important.

In this chapter, a number of approaches for measuring efficiency of microbial protein synthesis in vitro using the gas method are presented. In addition, variants of the gas method enabling measurement of fibre degradability and methane

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production are also dealt with. The theory behind the methods and explanations of the procedure by giving examples are also provided.

In Vitro Feed Evaluation Methods

In vitro methods for laboratory estimations of degraded feeds are important for ruminant nutritionists. An efficient laboratory method should be reproducible and the values obtained should correlate well with those actually measured in vivo. In vitro methods have the advantage of being less expensive, less time-consuming, and more ethical (minimizes the number of animals); and they allow maintaining experimental conditions more precisely than do in vivo trials.

The methods based on the digestion by rumen microbes are more meaningful since microorganisms are more sensitive to factors influencing the rate and extent of digestion than are chemical methods [50]. Four major techniques, based on the digestion by rumen microbes, are currently available for determining the nutritive value of ruminant feeds:

- in situ incubation of samples in nylon bags in the rumen [41],
- digestion with rumen microorganisms as in [49], and
- in vitro gas methods such as [18, 42, 44, 48],
- a modified in vitro gas method: gas production with concomitant microbial mass measurement [4, 8, 35].

Nylon Bag Technique

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 mean 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 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 [19, 40].

Tilley and Terry Technique

The technique [49] 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 [26], 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 [49] has been extensively validated with in vivo values [50], 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 feeds rich in plant secondary metabolites, such components are solubilized in both these systems but may or may not contribute to nutrient supply to animals [29, 30].

In Vitro Gas Method

General Description

A number of gas devices have been used to measure the gas volume in in vitro gas methods [20]. In this chapter, use of the in vitro gas method developed by Menke et al. [42] and based on incubation in calibrated syringes, is discussed; since, it is easier to use and a large set of values obtained using this method have been validated with the in vivo work. In the method of Menke et al. [42], 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 metabolisable energy of feeds. Aiple et al. [1] 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.

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 [4, 5, 15]. 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. [20]. The in vitro gas method based on syringes [7, 42] 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, in vitro gas methods can better monitor nutrient-antinutrient and antinutrient-antinutrient interactions [34].

Origin of Gas and Stoichiometry

On incubation of a feedstuff with buffered rumen fluid in vitro, the carbohydrates in the feed are fermented to produce short chain fatty acids, gases and microbial cells. Gas production is 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 2.0–2.8 mL of gas were produced while a similar amount of casein and cellulose produced about 23.4 and 80 mL gas in 24 h.

The gas produced in the gas technique is the direct gas produced as a result of fermentation plus the indirect gas produced from the buffering of short chain fatty acids. For roughages, when bicarbonate buffer is used, about 50% of the total gas is generated from buffering of the short chain fatty acids and the rest is evolved directly from fermentation. At very high molar propionate, the amount of CO_2 generated from buffering of short chain fatty acids is about 60% of total gas production. 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 short chain fatty acids produced is dependent on the type of substrate. Therefore, the molar ratio of acetate to 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 short chain fatty acids will be reflected by changes in gas production.

The gas produced on incubation of cereal straws [4], a wide range of feeds including many dairy compound feeds and their individual feed components whose protein and fat contents vary greatly [11], and tannin containing browses [25] 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 [51] stoichiometry, which is as follows:

$$\text{Fermentative CO2 (mmol)} = A/2 + P/4 + 1.5B \tag{7.1}$$

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

$$\text{Fermentative CH4 (mmol)} = (A + 2B) - CO2 \tag{7.2}$$

where A and B are mmol of acetate and butyrate respectively and CO_2 is mmol of CO_2 calculated from Eq. (7.1). Using these equations one can calculate, the waste products of fermentation: carbon dioxide and methane from acetate, butyrate and propionate produced during the fermentation.

In the in vitro gas method, the "expected gas volume" can also be calculated from acetate, butyrate and propionate produced during the fermentation.

Total volume of gas (mmol), calculated from short chain fatty acids production = (FG + BG)

FG = fermentative gas (mmol) (CO_2 + CH_4); calculated using Eqs. (7.1) and (7.2)
BG = gas volume (mmol) from buffering of short chain fatty acids
CF = correction factor for altitude and pressure

For the determination of BG, one requires the amount of short chain fatty acids (acetate + butyrate + propionate) produced during the fermentation. One mmol of short chain fatty acids releases one mmol of CO_2 from the bicarbonate-based buffer in the incubation medium, and is described as buffering CO_2. Therefore, mmol of buffering CO_2 is equal to mmol of total short chain fatty acids generated during incubation.

The total gas produced (mmol), which is the addition of mmol FG and mmol BG, can be converted to volume (mL) by the following equation:

$$\text{Gas volume at mean sea level} = \text{mmol of gas} \times \text{gas constant (R)} \times T$$

Where R = the ratio between molar volume of gas to temperature (Kelvin zero; K) i.e.

$(22.41\,l/273 = 0.0821)$,T = incubation temperature(Kelvin); $273 + 39^{\circ}C = 312\,K$

So the volume of 1 mmol of gas at 39°C at sea level would be: $1 \times 0.0821 \times 312 = 25.6$ mL.

The volume of gas depends on the altitude of a place, and hence a correction factor is required. If the incubation is conducted at Hohenheim, Germany, which is at an altitude of 400 m, the correction factor is 0.953 [11]. The volume of 1 mmol of gas at 39°C in Hohenheim would therefore be: $1 \times 0.082 \times 312/0.953 = 26.5$ mL.

The total volume of gas as mmol, calculated from mmol of FG and BG, can be converted to volume (mL) by multiplying with 26.5 for a place with an altitude of 400 m and by multiplying with 25.6 for a place at mean sea level. The detailed information on behaviour of gases at different temperature (as temperature increases, the volume increases) and pressure (as altitude increases, pressure decreases) can be obtained from a textbook on physical chemistry. The origin and stoichiometry of gas production, given above, have been described in details in [11] and [20].

The in vitro gas production measured after 24 h of incubation of tannin containing browses in the presence or absence of polyethylene glycol was strongly correlated with the gas volume stoichiometrically calculated from short chain fatty acids. The relationship between short chain fatty acid production (mmol) and gas volume (mL) after 24 h of incubation of browse species with a wide range of crude protein (5.4–27%) and phenolic compounds (1.8–25.3% and 0.2–21.4% total phenols and total tannins as tannic acid equivalent respectively) was [25]:

- In the absence of polyethylene glycol:

$$\text{Short chain fatty acids} = 0.0239 \times \text{Gas} - 0.0601;$$
$$\text{R2} = 0.953; \quad \text{N} = 39; \quad \text{P} < 0.001$$

- In the presence of polyethylene glycol

$$\text{Short chain fatty acids} = 0.0207 \times \text{Gas} + 0.0207;$$
$$\text{R2} = 0.925; \quad \text{N} = 37; \quad \text{P} < 0.001$$

- Overall (pooling the data)

$$\text{Short chain fatty acids} = -0.00425 + 0.0222 \times \text{Gas};$$
$$\text{R2} = 0.94; \quad \text{N} = 76; \quad \text{P} < 0.001$$

These relationships are similar to those obtained for wheat straw [6].

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

A modified in vitro gas method: gas production with concomitant microbial mass measurement

General Description

A simple in vitro approach is described here 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 production in the in vitro rumen fermentation system based on syringes [42]. This method does not require sophisticated equipment or the use of a large number of animals (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.

The method of Menke et al. [42] was modified by Blümmel and Ørskov [4] in that feeds were incubated in a thermostatically controlled water bath instead of a rotor in an incubator. Makkar et al. [35] and Blümmel et al. [8] 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 increased from 30 mL in the method of Menke et al. [42] 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 resulting from short chain fatty acid production; and in the 40 mL system, the linearity is lost when the gas volume exceeds 130 mL [21]. 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 [42] and 130 mL using the 40 mL system [7, 35], 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:

a) 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 organic matter degradability (see below),
b) almost no drop in temperature of the medium during the period of recording gas readings (when compared to the incubation of syringes in an incubator at 39°C as in the original method of Menke et al. [42]). This is useful for studying the kinetics of fermentation where gas volume must be recorded at various time intervals, and
c) drastic drop in the temperature of the incubation is prevented in case of power breakdown for a short duration because of large volume of water in the water bath and its higher temperature holding capacity.

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 (CO_2 and CH_4). In most studies, the rate and extent of gas production have been wrongly considered 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 in vitro gas methods. In vitro gas measurements reflect only short chain fatty acid production. The relationship between short chain fatty acids and microbial mass production is not constant and

the explanation for this resides in the variation of microbial mass production per unit ATP generated. This can impose an inverse relationship between gas volume (or short chain fatty acid production) 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. Blümmel et al. [7, 8] 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.

Partitioning Factor and Efficiency of Microbial Protein Synthesis

The partitioning factor is defined as the ratio of organic matter degraded in vitro (mg) to the volume of gas (mL) produced by it. A feed with higher partitioning factor means that proportionally more of the degraded matter is incorporated into microbial mass, i.e., the efficiency of microbial protein synthesis is higher. Different in vitro partitioning factor values are also reflected by in vivo microbial protein synthesis as estimated by purine derivatives (the higher the partitioning factor, the higher the excretion of urinary purine derivatives [12]), in methane production by ruminants (the higher the partitioning factor, the lower the methane output [12, 15]), and in predicting the dry matter intake (the higher the partitioning factor, higher the dry matter intake [8, 9]). These results show that the partitioning factor 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 organic matter degraded, and the calculation of the stoichiometrical factor; stoichiometrical relationship between short chain fatty acids and gas volume; and relationship between short chain fatty acid production, ATP production and microbial mass yield are given in Blümmel et al. [7] and Getachew et al. [20]. For roughages, partitioning factor values from 2.75 to 4.45 mg/mL approximately reflects ^{Y}ATP from 10 to 32. 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, fat or starch. Rymer and Givens [47] have shown that, as observed by [7], good quality feeds (grass silage, wheat, maize, molasses sugar-beet feed and fishmeal) which produce large amounts of gas and short chain fatty acids 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 high in vitro organic matter degradability but low gas production per unit organic matter degraded.

Microbial mass production in in vitro can be calculated as [7]:

$$\text{Microbial mass (mg)} = \text{mg substrate truly degraded} - (\text{mL gas volume} \times \text{stoichiometrical factor}) \tag{7.3}$$

The above equation becomes:

$$\text{Microbial mass (units)} = \text{gas volume (partitioning factor} - \text{stoichiometrical factor)} \quad (7.4)$$

For roughages, the stoichiometrical factor was 2.20.

The derivatization of these two equations, based on balancing of substrate supply and product formation in the in vitro gas method, is given in [7].

The partitioning factor reflected efficiency of microbial protein synthesis and microbial mass determined using the approach presented here (Eqs. 7.3 and 7.4) was found to be in good agreement with ^{15}N studies [8, 13]. This concept of partitioning factor analysis demands a close stoichiometric relationship between short chain fatty acids and gas production, and a reliable determination of organic matter degradability of the substrate. The partitioning factor based approach for prediction of efficiency of microbial protein synthesis is simpler compared to that suggested by the Cornell net carbohydrate and protein system [13].

The Partitioning Factor as a Reflection of Efficiency of Microbial Protein Synthesis for Tannin-Containing Feeds

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 partitioning factor for tannin-rich feeds (calculated as mg truly degraded substrate needed to produce one-mL gas) ranged from 3.1 to 16.1 [22], which is well above the theoretical partitioning factor range of 2.75–4.41 [7]. The high partitioning factor 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 of quantification of microbial mass redundant [36–38].

For the in vitro evaluation of tannin-rich feeds, the microbial mass should be quantified using diaminopimelic acid or purines as markers, or by ^{15}N incorporation into the microbes [34, 39], and the partitioning factor for tannin-rich feeds can be expressed as the microbial mass determined by these markers per mL of gas produced (or per mmol short chain fatty acids produced).

Using diaminopimelic acid, purines and ^{15}N approaches for measuring microbial mass it has been shown that for tannin-rich feeds the presence of polyethylene glycol (Molecular weight 4000 or 6000) – a tannin-inactivating agent, increased feed degradability and microbial mass production, but decreased the efficiency of

microbial protein synthesis [3, 23, 34, 39]. Similar results have also been obtained by following other approaches based on the gas method in which the rate of ammonia uptake [45] is taken as the efficiency of microbial protein synthesis [39] or microbial protein is determined by the nitrogen balance approach [23]. 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 the extents of "decrease in dry matter degraded" and "higher microbial mass produced per unit of dry matter degraded" in presence of tannins.

Incubation Time and Partitioning Factor

Another study [10], 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 digestibility, into short chain fatty acids, microbial mass yield, and ammonia, carbon dioxide and methane production were examined after 8, 12, 18 and 24 h of incubation in the gas method under both low and adequate nitrogen levels. Microbial synthesis was quantified gravimetrically [7], by nitrogen balance [23] and by purine analysis [31]. The short chain fatty acids 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 short chain fatty acid production were always inversely ($P < 0.05$) related to microbial mass yield regardless of incubation time and medium (low or adequate nitrogen). At later incubation times, continuously more short chain fatty acids 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 short chain fatty acids, gas and 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 [10].

For tannin containing feeds [33], the efficiency of microbial growth was higher for 16 h of incubation than 24 h when these were incubated in presence or absence of polyethylene glycol, a tannin-inactivating agent. Additional nitrogen in the medium also affected the efficiency of microbial protein synthesis from tannin-tannins feeds, both after 16 and 24 h [22]. For proper characterisation of feed and feed ingredients, approaches need to be developed for measuring the partitioning factor for the incubation time at which the lysis of microbes is minimal. Some possible simple approaches worth investigating to identify this incubation time are:

a) the time at which half of the maximum gas is produced (t/2), and
b) 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. Some efforts have been made to measure partitioning factor at t/2, 16 and 24 h for various feed resources and to correlate the values obtained with the measured in vivo efficiency of microbial protein synthesis [13,14].

In this chapter, the method for measuring partitioning factor, a reflection of efficiency of microbial protein synthesis is presented. Based on the available information in the literature, the suggested incubation times are: 16 h for concentrate based feeds/diets and 24 h for roughages. These incubation times would adequately serve the purpose of screening a large number of samples for efficiency of microbial protein synthesis. In addition, an error which could arise in the determination of truly degraded substrate through dissolution of the undegraded substrate in the neutral detergent solution (and thus overestimating truly degraded substrate) is expected to be minimal for most of the feed resources except those rich in starch such as maize grains [13]. Determination of truly degraded substrate required for determination of partitioning factor at incubation times lower than 16 h could substantially overestimate truly degraded substrate and the partitioning factor, leading to incorrect reflection of efficiency of microbial protein synthesis.

Method for Measuring Partitioning Factor

Sample Preparation

Dried sample should be passed through a 1 mm sieve.

Reagents

1. *Bicarbonate buffer solution:* Dissolve 35 g sodium bicarbonate ($NaHCO_3$) and 4 g ammonium carbonate (NH_4HCO_3) in approximately 500 mL distilled water and then make up the volume to 1 L with distilled water.
2. *Macromineral solution:* Dissolve 6.2 g potassium dihydrogen phosphate (KH_2PO_4), 5.7 g disodium hydrogen phosphate (Na_2HPO_4), and 0.6 g magnesium sulphate ($MgSO_4.7H_2O$) in approximately 500 mL distilled water and then make up the volume to 1 L with distilled water.
3. *Micromineral solution:* Dissolve 10 g manganese chloride ($MnCl_2.4H_2O$), 13.2 g calcium chloride ($CaCl_2.2H_2O$), 1 g cobalt chloride ($CoCl_2.6H_2O$), 8 g ferric chloride ($FeCl_3.6H_2O$) in approximately 50 mL distilled water and then make up the volume to 100 mL with distilled water.
4. *Resazurine:* Dissolve 0.1 g resazurine in 100 mL distilled water.
5. *Reducing solution:* Dissolve 996 mg sodium sulphide ($Na_2S.9H_2O$) in 94 mL distilled water and then add 6 mL of 1 N sodium hydroxide solution (dissolve 4 g sodium hydroxide in 100 mL distilled water for 1 N sodium hydroxide).

Weighing of Samples and Preparation of Syringes

Tear a specially made scoop (approximately 4 cm in length and 1 cm in depth/radius; standard sodium hydroxide-containing plastic container can be cut horizontally to half to form the scoop) on an analytical balance. Weigh 500 mg of air-dry feed sample in the scoop and then insert a 5 mL capacity pipette or a glass rod into the narrow end of the scoop and transfer the sample from the scoop into 100-mL calibrated glass syringes. The feedstuffs are incubated at least in triplicate. The blank syringes do not contain feed.

Preparation of In Vitro Rumen Fermentation Buffer Solution and Incubation

Collect the rumen fluid and particulate matter before the morning feed from two cattle, fed a diet of the type similar to that of the samples being analysed in vitro. Mix the contents taken from two cattle, homogenize, strain and filter them through four layers of cheesecloth. Keep all glassware at approximately 39°C and flush these with carbon dioxide before use. Carbon dioxide is heavier than air and hence it remains in the glassware for a reasonable period provided the container is not inverted up side down. The strained rumen fluid is kept at 39°C under carbon dioxide and should be prepared just before start of the incubation. As the amount of feed taken is 500 mg, composition of the medium is according to [49]. Menke et al. [42] reduced the rumen buffer volume per syringe by half as they used 200 mg of the substrate because of the limited volume of the syringes and the inconvenience of emptying the syringes. Here, besides recording the gas volume, we are interested to use the fermented material for various analyses; therefore, the amount of substrate taken is 500 mg [8, 35]. There is an inherent error associated with gravimetric determination of the fermented residue which (error) is large if 200 mg feed is taken in place of 500 mg.

Medium Composition

(According to [35])

Rumen buffer solution (bicarbonate buffer) 630 mL
Macromineral solution 315 mL
Micromineral solution 0.16 mL
Resazurine 1.6 mL
Distilled water 945 mL
Freshly prepared reducing solution 60 mL
The rumen fluid 660 mL
(see above for collection and preparation)

The above volume is sufficient for 60 syringes (40 mL/syringe) plus 10% extra.

Incubation Procedure

Mix, in the order mentioned above, all the above-mentioned solutions, except the reducing solution and rumen fluid, in a 3 or 5 L capacity glass container. The container is kept in a water bath adjusted at 39°C. This water bath is a plastic rectangular container (approximately 400 cm × 300 cm × 200 cm) filled with water, the temperature of which is adjusted at 39°C using a portable thermostat suspended from the top of the plastic container in water. This plastic water bath is kept on a magnetic stirrer. The contents are flushed with carbon dioxide and kept stirred using a magnetic stirrer. After about 5 min, add the reducing solution and keep the mixture stirring and flushing with carbon dioxide at 39°C. When the mixture has been reduced (blue colour of the dye changes to pink and then to colourless; it takes about 15–20 min for the reduction process to complete and during this time we generally homogenized and strained the rumen liquor and the particulate material collected from cattle), add 660 mL of the rumen fluid. Keep this mixture stirring and flushing with carbon dioxide for another 10 min. Transfer a portion (40 mL) of the rumen-fluid medium into each syringe using a dispenser, and incubate in a water bath at 39°C. After some practice, filling 60 syringes should take 30–35 min. After completion of the filling-up process, shake the syringes well and transfer them to the water bath. Shake all the syringes every hour for the first 4 h and then after every 2 h.

Generally, the incubation is started at about 7.30–8.0 a.m. and after 12 h of incubation, the syringes are not shaken until the termination of the incubation (24 h). When 500 mg of the air-dry sample is incubated with 40 mL of the medium containing rumen microbes, invariably the amount of gas in 24 h exceeds the capacity of 100-mL capacity syringes. After 8–10 h of incubation (depending on the fermentability of the feed), the amount of gas produced is registered and the piston is pushed back to 40 or 45 mL mark on the syringe (after pushing back the piston to 40 or 45 mL mark, shake the syringe contents after about 30 min; this will prevent sticking of feed particles on bottom of the piston, which otherwise will not get fermented). At 24 h, again the position of the piston is recorded. The addition of these two sets of values gives the total amount of gas production in 24 h (see an example below). The blanks (at least three in number) contain only the rumen-fluid medium and no feed material. For blanks, there is no need to push back the piston.

An Example

Suppose, at 0 h of incubation the piston was at 41-mL mark. After 8 h of incubation, the piston reached the 86-mL mark and it was pushed back to the 45-mL mark. The following morning after 24 h of incubation the piston was at 67-mL mark. Total gas produced during 24 h = (86 – 41) + (67 – 45) = 77 mL.

The operational aspects of the gas method are available at: http://www.iaea.org/programmes/nafa/d3/mtc/invitro-slideshowapr01.pdf

Notes:

1. *The procedure given here suggests termination of the incubation after 24 h; however, for concentrate-based feeds the incubation should be terminated after 16 h (see Incubation time and partitioning factor). Generally, the incubation is started in the morning (7.30–8.0 a.m.) and termination of the incubation after 24 h is convenient from the practical point of view. On the other hand, for 16 h of incubation (around mid-night), after recording the gas, the syringes can be kept in ice for determination of undegraded organic matter the following day; or better the syringe contents and the syringe washings (see* "Organic matter degradability*" and* "Preparation of apparent undegraded residue"*) could be transferred into a beaker for digestion the following day. For 16 h of digestion, the incubation could also be initiated at around 4 p.m. so that the incubation could be terminated at 8 a.m. the following day; however, variation in the activity of the rumen liquor taken at this hour could be higher than that of the rumen liquor taken before the morning feed. When methane proportion is to be measured in the gas (see Determination of other parameters), the syringes should not be kept in ice, since the solubility of methane and carbon dioxide is different at different temperature (solubility of carbon dioxide in water/buffered medium is higher than methane).*
2. *When the objective is to evaluate the effect of an additive on partitioning factor of a feed, a set of three syringes containing the feed, the additive and 40 mL of the incubation medium form a test set, and the corresponding blank contains a set of three syringes with the additive and 40 mL of the incubation medium (and no feed).*
3. *The 40 mL of the incubation medium consists of 10 mL each of rumen contents and bicarbonate buffer, 5 mL of macro-mineral solution (0.002 mL of which is micro-mineral solution) and 15 mL of distilled water. The nitrogen content in a syringe, delivered from the buffer is approximately 7 mg.*

Net Gas Production

The gas volume is recorded after 24 h in test and blank syringes for roughages and after 16 h for concentrate-based feeds. The net gas production is calculated by subtracting values of the blank from that of the test syringe. Let this net gas value be *x* mL.

Organic Matter Degradability

The syringe contents are digested with neural detergent solution to dissolve the microbes, leaving the undegraded residue. This residue is ashed to obtain undegraded organic matter (undegraded residue *minus* ash).

Reagents

Neutral detergent solution: Mix the Reagents 1 and 2, and dilute to 4 L with distilled water. Check the pH. It should be between 6.9 and 7.1.

Reagent 1: Weigh 74.4 g EDTA (disodium ethylenediamine tetraacetate dehydrate) and 27.2 g sodium teraborate ($Na_2B_4O_7.10.H_2O$) and place together in a large beaker, add distilled water (approximately 2 L) and dissolve with gentle heating, add to this solution 120 g sodium lauryl sulphate (also called as sodium dodecyl sulphate) and 40 mL of 2-ethoxyethanol (ethylene glycol -monoethylether) and mix.

Reagent 2: Weigh 18.24 g disodium hydrogen phosphate (anhydrous), add approximately 1 L of distilled water and dissolve with gentle heating.

Procedure

After measuring the gas volume after 24 h (or after 16 h for concentrate-based feeds), transfer the contents of the syringe quantitatively in a beaker (in practice, after emptying the syringe contents in a beaker, we rinse the syringe twice, each time with approximately 20 mL of double strength neutral detergent solution; each time the syringe is shaken to remove residual feed particles) and digest it with the neutral detergent solution for 1 h using the heating and refluxing unit generally used for estimation of neutral detergent fibre in feeds. The purpose of this treatment is to solubilize the microbes from the syringe contents and obtain only the undegraded feed. Filter the contents of the beaker through a crucible (porosity 2) and wash the residue on the crucible with hot water till the residue is free of the detergent. Dry the crucibles at 130°C for 2 h or at 100°C 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 undegraded feed in that particular syringe.

Note: This method of measuring undegraded residue does not work satisfactorily for tannin-containing feeds/samples (presence of tannin-protein complexes as artefacts in the residue) and for 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 undegraded residue). It may be noted that this method of measurement of undegraded 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 undegraded by microbes could be solubilized in the neutral detergent solution.

Let the weight of this residue (undegraded feed) be *a* mg. Now transfer the crucibles containing this residue to a Muffle furnace (550°C) 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* mg). Subtract *b* from *a* to obtain undegraded organic matter *(a–b)* in mg.

For determination of organic matter degraded in 24 h, one needs *(a–b)* value and organic matter in 500 mg of the air-dried sample incubated in the syringe.

Organic matter weighed into the syringe
= [500 × Feed DM content in percentage/100] minus [500 × (Feed DM content in percentage/100) × (Feed Ash content in percentage/100)]

or (500 × Feed DM content in percentage/100) × (1 – Feed Ash content in percentage/100)

Let this value be *c* in mg (ash content of the feed could be determined by burning in a Muffle furnace, 550°C)

$$\text{Organic matter degraded (mg)} = c - (a - b)$$

Determination of Partitioning Factor

For determination of partitioning factor, organic matter degraded and net gas values are required

$$\text{Partitioning factor} = (\text{mg organic matter degraded})/\text{mL gas}$$

or

Partitioning factor = *c – (a–b)* divided by mL net gas (*x*) produced in the syringe for which *c – (a–b)* has been calculated. Higher is this factor, higher the efficiency of microbial protein synthesis.

An Example

Scenario 1. Screening feeds for efficiency of microbial protein synthesis

Two samples (500 mg each) of Feed A and Feed B containing 95% and 93% dry matter (DM) and 5% and 4% ash respectively were incubated for 24 h in the in vitro gas method.

Organic matter weighed into the syringes, *c*:
Feed A = (500 × 95/100) (1 – 5/100) = 451 mg
Feed B = (500 × 93/100) (1 – 4/100) = 446 mg
Gas production at 24 h for blank syringes: 5 mL (average of three syringes)
Gas production at 24 h for Feed A (syringe 1): 115 mL
Gas production at 24 h for Feed B (syringe 1): 105 mL
Net gas production, *x*
Feed A = 115 – 5 = 110 mL
Feed B = 105 – 5 = 100 mL
Undegraded organic matter, *(a–b)*
Feed A (syringe 1) = 144 mg
Feed B (syringe 1) = 164 mg
Organic matter degraded (mg) = *c – (a–b)*
Feed A = 451 – 144 = 307 mg
Feed B = 446 – 164 = 282 mg
Partitioning Factor
Feed A = 307/110 = 2.79
Feed B = 282/100 = 2.82

On one day, three syringes are incubated for each feed, so one has three values of partitioning factor for each Feed A and Feed B.

Conclusion: Efficiency of microbial protein synthesis is higher for Feed B than Feed A. It may be noted that in this example, both the gas production and organic matter degradability are higher in Feed A. Therefore to, the efficiency of microbial protein synthesis may not follow the same pattern as the organic matter degradability or the net gas production. These results should not be looked in isolation. Ideally, a feed with higher organic matter degradability and higher partitioning factor should be preferred when the objective is to use the feed for increasing livestock productivity. On the other hand, in situations such as extreme cold where the aim is to keep the body generating heat and protect the animal from dying, or for draught animals where the energy requirement is high, a feed with higher organic matter degradability and lower partitioning factor could be preferred, since for this feed a higher proportion of the substrate would be partitioned to short chain fatty acids (main energy source for ruminants) and lower to microbial mass. In addition, in feeding situations below the maintenance requirements, high partitioning factor values might not be beneficial.

Some Data from the Literature

Values for partitioning factor and related parameters are presented in Table 7.1, showing the importance of measuring partitioning factor in an in vitro gas method. Sodium hydroxide and ammonia treatments of straws not only increased the true substrate degradability and gas production but also the efficiency of microbial protein synthesis. The efficiency of microbial production (as reflected by partitioning factor) was inversely related to methane production/kg digestible organic matter in vivo [15]. The reader is suggested to refer to [13] for partitioning factor of another set of roughages and mixed diets and the importance of partitioning factor values in predicting efficiency of microbial protein synthesis in vivo.

Table 7.1 In vitro gas production (GP), truly degraded substrate (TDS) and partitioning factor (PF) of untreated and NaOH- and ammonia-treated wheat straw and oat after 24 h of incubation (Partitioning factor derived from data of [15])

	TDS (mg)	GP (mL)	PF
Winter wheat variety Pastiche, untreated	240	98	2.45
Winter wheat variety Pastiche, NaOH-treated	307	110	2.79
Winter wheat variety Pastiche, NH_3-treated	280	103	2.72
Oat, untreated	235	91	2.58
Oat, NaOH-treated	298	108	2.76
Oat, NH_3-treated	279	102	2.74

TDS is not truly degraded organic matter since it has not been corrected for the ash content. This approach of calculating partitioning factor based on TDS could be used for comparative assessment of feeds (or of treatments on a feed) provided the ash content is similar in the samples tested. In the present case the presence of NaOH in the incubated material would lead to an overestimation of TDS and hence of partitioning factor.

Table 7.2 In vitro gas production (GP), truly degraded substrate (TDS) and partitioning factor (PF) of hybrid and local varieties of maize stover leaves (data from [12])

	TDS (mg)	GP (mL)	PF
Hybrid maize stover leaves	328.7	107.2	3.1
Local maize stover leaves	327.3	114.9	3.3.

Table 7.2 is from the data of [12]. It gives fermentation parameters, including partitioning factor of hybrid and local varieties of maize stover leaves. True substrate degradability of the two varieties at 24 h is the same; however, the gas production for the local varieties is lower, suggesting higher microbial mass production for this variety. The partitioning factor of local varieties was higher. This was reflected in vivo when microbial protein synthesis was estimated [17, 32] by urinary excretion of purine derivatives. Higher in vivo efficiency of microbial protein synthesis and higher microbial protein supply to the animal was recorded for local varieties.

Scenario 2. Study of the effect of an additive (water/buffer soluble) on efficiency of microbial protein synthesis

A sample (500 mg) of a feed containing 95% dry matter and 5% ash was incubated for 24 h in the in vitro gas method, without and with the additive (in three syringes each, with corresponding blanks)

Organic matter weighed into the syringes, *c*:
Feed = (500 × 95/100) (1 – 5/100) = 451 mg
Gas production after 24 h in blank syringes (without additive): 5 mL (average of three syringes)
Gas production after 24 h in blank syringes (with additive): 7 mL (average of three syringes)
Gas production after 24 h for feed, without additive (syringe 1): 95 mL
Gas production after 24 h for feed, with additive (syringe 1): 102 mL
Net gas production, *x*
Feed, without additive = 95 – 5 = 90 mL
Feed, with additive = 102 – 7 = 95 mL
Undegraded organic matter, *(a–b)*
Feed, without additive (syringe 1) = 140 mg
Feed, with additive (syringe 1) = 135 mg
Organic matter degraded (mg) = *c – (a–b)*
Feed, without additive = 451 – 140 = 311 mg
Feed, with additive = 451 – 135 = 316 mg
Partitioning Factor
Feed, without additive = 311/90 = 3.45
Feed, with additive = 316/95 = 3.32

On one day, three syringes are incubated for each feed, so one has three values of partitioning factor each for Feed, without additive and Feed, with additive.

Conclusion: The additive has increased gas production and true degradability but decreased the efficiency of microbial protein synthesis. Some relevant information from the literature is given in Table 7.2.

Note: The theoretical range for partitioning factor is 2.74–4.41. Any value above or below this range should be critically evaluated. For tannin-rich samples, the partitioning factor is normally above 4.41. The higher partitioning factor (for example 7.2) would mean that 7.2 mg of the truly degraded organic matter produce one mL of the gas. The reasons for the high values for tannin-rich feeds are given in the section above "The partitioning factor as a reflection of efficiency of microbial protein synthesis for tannin-containing feeds". For tannin-containing feeds, the syringe contents are not digested after incubation with the neutral detergent solution to determine truly undegraded organic matter. Instead, the syringe contents are taken for the determination of purines and/or ^{15}N incorporation studies (see section below "Methods for measurement of microbial mass, microbial-nitrogen, microbial purines and ^{15}N incorporation in microbes and determination of efficiency of microbial protein synthesis").

Determination of Other Parameters

From the above analyses, the following additional information can also be obtained:

i) Organic matter degradability (%) = $(c - (a–b))\ 100/c$
In the above two examples, organic matter degradability for:
Scenario 1

$$\text{Feed A} = (307) \times 100/451 = 68.1\%$$
$$\text{Feed B} = (282) \times 100/446 = 63.2\%$$

Scenario 2

$$\text{Feed, without additive} = (311) \times 100/451 = 69\%$$
$$\text{Feed, with additive} = (316) \times 100/451 = 70.1\%$$

ii) Neutral detergent fibre (NDF) degradability (or fibre degradability)
NDF degradability (fibre degradability) (%) = [{500 × (Feed DM content in percentage/100) × (Feed NDF content in percentage/100)} – {*(a–b)*}] 100/{500 × (Feed DM content in percentage/100) × (Feed NDF content in percentage/100)}
(Neutral detergent fibre content of the feed could be determined by cooking the sample for 1 h in neutral detergent solution, followed by filtration through a crucible of porosity 2)
Scenario 1
Let, neutral detergent fibre values for Feed A and Feed B are 50 and 55% respectively.
NDF degradability (fibre degradability) (%)

$$\text{Feed A} = (\{500 \times (95/100) \times (50/100)\} - \{135\}) \times 100/\{500 \times (95/100) \times (50/100)\}$$
$$= (237.5 - 135) \times 100/237.5 = 43.2$$

$$\text{Feed B} = (\{500 \times (93/100) \times (55/100)\} - \{142\}) \times 100/\{500 \times (93/100) \times (50/100)\}$$
$$= (255.8 - 142) \times 100/255.8 = 44.5$$

Scenario 2

Similarly, the effect of addition of additive on neutral detergent fibre degradability (fibre degradability) could be determined, once the neutral detergent fibre content of the feed incubated in the syringes is known.

iii) Estimated microbial mass

For most conventional feed resources (not the tannin-containing feeds or starch-rich feed ingredients), microbial mass production can be estimated at the time of termination of the incubation, using Eq. (7.3).

mg microbial mass production = ({*c – (a–b)*} – (net gas in mL x 2.2)); *c – (a–b)* is in mg and 2.2 is the stoichiometric factor

For the above example of Feeds A and B:

$$\text{mg microbial mass production for Feed A} = 307 - 110 \times 2.2 = 307 - 242 = 65\,\text{mg}$$
$$\text{mg microbial mass production for Feed B} = 282 - 100 \times 2.2 = 282 - 220 = 62\,\text{mg}$$

iv) Efficiency of microbial mass production = (({*c – (a–b)*} – (2.2 × net gas in mL)) × 100)/{*c – (a–b)*}

These values for Feed A and Feed B are: 65 × 100/307, and 62 × 100/282, or 21.2 and 22% respectively. The order for the efficiency of microbial protein synthesis calculated by this method is the same as obtained with partitioning factor (partitioning factor for Feed B is higher than Feed A).

Note: Based on the Cornell Net Carbohydrate and Protein System, a maximum incorporation of feed carbohydrate into microbial mass of 50% and 40% in the absence and presence of protozoa respectively has been suggested [46 *].*

v) Methane production

In our laboratory, we measure methane content of the gas in the syringe using an infrared-based methane analyser (0–30% range methane analyser from Pronova Analysentechnik GmbH & Co KG, Berlin, Germany). The methane analyser is calibrated against 10.6% or 12% standard methane. A gas chromatograph can also be used for methane measurement; however, the use of the infrared-based methane analyser is simple, convenient and takes less time and resources.

After measuring the total gas volume, the tubing of the syringe outlet is inserted into the inlet of the methane analyser; the piston is pushed to insert the accumulated gas into the analyser. The methane as percent of the gas is displayed on the methane analyser. This value is used for calculation of methane in the total gas volume. When the feed incubated is 500 mg, the volumes of total gas and methane are measured while pushing back the piston after 8 or 10 h of incubation, and similarly volumes of gas and methane are measured after 24 h. These two values are added to obtain the total methane production in 24 h of

incubation. Generally, we have observed that percent methane level in a syringe is lower for the period 0–8 or 10 h of incubation than for the period 8 or 10–24 h of incubation. If pushback of the piston is to be avoided, 200 mg of the sample can be incubated and the volumes of gas and methane can be measured after 24 h of incubation. These volumes could be multiplied by 2.5 to obtain the volumes corresponding to 500 mg of the sample. However, for determination of organic matter degraded in 24 h (required for expressing methane production based on per unit of organic matter degraded), incubation of 500 mg of air-dried sample is suggested.

The results on methane production can be expressed as:

1. Methane as percent of the total gas (on volume basis)
2. Methane (mL) produced/100 mg of organic matter degraded
   (Methane in the corresponding blank should be subtracted from that in the test syringe).

The in vitro gas method is a useful tool for screening various plants/plant extracts/plant compounds or any other additive having potential to reduce methane emission from ruminants. Lower is the methane produced per unit organic matter degraded, better the feed. In addition, effect of various supplementation strategies could also be evaluated for reducing methane production, enhancing efficiency of microbial protein synthesis or for achieving higher fibre degradability.

If the facilities for methane measurement do not exist, methane production can be calculated from the net short chain fatty acid production (after subtracting short chain fatty acids in the blank), using stoichiometric relationships. Using a gas chromatograph, short chain fatty acids are measured in the supernatant of the fermentation medium after 24 h (see section "Preparation of apparent undegraded residue"). High correlations between stoichiometrically calculated gas and actually observed values have been observed by many workers [20]. This forms the basis for the determination of carbon dioxide and methane from the amount and molar proportion of short chain fatty acids.

For example, for Feed 1 (roughage), net production of short chain fatty acids in 24 h is 1.2 mmol with a molar proportion of: 0.75 acetate, 0.19 propionate and 0.06 butyrate.

Using Eq. (7.1), fermentative CO_2 from 1 mmol short chain fatty acids for Feed 1$= 0.75/2 + 0.19/4 + 1.5 \times 0.06 = 0.5125$ mmol.

Using Eq. (7.2), fermentative CH_4 from 1 mmol short chain fatty acids for Feed 1 $= (0.75 + 2 \times 0.06) - 0.5125 = 0.3575$ mmol.

Total fermentative CH_4 from 1.2 mol short chain fatty acids for Feed 1 $= 0.3575 \times 1.2 = 0.429$ mmol.

Volume of methane = mmol methane × gas constant × temperature in Kelvin/atmospheric pressure.
$= 0.429 \times 0.0821 \times 312$(atmospheric pressure taken as 1 at mean sea level)
$= 10.99$ mL

If the experiment has been conducted at, for example, Hohenheim, Stuttgart which has an altitude of 400 metres, the p = 0.953, the volume of methane in the syringe would be 10.99/0.953 = 11.53 mL.

Let us assume that in in vitro the organic matter degraded in 24 h for Feed 1 is = 250 mg.

Estimated methane production (mL)/g organic matter degraded = (11.53/250) × 1000 = 46.12 mL

From the in vitro gas system, estimated methane production (mL)/kg organic matter degraded = 46.12 L.

If intake of the Feed 1 is known, using the values obtained from the in vitro gas method, methane excretion in vivo can be estimated [15].

If the organic matter intake (dry matter intake *minus* ash) of Feed 1 was 946 g/day in a sheep. Using the organic matter degradability value obtained after 24 h in the in vitro gas method (see section "Organic matter degradability"), the expected digestible organic matter in vivo could be calculated. Let us assume that the organic matter degradability (as directed in "Organic matter degradability") is 55%.

Expected digestible organic matter in vivo = 946 × 0.55 = 520 g.
Estimated methane production/day in vivo = (520 × 46.12)/1000 = 23.98 L.

From the amount of short chain fatty acids and their molar proportions, other parameters such as ATP produced, substrate required for microbial mass, total substrate required for formation of products (short chain fatty acids, microbial mass and fermentative gases), and partitioning factor can be calculated. The procedure for their calculation at ^{Y}ATP of 10 and 20 is given in Table 1 and Figure 1 of Getachew et al. [20].

Note: For Feed 2 (for example concentrate based), net production of short chain fatty acids in 24 h is 1 mmol with molar proportion: 0.46 acetate, 0.465 mmol propionate and 0.075 butyrate. For this feed, methane production would be only 3.4 mL at 1 atmospheric pressure. It may be noted that for the same total short chain fatty acid production of 1 mmol for Feed 1 and Feed 2, methane production could differ substantially, depending on the molar proportion of short chain fatty acids. Therefore, besides intake and digestibility, the nature of fermentation product formed from the digested feed determines the proportion of methane production. In the rumen, partitioning of the digested feed into microbial mass and short chain fatty acids, and within short chain fatty acids, the molar proportion of short chain fatty acids determines methane production.

Methods for Measurement of Microbial Mass, Microbial-Nitrogen, Microbial Purines and ^{15}N Incorporation in Microbes and Determination of Efficiency of Microbial Protein Synthesis

This section deals with measurement of efficiency of microbial protein determination for tannin-containing feeds, since the concept of determination of partitioning factor based on the determination of organic matter degraded does not hold true for

such feeds rich in tannins, saponins or in any other plant secondary compounds. However, the approaches listed below could be used for any feed resource.

The ratio of mg Microbial-N to mL net gas production in 24 h of incubation, as an index of efficiency of microbial protein synthesis

Microbial-N (MN) could also be measured after incubation by following two nitrogen balance approaches [23, 30]. The first approach is:

$$\text{MN} = \text{TN} - (\text{NDF-N} + \text{Ammonia-N}_{24\,\text{h}}) \tag{7.5}$$

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 neutral detergent fibre fraction following incubation (24 h) and Ammonia-N is the ammonia-N in the supernatant following the incubation (24 h). 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 TN at time 0, one required nitrogen content of the feed on dry matter basis. For a syringe containing 500 mg sample of 95% dry matter and nitrogen content of 4% on dry matter basis, the feed-N at time 0 will be: $(500 \times 0.95) \times 4/100 = 19$ mg. To this value, N in buffered rumen fluid in the syringe before incubation needs to be added. For determination of N in the buffered medium, we collect approximately 50 mL of the buffered medium while the syringes are being filled at time 0. It is centrifuged at approximately 20,000*g* for 20 min at 4°C to obtain the supernatant which is free of microbes. The supernatant is kept frozen until analysis for ammonia-N. The supernatant is thawed and an aliquot (10 mL) of this medium is taken in a Kjeldahl flask and to it is added 3 mL of 1 N sodium hydroxide and immediately steam distilled to liberate ammonia. The ammonia is absorbed in 2% boric acids solution and titrated with 0.1 N sulphuric acid. The volume of sulphuric acid (mL) used is converted to mg ammonia-N by multiplying with 1.4. This analysis in triplicate and average of the three values gives mg ammonia-N in 10 mL of the medium. This is multiplied by 4 to obtain mg ammonia-N in 40 mL syringe contents. The total-N is the sum of this ammonia-N and the feed-N incubated in the syringe.

Ammonia-$N_{24\,h}$ is calculated by centrifuging (20,000*g* for 20 min at 4°C) the contents of the syringe after 24 h of incubation and determination of ammonia-N as described for time 0.

For determination of neutral detergent fibre fraction for NDF-N after incubation, the syringe contents at 24 h of incubation (after recording the gas volume) are transferred into a 600 mL beaker and the syringes are washed twice with a total of 50 mL neutral detergent solution and emptied into the beaker. The contents are refluxed for 1 h, and then filtered through pre-tarred filter crucibles (porosity 2). The crucibles are dried overnight at 100°C and weighed. The residue after neutral detergent solution treatment (neutral detergent residue, NDF) on the crucibles is subjected to micro-Kjeldahl digestion for determination of nitrogen, NDF-N.

Equation (7.5) is used for determination of microbial-N.

The second approach for determination of microbial-N (MN) is:

$$\text{MN} = \text{APUR-N} - \text{NDF-N},$$

where APUR-N is N bound to apparent undegraded residue after incubation. The preparation of apparent undegraded residue is given in section "Preparation of apparent undegraded residue"). N bound to this fraction is determined in a manner similar to NDF-N determination, using the micro-Kjeldahl method.

The ratio of microbial-N (measured by any of the two methods) to the net gas produced in a syringe is a reflection of efficiency of microbial protein synthesis.

The ratio of microbial-N to net short chain fatty acids produced in 24 h is also a reflection of efficiency of microbial protein synthesis (using a gas chromatograph, short chain fatty acids are measured in the supernatant of the fermentation medium after 24 h; see section "Preparation of apparent undegraded residue").

On incubation of six tannin-rich browses in the presence or absence of polyethylene glycol, a strong correlation ($R^2 = 0.98$) occurred between the two N balance methods. In addition, the pattern observed using these methods was similar to that observed with purines [23].

Another approach for determination of microbial mass is the difference between the apparent undegraded residue after incubation and truly undegraded residue [8, 12]. The former contains undegraded feed and microbes and the latter only the undegraded feed since the microbes have been digested by the neutral detergent solution. Mathematically this could be described as:

$$\begin{aligned}\text{Microbial mass (mg)} = {} & ((\text{mg apparent undegraded residue of the test} - \text{mg apparent} \\ & \text{undegraded residue of the blank at 0 h}) - \text{mg truly} \\ & \text{undegraded residue}) = [(\text{y} - \text{y'}) - \text{a}]\end{aligned}$$

For determination of *y* and *a* see sections "Preparation of apparent undegraded residue" and "Organic matter degradability" respectively. The determination of *y'* is similar to that of *y*, except that 40 mL aliquot of the buffered medium containing rumen liquor which is added into the blank syringes at 0 h of incubation is centrifuged (20,000*g*, 20 min, 4°C) and the pellet washed, centrifuged and lyophilized, as for the 24 h sample (see "Preparation of apparent undegraded residue").

The ratio of the microbial mass to the net gas (or short chain fatty acids) produced in a syringe or $((y - y') - a) \times 100/c - (a–b)$ is the efficiency of microbial protein synthesis, as percent of organic matter degraded.

Since the values for apparent undegraded residue and truly undegraded residue are distorted by the presence of tannins [29, 30], the approach based on the difference between the apparent undegraded residue after incubation and the truly undegraded residue should not be used for tannin-rich feeds [36–38].

Determination of Microbial Protein Production and Efficiency of Microbial Protein Synthesis Using Purines as a Marker

This method involves the determination of purines in apparent undegraded residue left after fermentation (According to [31]).

Preparation of Apparent Undegraded Residue

After 24 h of fermentation, centrifuge (20,000*g*, 20 min, 4°C) the contents of the syringe (volume 40 mL) and discard the supernatant (if one has to measure short chain fatty acids and ammonia-N, retain this supernatant; generally we freeze it at –20°C till analysis). Wash the syringe three times with distilled water, by dispensing each time 15 mL through the spike into the syringe, shaking it to remove residual particles and transferring the contents to the centrifuge tube. After completing rinsing of the syringe, repeat the centrifugation (20,000*g*, 20 min, 4°C) and discard the supernatant. Wash the pellet with distilled water (see *Notes* below) followed by centrifugation (20,000*g*, 20 min, 4°C). Lyophilize the pellet, which consists of undegraded feed and microbial mass. If a lyophilizer is not available, dry the pellet in a vacuum over at approximately 50°C. The lyophilized residue is the apparent undegraded residue. The weight of this residue should be determined: (weight of centrifuge tube plus the residue) *minus* weight of empty centrifuge tube). This weight (let it be *y*) needs to be taken into account in the calculations at a later stage. Use a representative sub-sample of this preparation for analyses. In order to obtain a representative sub-sample, it is advised to grind the entire residue in a pestle and mortar or in a small rotating-ball grinding mill.

Notes:

1. *Saline solution instead of water has also been used for washing the pellet. In such a situation the pellet after drying contains sodium chloride, which will distort the value for apparently undegraded residue.*
2. *In our laboratory, short chain fatty acid determination is done using the procedure described in [27]. In brief, 1.8 mL sample and 0.2 mL formic acid containing internal standard (1 mL 2-methylvaleric acid dissolved in 99 mL formic acid) are incubated overnight at 4°C. The samples are centrifuged at approximately 20,000 g for 10 min at 4°C and about 1 mL of the supernatant is pipetted into 2 mL gas chromatograph vials. Short chain fatty acids are determined with a gas chromatograph (GC-14A, Shimazu Corporation) fitted with a flame ionization detector. Separation is carried out with Chromosorb WAW (100/200 mesh) containing a stainless column packed with GP 10% SP 1000 1% H_3PO_4. The analytical conditions are: N_2 60 mL/min, injection temperature 170°C, detection temperature 220°C, oven temperature with temperature program of 130–165°C with 2°C/min.*

Preparation of Lyophilized Rumen Microbial Fraction

Two hour after the morning feed, collect about 1 L rumen liquor from a cow fed a diet of the kind being analysed in the in vitro gas method. Pass the liquor through two layers of muslin cloth and then keep at 4°C for 30 min in a carbon dioxide-flushed cylinder of 1 L capacity. Separate the rumen fluid devoid of heavy and light particles by pipetting the liquor from the cylinder between the heavy particles that settle down and the light particles, which float on the top. This method for collection of rumen fluid is adapted from [52]. Centrifuge (20,000*g,* 20 min, 4°C) several portions (each 30–35 mL) of this liquor. Wash the pellets with distilled water followed by centrifugation (20,000*g,* 20 min, 4°C). Repeat this washing step two more times, lyophilize the pellets and pool them. Use a sub-sample of one preparation. The nitrogen content of this fraction should be approximately 7.7% [43]. In the lyophilized rumen, microbial fraction prepared in our laboratory in the manner as described above, the nitrogen content was 7.7–8%.

Spectrophotometric Method for Determination of Purines (Marker for Microbial Mass/Protein) ([53], With Some Modification as Described in [13])

Reagents

1. *Ammonium dihydrogen phosphate (0.2 M):* Dissolve 23 g ammonium dihydrogen phosphate in about 700 mL distilled water and then make up the volume to 1 L with distilled water.
2. *Sodium hydroxide (10 M):* Dissolve 40 g NaOH in approximately 70 mL distilled water and then make up the volume to 100 mL with distilled water.
3. *$AgNO_3$ (0.4 M):* Dissolve 1.6987g $AgNO_3$ in approximately 15mL distilled water and then make up the volume to 25 mL with distilled water. Protect the solution from light. Store in a brown bottle and surround the bottle with black paper.
4. *HCl (0.5 M):* Dilute 10 mL HCl (37%) to 240 mL with distilled water.
5. *Ammonium dihydrogen phosphate (0.2 M):* Dissolve 23 g ammonium dihydrogen phosphate in approximately 700 mL of distilled water and then make up the volume to 1 L with distilled water.
6. *Ammonium dihydrogen phosphate (28.5 mM):* Measure 100 mL of the above 0.2 M solution of ammonium dihydrogen phosphate and make up to 700 mL with distilled water.

Procedure

Weigh (25–75 mg) of the apparent undegraded residue (see above) or the lyophilized microbial fraction (see above) in 25 mL screw-cap tubes and add 2.5 mL of 0.6 M

perchloric acid (commercially available 70% phosphoric acid is 12 N). Incubate the mixture in a water bath at 90–95°C for 1 h. After cooling, add 7.5 mL of 28.5 mM ammonium dihydrogen phosphate and return the tubes to a water bath (90–95°C) for 15 min. After cooling, centrifuged (3000*g*, 10 min) the contents and collect the supernatant. Add an aliquot (0.25 mL) of the supernatant to 4.5 mL of 0.2 M ammonium dihydrogen phosphate and adjust the pH between 2 and 3 (generally to 2.5) using 10 M NaOH. After the pH adjustment, add 0.25 mL of $AgNO_3$ (0.4 M) and keep the mixture overnight at 5°C in the dark. Centrifuge (3000*g*, 10 min) the contents and discard the supernatant. Take care not to disturb the pellet. Wash the pellet with 4.5 mL distilled water adjusted to pH 2 (with sulphuric acid) followed by centrifugation. Suspend the pellet in 5 mL of 0.5 M HCl, vortex thoroughly and transfer to the 90–95°C water bath for 30 min after covering the tubes with marbles. Centrifuge (3000*g*, 10 min) the tubes and record absorbance of the supernatant at 260 nm against 0.5 M HCl. For studies with RNA in the range of 25–75 mg (instead of lyophilized microbial preparation or the apparent undegraded residue), read the absorbance at 260 nm after 1:10 dilution of the supernatant. Without adjustment of the pH (which is generally 3.4) to between 2 and 3 before addition of the $AgNO_3$ solution, the recovery of purine basis from yeast RNA (Sigma) is generally lower (80–90% vs. 94–99%), suggesting the importance of the pH-adjustment step in obtaining satisfactory recoveries. Addition of the $AgNO_3$ solution does not change the pH of the solution. Use o-phosphoric acid for adjustment of pH to 2.7.

Express results either based on RNA or lyophilized microbial preparation.

The value for microbial protein (as RNA equivalent or directly as adenine *plus* guanine content) or microbial mass (as lyophilized microbial preparation) obtained from an amount (say 25 mg of the apparent undegraded residue) is used for calculating purine basis in the total amount of the apparent undegraded residue (in *y*, see section "Preparation of apparent undegraded residue") obtained from the syringe (purine bases after 24 h in the syringe).

Similarly, microbial mass is calculated in the total apparent residue obtained by centrifuging (20,000*g*, 20 min, 4°C) 40 mL of the 0 h medium containing rumen liquor (used for filling the syringes for initiating the fermentation), washing with distilled water, followed by re-centrifugation (20,000*g*, 20 min, 4°C) and lyophilisation (purine bases at 0 h in the syringe).

Microbial protein produced in 24 h (as purine basis) = (purine bases in the apparently undegraded residue after 24 h – purine bases in the apparent residue at 0 h). It is assumed that microbial lysis is negligible during this period.

Microbial protein as RNA equivalent or (or microbial mass) produced after 24 h = (microbial protein (or microbial mass) in the apparently undegraded residue after 24 h – microbial protein (or microbial mass) in the apparent residue at 0 h). It is assumed that microbial lysis is negligible during this period.

Microbial protein as RNA equivalent (or microbial mass) produced after 24 *divided by* net gas produced (or short chain fatty acids produced) in 24 h is a measure of efficiency of microbial protein synthesis.

Some Data on Purines [31]

Purine determination (as A260 nm) using spectrophotometric method

	Absorbance at 260 nm	
	Mean	S.D.
25 mg LRM	0.215	0.006
50 mg LRM	0.456	0.005
75 mg LRM	0.675	0.005
25 mg RNA*	0.210	–
50 mg RNA*	0.414	–

LRM, lyophilized rumen microbes
A260 nm = (0.009207) mg LRM - 0.01178 ($r^2 = 0.99$; n = 3)
*Average of two values and after 1:10 dilution

High Performance Liquid Chromatograph (HPLC) Method for Determination of Purine Bases (Adenine and Guanine) ([2], with some modifications as described in [31])

Equipment, reagents, HPLC conditions and analysis: The HPLC equipment that we use consists of a Merck Hitachi L-7100 HPLC pump, an L-7450 photo diode array detector, an L-7200 autosampler, a D-700 interphase module and an LC organiser.

Analytical column: Reverse phase C18 (LiChrospher 100, endcapped 5 μm) 250 mm × 4 mm I.D. (Lichrocart; Merck, Darmstadt, Germany) protected by a guard column containing the material as in the main column.

HPLC solvent A: 10 mM $NH_4H_2PO_4$ and adjust pH to 6 with 10% NH_4OH.

(Dissolve 11.503 g $NH_4H_2PO_4$ in about 500 mL distilled water and then make the volume to 1 L with distilled water. It is 100 mM solution. Pipette 100 mL of this solution and dilute to 1 L to obtain 10 mM solution)

HPLC solvent B: Add 150 mL of acetonitrile to 600 mL of 12.5 mM $NH_4H_2PO_4$ (75 mL of 100 mM solution plus 525 mL of distilled water) and adjust pH to 6 with 10% NH_4OH.

(Filter solvents A and B through a 0.45 μm filter and degas by ultrasonication and by application of vacuum).

Purine bases and internal standard solution for converting integration units to the concentration: Prepare 1 mM stock solution. Put a measuring flask (250 mL capacity) containing approximately 50 mL distilled water on a magnetic stirrer fixed with a hot plate. Add to the flask 100 μl of 10 M sodium hydroxide solution. Heat at about 90°C and then transfer 33.77, 37.77 and 34.03 mg of adenine and guanine and allopurinol respectively to the flask. Wait (generally 30 min) until the contents dissolve. Cool the contents and make up the

volume to 250 mL with distilled water. This solution can be stored at 4°C for 10 days. Dilute this stock solution 12.5 times; pipette 2 mL of the stock solution into 25 mL measuring flask and make up the volume to 25 mL with buffer A of the HPLC. Inject 40 μl of this solution into the HPLC.

Preparation of 3 mM allopurinol solution: Take 100 mL measuring flask and weigh-in 40.83 mg allopurinol. Add approximately 50 mL of distilled water and 20 μl of 12 N (70%) perchloric acid. Heat the contents to approximately 90°C with stirring on a magnetic stirrer. Cool the contents to room temperature and make up the volume to 100 mL with distilled water.

Preparation of 8 mM caffeine solution: Dissolve 155.36 mg caffeine in 80 mL of distilled water and then make up the volume to 100 mL with distilled water.

Gradient: A 30-min linear gradient from 0 to 100% solvent B. After 40 min, increase solvent A to 100% in the following 5 min and equilibrate the column to the starting condition (100% A) in the next 15 min before injecting the next sample (Table 7.3).

Table 7.3 Gradient used for the HPLC

Time (min)	Solvent A (%)	Solvent B (%)
0.0	100	0
30	0	100
40	0	100
45	100	0
60	100	0

Detection wavelength: 254 nm with a full scale deflection set at 0.2 absorbance

Column temperature: Ambient (approximately 22°C)

Guanine and adenine appear at about 11 and 15.5 min respectively. Allopurinol or caffeine can be used as internal standards. These appear at about 13.5 and 29.5 min respectively. For tannin-containing feeds, do not use caffeine since it binds with tannins, which lowers the recovery of caffeine [31].

Sample Hydrolysis

Weigh 25–100 mg sample (the lyophilized microbial fraction or the apparent undegraded residue) in 25 mL screw-cap tubes and add 2.5 mL of perchloric acid (0.6 M) and 0.5 mL of an internal standard (3 mM allopurinol or 8 mM caffeine). Incubate the mixture in a water bath at 90–95°C for 1 h. After cooling, add 7.0 mL of Buffer A of the HPLC system, adjust the pH between 6.6 and 6.9 using concentrated KOH (approximately 8 M) and centrifuge (3,000*g*) to remove the precipitate

formed. Filter through 0.45 μm filter and inject appropriate volume (15–50 μl) into the HPLC.

Express results based on adenine plus guanine.

The value for purine bases (adenine plus guanine) obtained from an amount (say 25 mg of the apparent undegraded residue) is used for calculating purine basis in the total amount of the apparent undegraded residue (in *y*; see section "Preparation of apparent undegraded residue") obtained from the syringe (purine bases after 24 h in the syringe).

Similarly, purine bases are calculated in the total apparent residue obtained by centrifuging (20,000*g*, 20 min, 4°C) 40 mL of the 0 h medium containing rumen liquor (used for filling the syringes for initiating the fermentation), washing with distilled water, followed by recentrifugation (20,000*g*, 20 min, 4°C) and lyophilisation (purine bases at 0 h in the syringe).

Microbial protein produced in 24 h (as purine basis) = (purine bases in the apparently undegraded residue after 24 h – purine bases in the apparent residue at 0 h). It is assumed that microbial lysis is negligible during this period.

Microbial protein produced in 24 h (as purine basis) *divided by* net gas produced (or short chain fatty acids produced) in 24 h is a measure of efficiency of microbial protein synthesis.

Purine amount can be converted to microbial mass microbial-N by taking rumen fluid sample, centrifuging it at approximately 20,000*g* to obtain microbial pellet, washing it once with distilled water and recentrifuging, and lyophilising it [32]. In a weighed lyophilised pellet, purines can be determined by HPLC or using a spectrophotometeric 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.

Microbial mass (or microbial-N) produced in 24 h *divided by* net gas produced (or short chain fatty acids produced) in 24 h is also a measure of efficiency of microbial protein synthesis.

Some Data on Purine Bases [31]

Purine base determination using HPLC method

	Adenine (μmol)		Guanine (μmol)	
	Mean	SD (n=3)	Mean	SD (n=3)
50 mg LRM	2.35	0.03	2.95	0.02

LRM, lyophilized rumen microbes

Note: It may be noted that workers can optimize their own system for a particular material being studied once equipped with a sound grasp of the rationale behind these procedures.

Table 7.4 Effects of saponins on gas production, purine content (index of microbial protein), truly degraded substrate (TDS) and efficiency of microbial protein synthesis after 24 h of incubation

	TDS (mg)	Gas (mL)	Purines (μmol)	Efficiency of microbial protein synthesis	
				μmol purine/mL gas	μmol purine/mg TDS
Control	300.0 ± 10.3^{a}	95.3 ± 0.9^{a}	6.94 ± 0.36^{a}	0.0728	0.0231
Yucca saponins	320.7 ± 7.1^{b}	91.2 ± 0.9^{b}	9.11 ± 0.34^{b}	0.0998	0.0284
Quillaja saponins	323.0 ± 1.0^{b}	94.5 ± 1.9^{a}	7.99 ± 0.34^{c}	0.0845	0.0247
Acacia saponins	297.6 ± 6.0^{a}	83.5 ± 0.3^{c}	8.38 ± 0.23^{c}	0.1004	0.0282

Data are from Makkar et al. [39], 500 mg hay (475 mg DM) was incubated in the syringes. The means with different superscripts differ ($P < 0.05$).
Data on, and implications of, purines in apparently undegraded residues and efficiency of microbial protein synthesis as a ratio of purines to short chain fatty acids on incubation of tannin-rich feeds alone or with polyethylene glycol are available in Getachew et al. [22–24].

Table 7.4 presents a unique set of data on gas production, true dry matter degradability, microbial mass and efficiency of microbial protein synthesis on addition of potential additives (0.6 mg/mL of various saponins) in the gas method. These parameters were affected to different extents by saponins. For example, addition of Quillaja saponins did not affect gas production, but increased purine content and truly degraded substrate by about 7%. Since truly degraded substrate in an in vitro system can only lead to production of gas and microbial mass, implying that all of the increase in truly degraded substrate by Quillaja saponins resulted in higher microbial protein mass (i.e., saponins increased efficiency of microbial protein synthesis). Had only gas production been measured, the conclusion could have been that Quillaja saponins had no effect on fermentation. In contrast, Acacia saponins decreased gas production, but increased microbial protein synthesis without affecting true degradability. Thus saponins affected partitioning of degraded nutrients such that more microbial mass was produced at the cost of gas, and/or short chain fatty acid production; again reflecting higher microbial efficiency. This higher microbial efficiency would not have been detected had only gas production been measured. The effect of Yucca saponins differed from those of Quillaja or Acacia saponins. Yucca saponins decreased gas, increased microbial protein synthesis and increased true degradability [39], suggesting that measurement of gas only is not sufficient to describe the "true" response of saponins (or of any additive). This highlights the importance of measuring microbial protein (and efficiency of microbial protein synthesis) along with the gas in an in vitro gas method. Similar conclusions have been arrived at by using partitioning factor as a measure of efficiency of microbial protein synthesis [20, 30]. A holistic view of the effects of an additive on rumen fermentation could only be obtained by incorporating analysis of partitioning factor or of another indicator of efficiency of microbial protein synthesis.

Determination of Microbial Protein Production and Efficiency of Microbial Protein Synthesis Using ^{15}N Incorporation

Reagents

Same as in the "reagents" section under "Method for measuring Partitioning Factor", except for the following:

Bicarbonate buffer solution: Dissolve 35 g sodium bicarbonate ($NaHCO_3$) and 4 g ammonium carbonate (NH_4HCO_3) (consisting of 5% ^{15}N-enriched N) in approximately 500 mL distilled water and then make up the volume to 1 L with distilled water.

Notes:

1. *If 50% ^{15}N-enriched NH_4HCO_3 is available, add 0.4 g of this ^{15}N-enriched NH_4HCO_3 and 3.6 g of NH_4HCO_3 in 1 L solution.*
2. *If 96.5% ^{15}N-enriched NH_4HCO_3 is available, add 0.207 g of this ^{15}N-enriched NH_4HCO_3 and 3.793 g of NH_4HCO_3 in 1 L solution.*
3. *Using 5% ^{15}N-enriched bicarbonate buffer, the expected enrichment in the apparently undegraded residue would be approximately 1%. The enrichment will also depend on the nitrogen content of the feed incubated and the rumen liquor added to the incubation medium.*

Procedure

Other procedures such as "Weighing of samples and preparation of syringes", "Preparation of in vitro rumen fermentation buffer solution and incubation", and "Net gas production" were as described in sections "Weighing of samples and preparation of syringes", "Preparation of in vitro rumen fermentation buffer solution and incubation" and "Net gas production" respectively.

After recording the gas volume after 24 h, the syringe contents were subjected to the procedure given in "Preparation of apparent undegraded residue" to obtain apparent undegraded residues (undegraded feed plus microbes after 24 h of incubation). This residue should be quantitatively collected and ground to fine powder preferably using ball mill. This residue (1–3 mg containing approximately 100 μg N; could be lower depending on the N content of the residue and sensitivity of the mass spectrometer) is weighed into tarred tin foil cups using a microbalance, and subjected to Mass Spectroscopy to measure ^{15}N enrichment. Ammonium sulphate (or ammonium chloride) solution containing 50 μg N/μl of known ^{15}N enrichment (standard), covering the observed enrichment range, is included in the measurements. An amount of ^{15}N in excess of 0.366% is considered as enrichment.

The values obtained from the Mass Spectroscopy gives the proportion of the total nitrogen as ^{15}N (percent enrichment). If the mass spectrometer is combined with an elemental analyser, total N content can also be obtained. The N content can also

be determined separately using an elemental analyser or micro-Kjeldahl method (digestion followed by ammonia determination by steam distillation, absorption in boric acid and titration with sulphuric acid or by reaction with hypochlorite-nitroprusside and measurement of the blue colour spectrophotometrically [16]. Using these values, total ^{15}N enrichment in the total amount of apparently undegraded residue can be calculated:

^{15}N in apparently undegraded residue (mg) = ((weight of apparently undegraded residue in mg × % N)/100) × (% 15 N in apparently undegraded residue/100)

As described above, the apparently undegraded residue is composed of undegraded feed and microbes produced during the incubation. However, ^{15}N incorporation from ^{15}N-labelled ammonium bicarbonate added in the buffer is in the microbes, and hence it is an index of microbial protein (or microbial mass) synthesis. Higher is the ^{15}N incorporation in the residue, higher the microbial protein or microbial mass produced.

^{15}N incorporation in the microbes in 24 h (mg) = (mg ^{15}N in the apparently undegraded residue of the test syringe after 24 h – mg ^{15}N in the apparently undegraded residue of the corresponding blank after 24 h). It is assumed that microbial lysis is negligible during this period.

Efficiency of microbial protein synthesis = ^{15}N incorporation in the microbes in 24 h/net gas (or short chain fatty acids) produced in 24 h.

Do's and Don'ts for the Gas Method

1. The plunger should be properly lubricated using white Vaseline.
2. 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).
3. Reducing solution should be prepared fresh on the same day of conducting the experiment.
4. Start flushing the medium with carbon dioxide well before (approximately 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).
5. 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).
6. After dispensing 40 mL of the medium into the syringe, create a light vacuum by pushing back the plunger and then open the clip, for removing air from the syringe. 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.

7. After filling of the syringes has been completed (might take 30–40 min), shake the syringes. Shake them again after every 30 min until first 2 h of the incubation, and then after every two h till the first 10 or 12 h of the incubation. Make sure that all feed particles are taken into the medium while stirring (swirling shaking action might help).
8. 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.
9. Check temperature and level of water in the water bath at least twice a day.
10. 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.
11. When you push back the syringe in the evening, give a shake after approx. 30 min in order to prevent taking up the sample along with the bottom portion of the plunger and out from the incubation medium.
12. Use carbon dioxide gas cylinder with caution. Ask someone if you do not know its operation. Misuse could cause an accident.
13. 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.
14. 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.
15. 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.
16. 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.

Note: There are a number of in vivo methods for determination of net microbial protein synthesis in the rumen (and hence of the efficiency of microbial protein synthesis) 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 [17] 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 [28]. 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 has been further simplified using spot urine samples [32]. This technique does not require cannulated animals, but involves feeding of the diets under investigation to animals, and therefore is not suitable for screening a large numbers of samples or for developing feed supplementation strategies using various feed constituents.

Conclusions

The methods reported here in which gas production and microbial mass production are concomitantly measured have several major applications:

i) study of rumen modulators or in the screening plants or plant extracts for increasing efficiency of microbial protein synthesis and decreasing emission of methane and carbon dioxide,
ii) 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, and
iii) development of supplementation strategies using locally available conventional and unconventional feed constituents to achieve maximum microbial efficiency in the rumen; and
iv) study of roles of various nutrients (by changing the composition of the incubation medium) with respect to production of fermentative gases, short chain fatty acids and microbial mass.

The choice of methods for determination of microbial mass, and estimation of efficiency of microbial protein mass synthesis, and for investigation on the partitioning of nutrients to various products depends on the facilities available and objective of the experiment. The determination of partitioning factor as the ratio of organic matter degraded to gas production is simple and can be used for evaluation of conventional feeds or for studying the effects of plant extracts, plant compounds or any other additives provided these are not rich in tannins. The use of this partitioning factor-based approach should be used with caution for feeds rich in plant secondary metabolites. The determination of microbial mass using the nitrogen balance approaches, by measuring purine or ^{15}N incorporation in the microbes could be used for all type of feeds.

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