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In Vitro Gas Production Technique for Evaluation of Feed Resources

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Abstract

The *in vitro* rumen fermentation method in which gas production and microbial mass production are concomitantly measured has several major advantages: i) it has the potential for screening a large number of feed resources, for example in breeding programmes for the development of varieties and cultivars of good nutritional value, ii) it could also be of great value in the development of supplementation strategies using locally available conventional and unconventional feed constituents to achieving maximum microbial efficiency in the rumen; iii) it has an important role to play in the study of rumen modulators for increasing efficiency of microbial protein synthesis and decreasing emission of methane, an environmental polluting gas, and iv) it provides a better insight into nutrient-antinutrient and antinutrient-antinutrient interactions, and into the roles of various nutrients (by changing the composition of the incubation medium) with respect to production of fermentative gases, SCFA and microbial mass. The method is also being used increasingly to screen plant-derived rumen modulators. These products have a lower toxicity to animals and humans, and are environment friendly. Consequently, they are becoming increasingly popular with consumers.

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

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

Keywords: Scarcity; Unconventional feed resources; Ligno-cellulosic stovers; UMMB; Soil erosion; Food security.

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Introduction

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

Both growth and milk yield of ruminants are largely limited by forage quality which is mainly reflected in low voluntary intake and digestibility. The importance of these parameters in animal nutrition has long been recognized. The determination of intake and digestibility of feed stuffs *in vivo* is time-consuming, laborious, and expensive, requires large quantities of feed and is unsuitable for large-scale feed evaluation. Therefore, many attempts have been made to predict intake and digestibility using laboratory techniques. Much effort has been directed towards the development of regression equations to predict digestibility from forage chemical composition, but a regression equation that satisfactorily predicts a wide range of forages has not yet been derived.

In this seminar we are going to highlight the potential of a novel approach using an *in vitro* rumen fermentation technique for evaluation of the nutritional quality of conventional and unconventional feed resources.

Methods for evaluation of feed resources

Overview

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

There are a number of methods used to determine net microbial protein synthesis in the rumen based on the use of microbial markers. They require the use of post-rumen cannulated animals to determine flow of digesta. The cannulation approach is tedious and has several limitations[1]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.[2]This approach is being thoroughly investigated under a Joint FAO/ IAEA Coordinated Research Project.[3] Although the method is based on the collection of urine for determination of purine derivatives (allantoin and uric acid for cattle, and allantoin, uric acid, xanthine and hypoxanthine for sheep), the approach is being further simplified using spot urine samples. This technique does not require cannulated animals, but it involves feeding the diets under investigation to animals and therefore is not suitable for screening large numbers of samples or for developing feed supplementation strategies using various feed constituents.

A) In vivo methods

Total collection technique (direct method or conventional digestion trial)

The total collection (conventional digestion trial) is the most reliable method of measuring a feed's digestibility. Unfortunately, it is somewhat time consuming, tedious, and costly.

Usually, the animal is restrained in an individual cage so that a quantitative collection of feces can be made. Accurate records of feed intake, refusals and fecal output are kept, and a sub sample of each (usually 10% of daily output in the case of feces) is retained for analysis. When estimates of nitrogen balance are desired, urine output is also measured.

Three animals per feed are required as a minimum. The animals are usually allowed from 7 to 21 days (d) to adjust to the feed, followed by a collection. Samples can then be dried, ground, and analyzed for the nutrients of interest. Digestibility of any given nutrient can be calculated as follows:

Nutrient intake

$$\text{Nutrient digestibility (\%)} = \frac{\text{(Nutrient intake - Nutrient in feces)}}{\text{Nutrient intake}} \times 100$$

The most common arrangement for collecting the excreta of animals for digestibility experiments is through the use of metabolic crates. A metabolic crate is actually a stall or box large enough for the animal set on legs from 50 cm to 1 m high. It is so planned as to permit the quantitative collection of feces and urine. However, a common criticism of digestion estimation by total collection technique is that feed intake by animals is sometimes abnormally low and erratic. This lack of appetite is in many cases attributable to the fact that the animal may be too nervous or frightened to eat, resulting from the close confinement made necessary by the very nature of the equipment used. It is important that the experimental animals must be sufficiently comfortable during the adjustment period. The space allowed to the animal must be large enough to permit considerable freedom of movement. But conducting a digestion experiment may normally entail appreciable annoyance to the animal.

Some individual animals are temperamentally unsuited to be used in such experiments and are too nervous to be used in digestion trials. Mostly captive wild animals fall into this category. Even though conventional digestion trials are the standard with which all other measures of digestibility are compared, the values obtained still vary ± 1 to 4 % as a result of animal-to-animal variation, sampling procedures and analytical errors.

B) Indirect method

Apparent digestibility of a diet can be estimated using a natural constituent of the feed as an indicator. Acid insoluble ash (AIA) can be used in this way.The ratio between the concentration of AIA in the feed and the concentration of AIA in the faeces gives an estimate of digestibility.

For example, the digestibility of neutral

$$\text{Digestibility of DM} = 100 - \frac{[100\ (\text{AIA concentration in diet})}{(\text{AIA concentration in faeces})]}$$

detergent fibre (NDF) is calculated as:

$$\text{Digestibility of DM} = 100 - \left[\frac{100 \text{ (AIA concentration in diet X NDF concentration in faeces)}}{\text{(AIA concentration in faeces X NDF concentration in diet)}}\right]$$

In vitro methods

In vitro methods for laboratory estimations of degraded feeds are important for ruminant nutritionists. An efficient laboratory method should be reproducible and should correlate well with actually measured *in vivo* parameters. *In vitro* methods have the advantage not only of being less expensive and less time-consuming, but they allow to maintain experimental conditions more precisely than do *in vivo* trials. Three major biological digestion techniques are currently available to determine the nutritive value of ruminant feeds: 1) digestion with rumen microorganisms [4]or using a gas method[5] 2) *in situ* incubation of samples in nylon bags in the rumen [6], and 3) cell-free fungal cellulose[7] These biological methods are more meaningful since microorganisms and enzymes are more sensitive to factors influencing the rate and extent of digestion than are chemical methods. [8] The nylon bag technique has been used for many years to provide estimates of both the rate and extent of disappearance of feed constituents. This technique provides a useful means to estimate rates of disappearance and potential ruminal degradability of feed stuffs and feed constituents whilst incorporating effects of particulate passage rate from the rumen. The disadvantage of the method is that only a small number of forage samples can be assessed at any one time, and it also requires at least three fistulated animals to account for variations due to animal. It is therefore of limited value in laboratories undertaking routine screening of a large numbers of samples. It is also laborious, and requires large amounts of samples. Substantial error could result in values obtained at early stages of digestion due to a low weight loss; and for poor quality roughages, adherence of microbes at early stages can even lead to higher weights and thus distortion of results if kinetic modelling does not incorporate the lag-phase. [9,10]

The technique[4] is used widely because of its convenience, particularly when large-scale testing of feedstuffs is required. This method is employed in many forage evaluation laboratories and involves two stages in which forages are subjected to 48 h fermentation in a buffer solution containing rumen fluid, followed by 48 h of digestion with pepsin in an acid solution. The method was modified by Goering and Van Soest (1970)[11], in that the residue after 48 h incubation was treated with neutral detergent solution to estimate true dry matter digestibility. Although the method [4] has been extensively validated with *in vivo* values[8] , 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 digestibilities for tannin-rich feeds, since tannins are solubilised in both these systems but might be indigestible and do not contribute to nutrient supply to animals.[12]

In vitro gas production technique

The gas measuring technique has been widely used for evaluation of nutritive value of feeds. More recently, the increased interest in the efficient utilization of roughage diets has led to an increase in the use of this technique due to the advantage in studying fermentation kinetics. Gas measurement provides a useful data on digestion kinetics of both soluble and insoluble fractions of feedstuffs. Several gas measuring techniques and *in vitro* gas methods are in use by several groups. Advantages and disadvantages of these methods are discussed. [13]The *in vitro* gas method based on syringes [14,5]appears to be the most suitable for use in

developing countries. Other *in vitro* methods such as Tilley and Terry and nylon bag methods are based on gravimetric measurements which follow disappearance of the substrate (the components which may or may not necessarily contribute to fermentation), whereas gas measurement focuses on the appearances of fermentation products (soluble but not fermentable products do not contribute to gas production). In the gas method, kinetics of fermentation can be studied on a single sample and therefore a relatively small amount of sample is required or a larger number of samples can be evaluated at a time. The *in vitro* gas method is more efficient than the *in sacco* method in evaluating the effects of tannins or other anti-nutritive factors. In the *in sacco* method these factors are diluted in the rumen after getting released from the nylon bag and therefore do not affect rumen fermentation appreciably. In addition, the *in vitro* gas method can better monitor nutrient-antinutrient and antinutrient-antinutrient interactions.[15]

A simple *in vitro* approach is described below which is convenient and fast, and allows a large number of samples to be handled at a time. It is based on the quantification of substrate degraded or microbial protein produced using internal or external markers and of gas or short chain fatty acid (SCFA) production in an *in vitro* rumen fermentation system based on syringes. [5]This method does not require sophisticated equipment or the use of a large number of animals (but one or preferably two fistulated animals are required) and helps selection of feeds or feed constituents based not only on the dry matter digestibility but also on the efficiency of microbial protein synthesis.

In the method of Menke *et al* (1979)[5], fermentations are conducted in 100 ml capacity calibrated glass syringes containing feed stuff and a buffered rumen fluid. The gas produced on incubation of 200 mg feed dry matter after 24 h of incubation together with the levels of other chemical constituents are used to predict digestibility of organic matter determined *in vivo* and metabolizable energy.

For roughages, the relationships are:

ME (MJ / Kg DM) = 2.20 + 0.136 Gp + 0.057 CP, R^2= 0.94

OMD (%) = 14.88 + 0.889 Gp + 0.45 CP + 0.0651 XA, R^2=0.92

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

Aiple et al (1996) compared three laboratory methods (enzymatic, crude nutrient and gas measuring technique) as predictors of net energy (as estimated by equations based on *in vivo* digestibility) content of feeds and found that for predicting net energy content of individual feeds, the gas method was superior to the other two methods.[16]

The method of Menke *et al* (1979)[5] was modified by Blümmel and Ørskov (1993) [17]in that feeds were incubated in a thermostatically controlled water bath instead of a rotor in an incubator. Makkar *et al* (1995b) and Blümmel *et al* (1997)[14,18] modified the method further by increasing the amount of sample from 200 to 500 mg and increasing the amount of buffer two-fold as a result the incubation volume increase from 30 ml in the method [5] to 40 ml in the modified method. In the 30 ml system, the linearity between the amount of substrate incubated and the amount of gas produced is lost when the gas volume exceeds 90 ml because of the exhaustion of buffer of the medium; and in 40 ml system, the linearity is lost when the gas volume exceeds 130 ml.[19] The exhaustion of the buffer decreases p^H of the incubation medium; consequently the fermentation is inhibited. If the amount of gas production exceeds 90 ml using the 30 ml system and 130 ml using the 40 ml system, the amount of feed being incubated should be reduced.

The main advantages of the modified method (the 40 ml system and incubation in a water bath) are: i) there is only a minimum drop in temperature of the medium during the period of recording gas readings on incubation of syringes in a water bath. This is useful for studying the kinetics of fermentation where gas

volume must be recorded at various time intervals, ii) because of large volume of water in the water bath and also its higher temperature holding capacity, drastic drop in the temperature of the incubation is prevented in case of power breakdown for a short duration, and iii) an increase in amount of sample from 200 to 500 mg reduces the inherent error associated with gravimetric determination needed to determine concomitant *in vitro* apparent and true digestibility.

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

The gas produced in the gas technique is the direct gas produced as a result of fermentation and the indirect gas produced from the buffering of SCFA. For roughages, when bicarbonate buffer is used, about 50% of the total gas is generated from buffering of the SCFA and the rest is evolved directly from fermentation. At very high molar propionate the amount of CO_2 generated from buffering of SCFA is about 60% of total gas production. Each mmol of SCFA produced from fermentation releases 0.8-1.0 mmol of CO_2 from the buffered rumen fluid solution, depending on the amount of phosphate buffer present. Highly significant correlation has been observed between SCFA and gas production.

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

The gas produced on incubation of cereal straws[17] , a wide range of feeds including many dairy compound feeds and their individual feed components whose protein and fat contents vary greatly[20],and tannin containing browses[17]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 (SCFA) as per Wolin (1960) stoichiometry[22], which is as follows:

Fermentative CO_2 = A/2+P/4+1.5B

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

Fermentative CH_4 = (A+2B)-CO_2

where A and B are moles of acetate and butyrate respectively and CO_2 is moles of CO_2 calculated from previous equation.

Assumption: one mole of SCFA releases one mole of CO_2 from bicarbonate-based buffer described as buffering CO_2 and therefore, mmol of buffering CO_2 is equal to mmol of total SCFA generated during incubation.

Gas volume = mmol of gas x gas constant (R) x T

Where

R = the ratio between molar volume of gas to

temperature (Kelvin zero; K) i.e. (22.411/273 = 0.082),

T = incubation temperature (Kelvin); 273 + 39° C = 312 K

Total volume of gas (ml) calculated from SCFA production = (BG + FG) x CF

BG = gas volume (ml) from buffering of SCFA,

FG = fermentative gas (ml) (CO_2 + CH_4),

CF = correction factor for altitude and pressure which is 0.953 for Hohenheim at altitude 400m above sea level[23]

(The volume of 1 mmol of gas at 39° C in Hohenheim would be;

1 x 0.082 x 312 x 0.953=24.4 ml).

The origin and stoichiometry of gas production have been described in details[13,14]

The *in vitro* gas production measured after 24 h incubation of tannin containing browses in the presence or absence of PEG was strongly correlated with gas volume stoichiometrically calculated from SCFA. The relationship between SCFA production (mmol) and gas volume (ml) after 24 h of incubation of browse species with a wide range of crude protein (5.4-27 %) and phenolic compounds (1.8-25.3 % and 0.2-21-4 % total phenols and total tannins as tannic acid equivalent respectively) was [21]:

In the absence of PEG

SCFA = 0.0239.Gas -0.0601; R^2 = 0.953; N = 39; P<0.001 (I)

In the presence of PEG

SCFA = 0.0207.Gas + 0.0207; R^2 = 0.925; N = 37; P<0.001 (II)

These relationships are similar to that obtained for wheat straw.[24]

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

The stoichiometric balance also allows calculation of the CH_4 and CO_2 expected from the rumen fermentation if the molar proportions and amount of SCFA are known.

Kinetics of fermentation feedstuffs can be determined from fermentative gas and the gas released from buffering of SCFA. Kinetics of gas production is dependent on the relative proportion of soluble, insoluble but degradable, and undegradable particles of the feed. Mathematical descriptions of gas production profiles allows analysis of data, evaluation of substrate- and media-related differences, and fermentability of soluble and slowly fermentable components of feeds. Various models have been used to describe gas production profiles. France *et al* (1993; 2000) [25,26]combine modelling of the gas profiles with estimates of substrate loss and ruminal rate of passage and derive estimates of ruminal extent of degradation thus linking gas production to events in the rumen proper.

Procedure

Preparation and weighing the feed sample

Before weighing, grind the dry material through a 1 mm screen. Avoid very fine grinding because of observed differences in digestibility (*in vivo*) and gas production (*in vitro*) between coarse and finely ground roughage.

For fresh samples, use a cutting mill, a slow rotating meat cutter or a pair of scissors to chop the roughage.

Weigh about 200 mg DM of the sample on a weighing boat. 2 Push the piston (greased with vaseline to ensure easy movement and precise fitting) down the cylinder. Close the silicon rubber tube attached to the capillary attachment (needle) of the syringe with a plastic clip. Fermentation is carried out in this glass syringe.

Rumen fluid

Not more than 15 minutes before the trial starts, collect rumen fluid (about 1 litre) in equal proportions from two rumen-fistulated donor cows/small ruminants under the same feeding regime (at Debre Zeit, grass hay given *ad libitum* and a total of 2.4 kg cotton seed cake given in two meals daily). Filter the sample through two layers of cheese cloth into a warm flask (kept in a bucket of water at 37–38°C) and flush with carbon dioxide (CO_2). Take the rumen fluid before the morning feed or before feeding the diet supplement.

Solutions

Prepare five different solutions as media and mix with rumen liquor.

The composition of the solutions are as follows:

Solution A (Micro mineral solution)

13.2 g calcium chloride ($CaCl_2.2H_2O$)

10.0 g manganese chloride ($MnCl_2.4H_2O$)

1.0 g cobalt chloride ($CoCl_2.6H_2O$)

8.0 g iron chloride ($FeCl_3.6H_2O$)

made up to 100 ml with distilled water.

Solution B (Buffer solution)

39.0 g sodium hydrogen carbonate ($NaHCO_3$)
or

35.0 g $NaHCO_3$ + 4.0 g ammonium hydrogen carbonate ($(NH_4)HCO_3$)

made up to 1 liter with distilled water.

Solution C (Macro mineral solution)

5.7 g disodium hydrogen phosphate (Na_2HPO_4)

6.2 g potassium dihydrogen phosphate (KH_2PO_4)

0.6 g magnesium sulphate ($Mg\ SO_4.7H_2O$)

made up to 1 litre with distilled water.

Resazurin solution

100 mg resazurin made up to 100 ml with distilled water.

Reducing solution

4 ml sodium hydroxide (1N NaOH)

625 mg sodium sulphide ($Na_2S.9H_2O$)

added to 95 ml distilled water.

The reducing solution must be freshly prepared each time shortly before the rumen fluid is taken from the animal. The other solutions can be made up and stored.

Preparation of media

Pour 400 ml distilled water, 0.1 ml solution A, 200 ml solution B, 200 ml solution C and 1 ml resazurin into a Buckner flask. You will observe a bluish colour. Add 40 ml reducing solution while mixing with a magnetic stirrer. Flush the mixture with CO_2 gas while the reducing solution is being added. The colour will change from bluish through a reddish colour (oxidised) to colourless (reduced).

Add the rumen fluid. The ratio of rumen fluid to buffer medium is 1:2 (v/v).

Preparing syringes for incubation

Place the glass syringes containing the substrates in a water bath at 38–39°C an hour before incubation starts.

During incubation, remove the glass syringe from the water bath and firmly fix the rubber tube on to the needle of the automatic syringe.

Pipette 30 ml of the rumen fluid/medium mixture with an automatic syringe into each of the pre-warmed glass syringes. Bring any air bubbles trapped in the syringe to the surface by gentle shaking and remove them through the capillary attachment by careful upward orientation and pushing the piston. Close the clip on the tube, read the initial volume and record it as V0. Place the syringe back in the

syringe rack for incubation in the water bath at 38–39°C.

Twenty-four hour incubation

Incubate the feeds in triplicate in at least two different sessions (with different rumen fluids), yielding six parallel measurements. Include four glass syringes containing rumen fluid/ media mixture without substrate (blank), three glass syringes containing Sululta hay (200 mg DM), i.e. the standard, and three syringes containing 140 mg DM Sululta hay and 60 mg starch in every set to control differences in composition and activity of the rumen fluid (control incubations). The readings from the blank, grass hay and grass hay + starch are GP0, GPH and GPHS, respectively. The exact reading where the end mark on the piston lies is regarded as the initial volume (V0).

Read the position of the piston 6 h after incubation begins and record it as intermediate volume (Vint.).

Move the piston gently beforehand to make sure that it is not sticking. If gas production exceeds 60 ml, open the clip and move the piston back to the 30 ml mark, while keeping it vertical, thus allowing most of the gas which has formed to escape. Record the exact reading before the piston is moved back to 30 ml as V1 for the next incubation hour. Continue the incubation and take the final reading after 24 hours (Vfinal).

Sequential incubation (3, 6, 12, 24, 48, 72, 96 and 120 h)

To determine the volume of gas produced at 3, 6, 12, 24, 48, 72, 96 and 120 hours, a slightly modified procedure is followed regarding the number of parallel measurements. Except for the blank which is incubated in triplicate, the substrates and both standards are all incubated in duplicate for every incubation period (time).

In the 3- and 6-hour incubations, the gas produced is not expected to exceed 60 ml and thus there is no V1. For the rest of the incubation periods conducted in series, i.e. 12, 24, 48, 72, 96 and 120 hours, consider the 12-hour reading as the first calibrated volume (1V1). During calibration reset the piston to the 30 ml position for all of the syringes except for the blanks. Since there is no calibration before the 12-hour reading, the net gas production at 3, 6 and 12 h incubation periods is simply the final reading of gas produced minus the sum of V0 and blanks at these hours.

For all other readings taken at and after 24 hours of incubation, calibrate only when the gas produced exceeds 60 ml. Release the gas produced and set the piston back to 30 ml (second calibration). The second calibrated volume (2V1) is the sum of the 1V1 and the most recent reading taken before the second calibration. Use the 2V1, like the 1V1, only for the calculation of the net gas production for the succeeding incubation hour. Likewise, if there is a need to release the gas for the third time, the 3V1 is the sum of the 2V1 and the most recent reading before the gas is released. The same method of calculation applies in this case.

Calculations

Use the volumes of gas recorded at different times to estimate the *in vitro* gas production during incubation of the feeds. Gas production (GP) is defined as the total increase in volume minus the blank (GP0). Subtract the mean blank value (GP0) from the recorded gas production of all samples and standards to give the net gas production. Relate the gas volume from which the blank value has been deducted to the weight of exactly 200 mg DM of the sample taken.

After collecting ample data for the standards, calculate the standard value for, in the case of Debre Zeit, the Sululta grass hay and the Sululta hay + starch. Estimate the mean gas production (in ml/200 mg DM) with each of the standard feeds at each incubation period and calculate the correction factor for the corresponding periods. Divide the standard value for the Sululta grass hay by the measured net value of the same standard hay for the particular incubation session to give the correction factor (FH). The correction factor for hay + starch standard (FHS) is derived similarly. Use the

mean of these two factors FH and FHS for correction of the sample measurements.

It is necessary to check from the standards included in every set how far the recorded values deviate from the standard values. The difference between FH and FHS is expected to be insignificant. The theoretically accepted values for FH and FHS lie between 0.9 and 1.1. If the factors do not fall within this range, the test must be repeated.

The general formula for calculating the corrected gas production is:

GP (ml/200 mg DM) = (XV1 - 30X + Vfinal - V0 - GP0) x 200 ((FH + FHS) 2)

weight in mg DM where:

X = the number of times that the gas is released from the syringe and the volume is set back to 30 ml

V0 = the initial volume of gas recorded before incubation starts

V1 = the volume of gas recorded before the gas is released from the syringe and the volume is set back to 30 ml

Vfinal = the final volume of gas recorded at the end of incubation time

GP0 = the mean blank value

FH = the correction factor for the standard grass hay

FHS = the correction factor for the grass hay/ starch standard

DM = dry matter.

Data from gas production may be processed like data obtained with the nylon bag technique. More often the following model is fitted to the data:

$Y = b (1–e–ct)$

where:

Y = the volume of gas produced with time (t)

c = the gas production rate

b = the potential extent of gas production.

The intercept is not included in the model with the understanding that no gas is produced from unfermented feed.

Applicability of the in vitro gas production technique

Determination of microbial mass

In vitro gas tests are attractive for ruminant nutritionists since it is very easy to measure the volume of gas production with time, but the measurement of gas only implies the measurement of nutritionally wasteful and environmentally hazardous products. In most studies the rate and extent of gas production has been wrongly considered to be equivalent to the rate and extent of substrate (feed) degradation. Current nutritional concepts aim at high microbial efficiency, which can not be achieved by measurement of gas only. *In vitro* gas measurements reflect only SCFA production. The relationship between SCFA and microbial mass production is not constant and the explanation for this resides in the variation of biomass production per unit ATP generated. This can impose an inverse relationship between gas volume (or SCFA 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* (1997) have demonstrated how a combination of *in vitro* gas production measurements with a concomitant quantification of the truly degraded substrate provides important information about partitioning of fermentation products, and the *in vitro* microbial mass production can be calculated as:

Microbial mass (mg) = mg substrate truly degraded - (ml gas volume x stoichiometrical factor)

For roughages, the stoichiometrical factor was 2.20. [14]

Partitioning factor

The parameters in the above equation also allow the calculation of a partitioning factor (PF). The PF is defined as the ratio of substrate truly degraded *in vitro* (mg) to the volume of

gas (ml) produced by it (equivalent to the reciprocal of parameter *Y*.[25] The above equation becomes

Microbial mass (units) = gas volume (PF - stoichiometrical factor)

A feed with higher PF means that proportionally more of the degraded matter is incorporated into microbial mass, i.e., the efficiency of microbial protein synthesis is higher. Roughages with higher PF have been shown to have higher dry matter intake. Different *in vitro* PF values are also reflected by *in vivo* microbial protein synthesis as estimated by purine derivatives (the higher the PF, the higher the excretion of urinary purine derivatives; and in methane production by ruminants (the higher the PF, the lower the methane output.[23] These results show that the PF calculated *in vitro* provides meaningful information for predicting the dry matter intake, the microbial mass production in the rumen, and the methane emission of the whole ruminant animal.

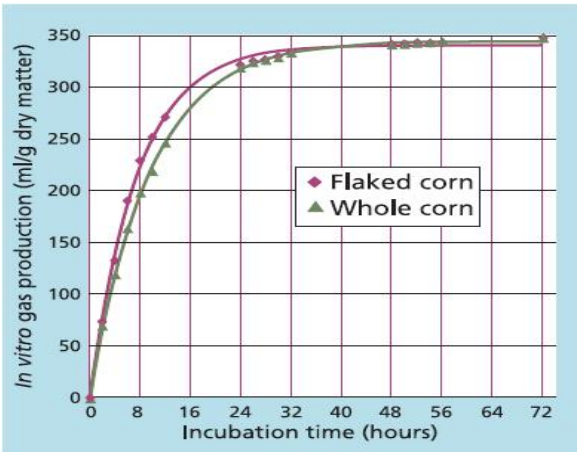
The procedures for the determination of truly degraded substrate and the calculation of the stoichiometrical factor; stoichiometrical relationship between SCFAs and gas volume; and relationship between SCFA production, ATP production and microbial mass yield can be obtained.[13,14] It may be noted that these procedures and relationships are valid for substrates consisting predominantly of structural carbohydrates, and the findings might not extend to substrates such as those high in soluble carbohydrate, protein or fat. Rymer and Givens (1999) have shown that, as observed by Blümmel *et al* (1997), good quality feeds (grass silage, wheat, maize, molasses sugarbeet feed and fishmeal) which produce large amounts of gas and SCFA yield small amounts of microbial mass per unit of feed truly degraded.[27,14]

It seems therefore justified to suggest that feeds or feed ingredients should be selected that have a high *in vitro* true degradability but low gas production per unit of truly degraded substrate. Dijkstra *et al* (2000) have described modelling of microbial protein synthesis *in vivo* from the *in vitro* gas parameters.[28]

Digestion kinetics of neutral detergent-soluble fraction

The gas measurement method has also been used to study digestion kinetics of the neutral detergent soluble fraction of forages, starch-rich feeds and other highly digestible carbohydrate components, which was obtained by subtracting the average gas production curve for the digestible neutral detergent fibre (NDF) from that of the unfractionated whole feed.[29]The subtraction procedure might give some useful information relevant to low-NDF fibre feeds, e.g., corn grain [29] but it is not suitable for forages rich in NDF.[30] Blümmel *et al* (1998b) examined the rate and extent of fermentation of whole roughage and extracted NDF, dry matter degradability of extracted NDF and the PF for whole roughage and the extracted NDF of 54 roughages.[30] The 24-h degradabilities of extracted NDF were higher than NDF degradabilities in whole roughages, and the PF values were lower for extracted NDF than for whole roughages (2.5 vs 3.1; i.e. the efficiency of microbial protein synthesis with extracted NDF was lower). Both the higher degradability and lower PF contributed to higher gas volumes obtained from extracted NDF compared with whole roughage. Supplementation of amino acids and sugars, which essentially constitute the solubles, may increase the efficiency of microbial synthesis from cell walls during fermentation (a situation similar to that in unfractionated forages) and

Fig 1: Kinetics of *in vitro* gas production during incubation of flaked and whole corn grain (Getachew et al. 2001)



the removal of solubles may result in lower microbial efficiencies. A considerable effect of cell solubles on partitioning of nutrients from the NDF raises doubts as to the significance *in vivo* of the kinetic parameters calculated using the subtraction procedure.[30]

Voluntary intake prediction

The main constraint to the utilisation of roughages by ruminants is voluntary feed intake so prediction of feed intake, particularly of fibrous roughage, is one of the important aspects of ruminant nutrition. *In vitro* gas production has been used to predict dry matter intake. Various workers have reported significant correlation between in vitro gas production and dry matter intake. Forage cell walls have considerable influence on voluntary feed intake through rumen fill mechanism.[8] Gas production from extracted neutral detergent fibre was shown to be better correlated to voluntary feed intake than the values obtained from the incubation of whole roughage. The use of various models for intake prediction was investigated and it currently appears that combination of gas volume measurements (4-8h) with concomitant determination of the amount of substrate degraded (> 24 h) is superior to the models based on kinetics of gas production only. The *in vitro* gas production from NDF explained more (82 % vs. 75 %) of the variation in dry matter intake than gas production from whole roughage.[13]

Interaction between dietary constituents

Gas measurement was also employed for evaluation of the interaction between basal and supplementary diets by incubating basal diet and supplementary diet separately as well as in combination and monitoring gas production at different hours of incubation using the pressure transducer system.[31]This will indicate the availability of readily fermentable material as a ready energy source, which will stimulate the activity of the rumen micro-organisms which in turn would accelerate the digestion of roughages. These workers, by incubating the basal diet and the supplement, observed a positive interaction in gas production in the early hours of incubation, which according to the authors can be an approach to study the synergetic effects of supplementation. However, it must be pointed out that measurement of gas only, could lead to misleading results. It is suggested to determine microbial mass production in addition to the gas measurement for such studies.

Organic matter digestibility

The digestibility of measured organic matter is closely correlated with that predicted from gas production and the crude protein and ash contents of feeds. Therefore, the method can be used to predict the extent of digestion for various feeds as below:

OMD (%) = 14.88 + 0.889 Gp + 0.45 CP + 0.0651 XA, R^2=0.92

Energy contents of feeds

The gas method has also been used successfully to predict the ME content of feeds. A regression equation has been developed with data generated by *in vivo* studies conducted with a variety of feeds and *in vitro* gas production. The gas measurement provides a better estimate of the ME level of feeds, when combined with some chemical constituents, compared with calculations based on chemical constituents only (see below).

ME (MJ / Kg DM) = 2.20 + 0.136 Gp + 0.057 CP, R^2= 0.94

Effects of added fat on feed degradation

Tallow and yellow grease (YG), both rendering byproducts, are typical fats used in the diets of lactating dairy cows. The gas technique was used at UC Davis to examine the effect of sources and levels of added fat on gas production and rumen fermentation of a total mixed ration.[32]Fatty acids in the form of triglyceride (YG) had no effect (when comprising up to 25% of the diet) on gas

production, but fatty acids in the form of potassium salts (YG soap) significantly depressed gas production. In the animal, however, there is a limit to the amount of fatty acids that can be successfully fed, and this is lower than *in vitro*. The fatty acids in potassium salts are quickly available to microbes as free fatty acids in ruminal fluid, and have detrimental effects on microbial growth.

In contrast, the fatty acids in the triglyceride form must be released through hydrolysis of the ester bond and therefore are available at a slower rate. Hydrolysis refers to breaking the chemical bond between the individual fatty acid and the glycerol backbone of the triglyceride.

The effects of fatty acids on rumen fermentation are important because feeds with high levels of residual fat, for example rice bran created in the production of white rice, are commonly fed to ruminants.

Anti-nutritive factors

The gas method can be used to measure how microbial activity lowers feed digestibility. Some feeds, such as forage legumes and cotton seed, contain phenolics, alkaloids and saponins that have negative biological effects on microbes and reduce microbial growth in rumen. Tannins are naturally occurring polyphenolic compounds found in plants, which form complexes with feed and microbial proteins and can depress feed digestibility in the rumen.

Fig 2: Effect of Yellow Grease (YG) and Yellow Grease Soap (YG Soap) on in vitro gas production (Getachew *et al*, 2001)

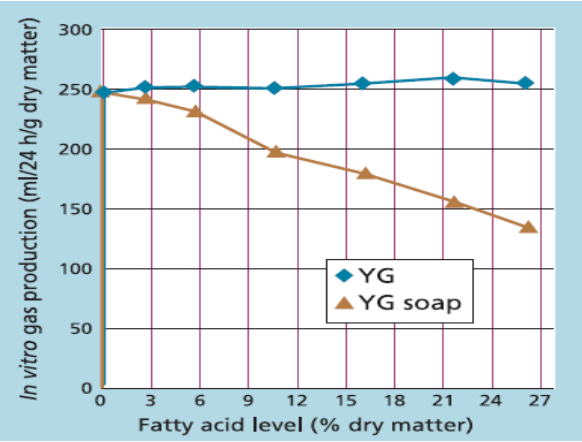
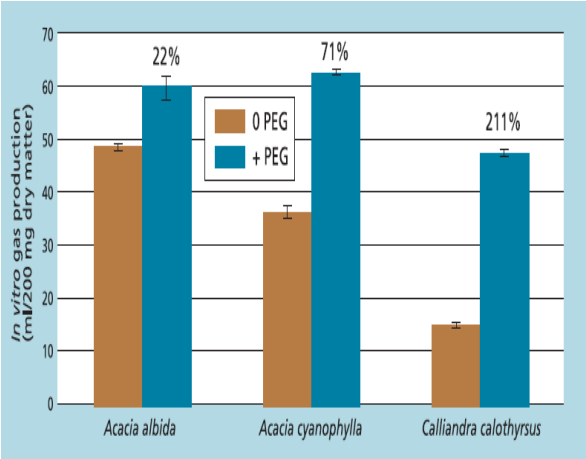


Fig 3: In vitro gas production of tannin-containing leaves in absence of PEG (0 PEG) and presence of PEG (+ PEG)



The effect of tannins on the nutritive value of feeds can be studied using tannin-binding agents, such as polyethylene glycol (PEG), which strongly binds to tannins and inhibits their biological effects. The percent increase in gas production when PEG is present indicates the rate at which tannins depress rumen fermentation of feeds.

After adding PEG to limit tannin effects, gas production increased by 22%, 71% and 211% in apple ring acacia *(Acacia albida)*, beach acacia *(Acacia cyanophylla)* and red calliandra *(Calliandra calothyrsus)*, respectively, which are browse plants.[33]

Rumen modifiers

The gas method is also utilized to study feed additives and rumen fermentation modifiers, such as monensin sodium, by incubating feeds in the presence or absence of these compounds. Rumen modifiers are compounds that are added to the diet to modify the populations of bacteria in the rumen. For example, some compounds are fed to reduce methanogenic bacteria to reduce methane production in the rumen. Previous studies have shown that the addition of saponins and tannins in an *in vitro* system increases microbial protein synthesis .[15] Yeast and yeast fermentation products are routinely added to the diets of lactating dairy cows, although their mode of action has not been clearly identified. By studying the impact

of various rumen modifiers on microbial fermentation, effects important to milk production in commercial dairy farms can be quantified.

Feed associative effects

The *in vitro* gas production method is currently being used to assess "associative" effects of feeds used in rations. Rations are mixtures of individual feeds, with a multitude of possible combinations. The energy value of a ration is generally calculated by adding up the energy values of the individual feeds in the ration, on the assumption that the individual energy value of any particular feed is the same in every possible combination with other feeds. However, this is not always true. For example, when poor quality forage — such as wheat straw — is fed to a ruminant, its digestibility is low, but by adding nitrogen in the form of urea or protein, the digestibility of the straw will be increased and in turn, the energy derived from straw organic matter in the diet will be increased. Recent studies indicate that positive associative effects on *in vitro* gas production occurred when rice straw was incubated in mixtures with hay or mulberry leaves.

Monitoring rumen microbial change

In addition to rates and extents of digestion, the gas production method can be used to study substrate related factors that influence microbial populations in the rumen. This enables manipulation of rumen microflora to increase the utilization of feeds through degradation of fiber and lignin, increasing the efficiency of nitrogen utilization or allowing the degradation of anti-nutritional and toxic components of feeds. Such controlled fermentation systems could potentially be used with genetic engineering of plants to solve animal productivity problems.

The technique is suitable for application of molecular-based assays, such as polymerase chain reaction (PCR) and ribonucleic acid (RNA)–targeted oligonucleotide probes, to study and measure rumen microbial growth, with the goal of increasing the efficient utilization of feeds and reducing environmental impacts. Recently, Muetzel and Becker (2003) used the gas technique, in combination with ribosomal RNA targeted probes, to measure the efficiency of microbial growth when barley straw was supplemented with legume leaves.

Nutrient synchronization

Carbohydrate and nitrogen sources must be available simultaneously in order to maximize microbial growth. Ruminal ammonia concentrations can be influenced by the degradation rates of carbohydrates and nitrogen-containing compounds. For a given level of dietary protein, an increased rate of protein degradation enhances the ruminal ammonia concentration while an increased rate of carbohydrate degradation decreases it. Increased carbohydrate availability for fermentation promotes microbial growth and as a result less nitrogen is lost from the rumen in the form of ammonia-nitrogen.[21] The gas method offers an opportunity to study microbial requirements for nitrogen and carbohydrate to enable efficient fermentative activity and accumulation in the rumen. Using this technique, studies have been conducted to assess rumen microbial requirements for nitrogen when different types of carbohydrate sources are incubated.

Plant breeding and biotechnology

We believe that animal nutritionists and plant geneticists should collaborate to select genetic materials that have better agronomic performance and superior nutritional qualities. Siaw *et al* (1993) used the gas technique to evaluate large numbers of browse species in order to select those high in feeding value. Browse is the edible parts of woody vegetation such as leaves, stems and twigs from bushes common on California foothills; they have been identified as integral to the development of fires that ravaged Southern California in the fall of 2003. Several forage and cereal crops have been genetically modified to increase yield, or produce chemical constituents normally deficient in a particular plant.

Plants have also been genetically engineered to produce human lysozyme, but it is unclear what effect lysozyme has on microbes in the rumen. Although many genetically engineered plants are intended for human consumption, their byproducts will be fed to animals as a means of disposal. The starch contained in cereals, including corn and milo, is found in granules surrounded by a tough protein matrix that reduces enzymatic degradation. There are new genetic varieties of these cereals with modified protein matrices. We are currently using the gas production method to explore whether these new varieties increase the extent and rate of starch digestion.

Environmental degradation

More than half of the nutrients consumed by ruminants leave the animal unutilized and undigested, and are excreted in feces, urine and gases. This increases animal production costs as well as environmental impacts, by contaminating surface and groundwater and contributing to air pollution. The nitrogenous and organic compounds excreted are further decomposed and can cause odors in residential areas. Increasing the efficiency of feed utilization reduces the amount of unutilized nutrients leaving the animal. Significant reductions in nitrogenous compounds and in methane can be achieved by manipulating animal diets. The *in vitro* gas method can be used to study the efficiency of feed utilization and to examine animal waste components that impact the environment in order to develop appropriate mitigation strategies.

Factors affecting the accuracy of the IVGPT

Many factors such as sample preparation and size, buffer and media, incubation conditions and time of reading, host animal management or material sampled, combine to influence activity of the microbial inoculum. That the prime purpose of the inoculum is to provide a suitable microflora with which to ferment or degrade a feed over time, and to use the outcome, for example, to provide an estimate of rate of in vivo digestibility, seems to have been overlooked. This has resulted in a series of statistically significant correlations, which are of little predictive use.

Considerable research has been conducted to reduce the requirement to surgically modify animals, and this is to be applauded. However, and while of no direct consequence, it should be recognised that faecal and rumen inocula are dissimilar. In addition, the methodologies used need to be fully described and appropriate conclusions drawn. It would appear that under certain conditions, for example, where long term in vitro end-point degradation assays are completed, that faeces have the potential to replace rumen fluid.

However, where precise fermentation kinetic data are required, the data suggests that fresh rumen fluid must be used. In vitro methodologies, in particular those such as gas production that are based on estimating the rate of fermentation, are highly adaptable and powerful research tools. To ensure their optimum application, it is vital that controls exist on the type and quality of inoculum. To this end, a series of research programs is required to address specific issues.

Considering the range of *in vitro* methodologies and equipment employed, it is unlikely that a particular system will be accepted as the 'standard' procedure. However, a great deal can be done to reduce variation among inocula by adopting an agreed upon set of guidelines relating to the host animal, sampling technique and inoculum preparation. A method to assess inoculum 'quality' needs to be developed with respect to its fermentative and/or degradative activity. This would allow the impact of preparation techniques to be accurately assessed. Equally a technique to store rumen fluid without loss of efficacy would allow use of rumen contents obtained following slaughter of known donor animals to be used, so obviating the requirement for surgically modified animals or use of faecal inocula. Finally, alternative approaches such as statistical mapping of fermentation profiles to generate estimates of degradation, need further investigation.

Advantages and limitations of the IGPT

The *in vitro* rumen fermentation method in which gas production and microbial mass

production are concomitantly measured has several major advantages:

It has the potential for screening a large number of feed resources, for example in breeding programmes for the development of varieties and cultivars of good nutritional value?it could also be of great value in the development of supplementation strategies using locally available conventional and non-conventional feed constituents to achieving maximum microbial efficiency in the rumen.

It has an important role to play in the study of rumen modulators for increasing efficiency of microbial protein synthesis and decreasing emission of methane, an environmental polluting gas. It provides a better insight into nutrient-anti-nutrient and anti-nutrient-anti-nutrient interactions. The method is also being used increasingly to screen plant-derived rumen modulators. These products have a lower toxicity to animals and humans, and are environmentally friendly. Consequently, they are becoming increasingly popular with consumers.

Further studies are required on:

- the development of simple approaches for identifying the incubation time in the *in vitro* gas system at which the PF (a measure of the proportion of fermented substrate which leads to microbial mass production) is maximum,
- the effect of nitrogen in the incubation medium on the PF,
- the *in vivo* significance of the PF so obtained.

The results of the limited experiments conducted so far have shown that simple models employing gas kinetic parameters and the PF are capable of predicting the dry matter intake of roughages and level of emission of methane by ruminants. Experiments also need to be done to test whether, for any given feed, the microbial protein synthesis as derived from digestion kinetic parameters (including PF) *in vitro* is sufficient to explain the observed microbial protein supply to the small intestine *in vivo*. At present, the simplest way of determining the latter parameter is to calculate it from the level of urinary purine derivatives.

This validation exercise should be conducted for a wide range of feed constituents and diets which should enable the above mentioned simple technique of measuring gas and microbial mass to be a routine and powerful tool for feed evaluation thus avoiding the need for timeconsuming, laborious and expensive feeding studies. Lately, much emphasis has been given to the development of statistical or mathematical models, which fit best the gas production profiles and describe the gas evolution with high accuracy.

Experiments must be designed to understand the biological significance of the various statistical and functional parameters being calculated using these models, and also to incorporate a measure of microbial mass into these mathematical descriptions.

Enhancement of the feeding value of tannin-rich feeds can be achieved by anaerobic storage in the presence or absence of urea, by the use of oxidising agents, by the treatment with white-rot fungi or by the use of PEG, preferably in a slow release form. PEG can be added to forages rich in tannins along with an energy supplements or to tannin-rich byproducts low in energy with the aim of synchronising nitrogen degradability and availability of energy and thus increasing the efficiency of microbial protein synthesis.

PEG is best given as an ingredient of nutrient blocks. so that not only will it enhance the incorporation of the feed nitrogen into microbial mass but will also allow the livestock to self-regulate the intake of PEG, thereby decreasing the cost of the treatment. The aim of future studies should be to explore the potential of these approaches for a wide range of tannin-containing feeds, and then to develop simple and economically viable detanninification approaches for use by farmers for feed resources such as foliage from trees and shrubs and for other available by-products.

Other techniques will be required for use by small-scale industry to treat agro-industrial and forestry by-products which are available in large quantities in one place. These approaches will help to alleviate the problems posed by the disposal of various agro-industrial byproducts and the shortages of conventional feeds.

Conclusions

The *in vitro* rumen fermentation method in which gas production and microbial mass production are concomitantly measured has several major advantages:

i) it has the potential for screening a large number of feed resources, for example in breeding programmes for the development of varieties and cultivars of good nutritional value,

ii) it could also be of great value in the development of supplementation strategies using locally available conventional and unconventional feed constituents to achieving maximum microbial efficiency in the rumen;

iii) it has an important role to play in the study of rumen modulators for increasing efficiency of microbial protein synthesis and decreasing emission of methane, an environmental polluting gas, and

iv) it provides a better insight into nutrient-antinutrient and antinutrient-antinutrient interactions, and into the roles of various nutrients (by changing the composition of the incubation medium) with respect to production of fermentative gases, SCFA and microbial mass. The method is also being used increasingly to screen plant-derived rumen modulators. These products have a lower toxicity to animals and humans, and are environmentally friendly. Consequently, they are becoming increasingly popular with consumers.

Further studies are required on:

i) the development of simple approaches for identifying the incubation time in the *in vitro* gas system at which the PF (a measure of the proportion of fermented substrate which leads to microbial mass production) is maximum,

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

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

biological activities of tannins as well as measuring tannin levels by chemical methods.

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