APPLICATIONS OF THE *IN VITRO* GAS METHOD IN THE EVALUATION OF FEED RESOURCES, AND ENHANCEMENT OF NUTRITIONAL VALUE OF TANNIN-RICH TREE/BROWSE LEAVES AND AGRO-INDUSTRIAL BY-PRODUCTS

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Abstract

APPLICATIONS OF THE *IN VITRO* GAS METHOD IN THE EVALUATION OF FEED RESOURCES, AND ENHANCEMENT OF NUTRITIONAL VALUE OF TANNIN-RICH TREE/BROWSE LEAVES AND AGRO-INDUSTRIAL BY-PRODUCTS.

A major constraint to increasing livestock productivity in developing countries is the scarcity and fluctuating quantity and quality of the year-round supply of conventional feeds. In order to meet the projected high demand of livestock products, and to fulfil the future hopes of feeding the millions and safeguarding their food security, the better utilisation of non-conventional feed resources which do not compete with human food is imperative. There is also a need to identify and introduce new and lesser known food and feed crops capable of growing in poor soils, which can play a vital role in control of soil erosion, bring economic benefits to farmers, enhance biodiversity, create jobs and bridge the wide gap between supply and demand for animal feeds. This paper highlights the potential of a novel approach using an *in vitro* rumen fermentation technique for evaluation of nutritional quality of non-conventional feed resources; many of the considerations discussed also apply to conventional feeds. This technique enables selection of a feed for high efficiency of microbial protein synthesis in the rumen along with high dry matter digestibility, and provides a basis for development of feeding strategies to better synchronise energy and nutrient release to maximise substrate fixation into microbial cells. This could lead to increase in the supply of protein to intestine and reduce methane production from ruminants. Tannins are the most widely occurring anti-nutritional factor in non-conventional feeds. General approaches for detanninification and increasing nutritional value of tannin-rich feed resources are also discussed.

1. INTRODUCTION

A major constraint to livestock production in developing countries is the scarcity and fluctuating quantity and quality of the year-round feed supply. These countries experience serious shortages in animal feeds of the conventional type. Food grains are required almost exclusively for human consumption. The world population is increasing at a very high rate and most of the population growth is occurring in developing countries. With increasing demand for livestock products as a result of rapid growth in the world economies and shrinking land area, future hopes of feeding the millions and safeguarding their food security will depend on the better utilisation of non-conventional feed resources, which can not be used as food for humans. 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 better utilisation of already known non-conventional resources but also 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, bring economic benefits to farmers, create jobs and bridge the wide gap between supply and demand for animal feeds. An important class of non-conventional feeds is by-product feedstuffs which are obtained during harvesting or processing of a commodity in which human food or fibre is derived. The amount of by-product feedstuffs generally increases as the human population increases and economies grow. The role of

ruminants must increasingly be one of scavenging to make use of by-products, residues and other non-conventional feeds. In developing countries, ruminants are fed low quality roughages in various proportions depending on the type of animal and season. These feeds are poor in protein, energy, minerals and vitamins. Addition of foliage from tree spp. *Leucaena, Glyricidia, Calliandra, Acacia*, etc. in ruminant diets can improve the utilisation of low quality roughages mainly through the supply of protein to rumen microbes, but the presence of tannins in these tree foliages prevents not only their optimal utilisation but also that of the roughages and by-products. Tannins are generally present in high amounts in tree foliage and agro-industrial by-products [1, 2].

This paper highlights the potential of a novel approach using an *in vitro* rumen fermentation technique for evaluation of the nutritional quality of conventional and non-conventional feed resources, and to describe general approaches for improving the nutritional quality of tannin-rich non-conventional feed resources such as agro-industrial and forestry by-products and tree and shrub foliage. The reason for choosing tannin-rich non-conventional feed resources is that tannins are the most widely occurring anti-nutritional factors found in plants.

2. EVALUATION OF FEED RESOURCES

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

There are a number of methods used to determine net microbial protein synthesis in the rumen based on the use of microbial markers. They require the use of post-ruminally cannulated animals to determine flow rate of digesta. The cannulation approach is tedious and has several limitations [3] 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 [4]. This approach is being thoroughly investigated under a joint FAO/IAEA Coordinated Research Project [5]. 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.

2.1. *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 one 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 as in Tilley and Terry [6] or using a gas method [7], 2) *in situ* incubation of samples in nylon bags in the rumen [8], and 3) cell-free fungal cellulase [9]. 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 [10].

2.2. *In vitro* gas method

Several gas measuring techniques and *in vitro* gas methods are in use by several groups. Advantages and disadvantages of these methods are discussed by Gatechew et al. [11]. The *in vitro* gas method based on syringes [7, 12] appears to be the most suitable for use in developing countries. The *in vitro* gas method is more efficient than the *in sacco* method in evaluating the effects of tannins or other anti-nutritional factors. In the *in sacco* method these factors are diluted in the rumen after getting released from the nylon bag and therefore do not affect rumen fermentation appreciably. In addition, the *in vitro* gas method can better monitor nutrient-anti-nutrient and anti-nutrient-anti-nutrient interactions [13].

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 [7]. 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. [7], fermentation is conducted in 100 mL capacity calibrated glass syringes containing the feedstuff and a buffered rumen fluid. The gas produced on incubation of 200 mg feed dry matter after 24 h of incubation together with the levels of other chemical constituents are used to predict digestibility of organic matter determined in vivo and metabolizable energy.

For roughages, the relationships are:

$$OMD\ (\%) = 14.88 + 0.889 * Gv + 0.45 * CP$$
$$ME\ (MJ / kg\ DM) = 2.20 + 0.136 * Gv + 0.057 * CP$$

where, OMD is organic matter digestibility (%); ME, metabolizable energy; CP, crude protein in percent; and Gv, 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.

The method of Menke et al. [7] was modified by Blümmel and Orskov [14] in that feeds were incubated in a thermostatically controlled water bath instead of a rotor in an incubator. Blümmel et al. [12] and Makkar et al. [15] 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. [7] 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. In the 40 mL system, the linearity is lost when the gas volume exceeds 130 mL [16]. 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 in the 30 mL system and 130 mL in 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:

- 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,
- 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 a power failure for a short duration,
- an increase in amount of sample from 200 to 500 mg reduces the inherent error associated with gravimetric determination needed to determine concomitant *in vitro* apparent and true digestibility (see below).

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

The gas produced in the gas technique is the direct gas produced as a result of fermentation and the indirect gas produced from the buffering of short chain fatty acids (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 levels 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. A highly significant correlation has been observed between SCFA and gas production (see below).

Gas is produced mainly when substrate is fermented to acetate and butyrate. Substrate fermentation to propionate yields gas only from buffering of the acid and, therefore, relatively lower gas production is associated with propionate production. The gas which 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: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 a lower acetate to propionate ratio in the ruminal fluid of cows fed a high grain diet. If fermentation of a feed leads to a higher proportion of acetate, there will be a concomitant increase in gas production compared with a feed producing 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 [14], a wide range of feeds including many dairy compound feeds and their individual feed components, whose protein and fat contents vary greatly [17], and tannin containing browses [18] in the 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 [19] stoichiometry, which is as follows:

Fermentative CO_2 = A/2+P/4+1.5*B; where A, P and B are moles of acetate, propionate, and butyrate respectively.

Fermentative CH_4 = [A+2*B]-CO_2; where A and B are moles of acetate and butyrate respectively and CO_2 is moles of CO_2 calculated from 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*gas constant (R)*T;
where
R = the ratio between molar volume of gas to temperature (k) [22.41L/273 = 0.082],
T = incubation temperature; 273 + 39°C = 312 k.
Total volume of gas (ml) calculated from SCFA production = [BG + FG]*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 [17].
(The volume of 1 mmol of gas at 39°C in Hohenheim would be; 1*0.082*312*0.953 = 24.4 mL).

The origin and stoichiometry of gas production have been described in details by Blümmel et al. [12] and Gatechew et al. [11].

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) was [18]:

In the absence of PEG; SCFA = –0.0601 + 0.0239 * Gas; R^2 = 0.953; n = 39; P <0.001
In the presence of PEG, SCFA = 0.0521 + 0.0207 * Gas; R^2 = 0.925; n = 37; P <0.001
Overall, SCFA = –0.00425 + 0.0222 * Gas; R^2 = 0.935; n = 76; P <0.001
These relationships are similar to that obtained for wheat straw [20].

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 equipment 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.

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

2.3.1. Need for determination of microbial mass

Lately, the surge of interest in the efficient utilisation of roughage diets has caused an increase in the use of gas methods because of the possibility of estimating the extent and rate of gas production in one sample by time series measurements of the accumulating gas volume. *In vitro* gas tests are attractive for ruminant nutritionists since it is very easy to measure the volume of gas production with time, but the measurement of gas only implies the measurement of nutritionally wasteful and environmentally hazardous products. In most studies the rate and extent of gas production has been wrongly considered to be equivalent to the rate and extent of substrate (feed) degradation. Current nutritional concepts aim at high microbial efficiency, which cannot be achieved by measurement of gas only. *In vitro* gas

measurements reflect only SCFA production. The relationship between SCFA and microbial mass production is not constant and the explanation for this resides in the variation of biomass production per unit 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. [12] 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. The *in vitro* microbial mass production can be calculated as:

Microbial mass = Substrate truly degraded — (gas volume × stoichiometrical factor).

For roughages, the stoichiometrical factor was 2.20.

2.3.2. 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. 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; [21]) and in methane production by ruminants (the higher the PF, the lower the methane output; [22]). These results show that the PF calculated *in vitro* provides meaningful information for predicting the dry matter intake, the microbial mass production in the rumen, and the methane emission of the whole ruminant animal.

The procedures for the determination of truly degraded substrate and the calculation of the stoichiometrical factor; stoichiometrical relationship between SCFAs and gas volume; and relationship between SCFA production, ATP production and microbial mass yield can be obtained from Blümmel et al. [12] and Getachew et al. [11]. 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 [23] have shown that, as observed by Blümmel et al. [12], 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 which is truly degraded.

It seems therefore justified to suggest that feeds or feed ingredients should be selected that have a high *in vitro* true degradability but low gas production per unit of truly degraded substrate.

2.3.3. Role of incubation time in PF determination

Another study by Blümmel et al. [24], in addition to once again describing the importance of measuring microbial mass, has highlighted the importance of the fermentation time at which the microbial mass should be measured. In this study, substrate degradation and kinetics of *in vitro* partitioning of three hays, with similar *in vivo* digestibilities, into SCFA, microbial mass yield, and ammonia, carbon dioxide and methane production was examined at 8, 12, 18 and 24 h of incubation in the gas method under both low and adequate nitrogen levels. Microbial synthesis was quantified gravimetrically [12], by nitrogen balance [25] and by purine analysis [26]. SCFA and gas production were positively correlated ($P < 0.01$) and cumulative at all times of incubation under both low and adequate nitrogen levels. On the

other hand, microbial mass, microbial nitrogen and microbial purine yields declined after 12 h of incubation while ammonia production increased. Per unit of substrate degraded gas and SCFA production were always inversely ($P < 0.05$) related to microbial mass yield regardless of incubation time and medium (low or adequate nitrogen). At later incubation times, continuously more SCFA or gas and less microbial mass were produced reflecting microbial lysis and probably increasing microbial energy spilling. All three hays differed ($P < 0.05$) consistently in how the degraded substrate was partitioned into SCFA and gas and into microbial mass in both the low and adequate nitrogen medium. Purine analysis indicated substantial differences in microbial composition across treatments, which might be one explanation for these different microbial efficiencies [24].

The efficiency of microbial growth was higher for 16 h incubation than 24 h for tannin-rich feeds when these were incubated in the presence or absence of PEG, a tannin-inactivating agent. Additional nitrogen in the medium also affected the efficiency of microbial protein synthesis from tannin-tannins feeds both at 16 h and 24 h [27]. Approaches need to be developed for measuring the PF at the incubation time at which the lysis of microbes is minimal. Some possible approaches worth investigating to identify this incubation time are:

i) the time at which half of the maximum gas is produced, and
ii) the inflection point at which the rate of gas production is maximum (the rate increases up to a certain incubation time and thereafter decreases as the incubation progresses).

Both these parameters can be mathematically calculated from the gas profiles. The effect of the nitrogen level in the medium on the PF and the significance *in vivo* of these PFs also need to be investigated.

2.3.4. 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 [28]. The subtraction procedure might give some useful information relevant to low-NDF fibre feeds, e.g., corn grain [28], but it is not suitable for forages rich in NDF [29]. Blümmel et al. [29] examined the rate and extent of fermentation of whole roughage and extracted NDF, dry matter degradability of extracted NDF and the PF for whole roughage and the extracted NDF of 54 roughages. The 24-h degradabilities of extracted NDF were higher than NDF degradabilities in whole roughages, and the PF values were lower for extracted NDF than for whole roughages (2.5 vs 3.1; i.e. the efficiency of microbial protein synthesis with extracted NDF was lower). Both the higher degradability and lower PF contributed to higher gas volumes obtained from extracted NDF compared with whole roughage. Supplementation of amino acids and sugars, which essentially constitute the solubles, may increase the efficiency of microbial synthesis from cell walls during fermentation (a situation similar to that in unfractionated forages) and the removal of solubles may result in lower microbial efficiencies. A considerable effect of cell solubles on partitioning of nutrients from the NDF raises doubts as to the significance *in vivo* of the kinetic parameters calculated using the subtraction procedure [29].

2.4. Evaluation of silages and by-products rich in acids by the gas methods

Caution is required in the evaluation of acid-containing silages and by-products using the gas methods. Some silages [30] and by-products such as citrus pulp and distillery by-products can contain a substantial amount of acids. Since the gas methods [11, 12] are based

on incubation of the feed in a bicarbonate-based buffer, the acid present in the feed will release carbon dioxide from the buffer, which might be mistaken for fermentation gases, leading to erroneous results. This acid-base reaction is spontaneous and the gas released due to the neutralisation of acids can be measured within 5 to 10 min of the start of the incubation. It should therefore be subtracted from the total gas production volumes measured at different times of fermentation before these gas values are used for evaluation of such feeds.

2.5. Protein degradability using the gas method

A method, based on the gas method [12] and that of Raab et al. [31], which measures gas production and ammonia in the medium has also been developed for measuring rumen degradability of nitrogen from low quality feeds [11]. This method could be useful in developing sound supplementation strategies and increasing the efficiency of utilisation of non-conventional feed resources.

Although the 24-h *in vitro* degradable nitrogen values obtained from tannin-rich browse and herbaceous legumes were lower than those reported for low quality roughages [11, 25], the relatively high crude protein content of these browses and herbaceous legumes could play a significant role in supplying rumen degradable nitrogen. In the presence of a tannin-inactivating agent, PEG, their *in vitro* degradable nitrogen values were raised. The difference between these values observed in the presence and absence of PEG indicates the amount of protein protected by tannins from degradation in the rumen [25]. Whether the protein protected by tannins from microbial degradation is fully available to animals post-ruminally requires further research. Raab et al. [31] reported a close relationship between *in vivo* and *in vitro* values when incubation was terminated after 17 h. When normal protein feeds were tested about 80% of the 24 h value was degraded in the first 8 h incubation whereas in protected protein feed only 60% of the 24 h value was degraded in this time [31]. The appropriate incubation time for *in vitro* degradability studies in the presence and absence of PEG for tannin-rich feeds may depend on the nature of protein and tannins, which should be identified. Also, this method for quantifying feed proteins that have been protected by tannins from degradation in the rumen needs to be validated *in vivo*.

Measurement of feed proteins during fermentation using polyacrylaminde gel electrophoresis coupled with the use of an image analyser could be another attractive approach for measuring the rumen degradability of nitrogen, and for studying the influence of various natural plant products (e.g., tannins, saponins, alkaloids [32]).

2.6. Evaluation of tannin-containing by-products and forage

2.6.1. The need to determine microbial mass using internal or external markers

The approach mentioned above used data from the gas method and the detergent system of fibre analysis to calculate the microbial mass produced during the fermentation of fibrous feeds. Unfortunately, this method did not work at all for tannin-rich feeds. The PF for tannin-rich feeds (calculated as mg truly degraded substrate needed to produce one-ml gas) ranged from 3.1 to 16.1 [25] which is well above the theoretical range of PF (2.75 to 4.41) [12]. The high PF could be due to: a) solubilization of tannins from the feed. These tannins would make no contribution to gas or energy in the system but would contribute to dry matter loss, b) the cell solubles contributing to dry matter loss but not to gas production because the gas production is inhibited by tannins or c) a combination of a) and b). In addition, the appearance of tannin-protein complexes as artefacts in the true residue also makes the gravimetric approach [12] of quantification of microbial mass redundant [33]. The presence of

tannin-protein complexes in faecal samples (the origin of proteins could be microbes, feed or endogenous secretion from the gastro-intestinal tract) from animals fed tannin-rich forage and their non-removal by the detergent system of fibre analysis [34] leads to misleading values of fibre and also causes problems in the *in vivo* evaluation of tannin-rich feeds [35, 36]. Therefore, caution is required in interpreting results obtained from *in vivo* or *in vitro* experiments on the evaluation of tannin-containing feeds using the detergent system of fibre analysis.

For the *in vitro* evaluation of tannin-rich feeds, the microbial mass should be quantified using diaminopimelic acid or purines as markers, or by ^{15}N incorporation into the microbes [32], and the PF for tannin-rich feeds can be expressed as the microbial mass determined by these markers per ml of gas produced (or per mmol SCFA produced). The system developed for evaluation of tannin-containing feeds depends on incubation of the feed in the presence and absence of polyethylene glycol (PEG MW 4000 or 6000 preferably of 6000; [15]) and measurement of gas (or SCFA) and microbial mass using the above-mentioned markers. PEG has a high affinity for tannins. Addition of PEG results in the formation of PEG-tannin complexes which inactivates tannins. The changes in gas (or SCFA) and microbial mass as a result of PEG addition represents 'in totality' the tannin effects (biological) as a function of the rumen fermentation parameters. This bioassay based on an *in vitro* rumen fermentation system coupled with the use of a tannin-complexing agent, polyethylene glycol (PEG) could be complementary to other tannin assays [37, 38] in evaluating the nutritional quality of tanniniferous feeds.

2.6.2. Significance of bound tannins and the efficiency of microbial protein synthesis

The above approach of incubating tannin-containing feeds in the presence and absence of PEG also enables studies to be made on the nutritional significance of both extractable and unextractable (bound) tannins. Addition of PEG during the incubation of tannin-rich NDF led to an increase in gas production, suggesting that tannins released as a result of NDF degradation by rumen microbes are biologically active and have the potential to influence rumen fermentation [39]. Similar results were obtained on incubation of tannin-rich browses made free of extractable tannins by repeated use of 70% aqueous acetone. Another application of this method is to study the effect of tannins on the partitioning of nutrients between microbial protein and SCFAs or gases or to study the efficiency of rumen microbial protein synthesis. Using DAPA, purines and ^{15}N approaches for measuring microbial mass it has been shown that in the presence of PEG, the degradabilities of substrate and microbial mass production were higher, the efficiency of microbial protein synthesis was lower. Similar results have also been obtained by another approach based on the gas method in which the rate of ammonia uptake is taken as the efficiency of microbial protein synthesis [32]. Conversely, efficiency of microbial protein synthesis is expected to be higher in the presence of tannins. The net microbial mass production would depend on the balance between decreased degradable dry matter and higher microbial mass production per unit of dry matter digested in presence of tannins.

3. ENHANCEMENT OF FEEDING VALUE

Various studies have been conducted aimed at the detanninification of tannin-rich feeds. Two types of approaches were developed, one for farmers and the other for small-scale industries. The capabilities of farmers and small-scale industries were kept in mind so that the approaches could be easily adopted by the end users.

3.1. Farmers-based approaches

Oak and pine wood are generally used as fuel in rural areas of the Himalayan region in India and adjoining countries. A 10% solution of ash (pH, 10.5–11) decreased tannin levels in mature oak leaves by up to 80% [40]. Magadi soda (sodium sesquicarbonate, sodium carbonate and sodium bicarbonate), another unrefined material containing alkalis has also been shown to reduce assayable tannins in sorghum by 40–50%. Wood ash solutions have also been used traditionally for the treatment of high-tannin containing sorghum and millet for human consumption (for references see [40]). The use of wood ash, a cheap source of alkali holds potential for the detanninification of tannin-rich feedstuffs.

Other major findings were: i) a substantial reduction in the tannin content (72–89%) on storage of fresh leaves, containing 55% moisture and 4% urea, for five days, and ii) a reduction in tannin content of 46–60% on storage of chopped leaves for 10 day [41]. The 'chopping of leaves followed by storage' can find practical application for the farmers and can be adopted easily as it requires only a slight change in normal management practices. Instead of feeding the leaves on the same day as they are lopped, chopped and stored for about five to ten days before feeding. The use of urea calls for resources and some degree of education, the lack of which has hindered the adoption of this approach. The higher extent of inactivation of tannins by chopping leaves could be due to oxidation of tannins by phenol oxidases present in leaves because chopping should increase the availability of tannins to the enzyme. In addition, inactivation of tannins during storage is due to their polymerization to higher 'inert' polymers [41, 42]. Another factor responsible for enhanced effects observed on addition of urea could be the higher pH caused [43] by the evolution of ammonia from urea. Similarly, inactivation of tannins observed when using wood ash is also mediated by high pH-mediated oxidation of tannins.

3.1.1. Approaches for small-scale industries.

Drying under different conditions [44], steaming and autoclaving [45] were found to be not very effective for oak leaves. Extraction with organic solvents (acetone, methanol, ethanol) and treatment with oxidising agents (potassium dichromate, potassium permanganate and alkaline hydrogen peroxide) were very effective and removed/inactivated up to 90% of the tannins in oak leaves [46] and up to 99% in agro-industrial and forestry by-products [1]. The use of oxidising agents holds promise for the large-scale detoxification of tannin-rich feedstuffs because of its low cost. These approaches are very simple, do not require complex equipment and are likely to be adopted by the feed industry in the future both in developing and developed countries. In addition, potassium permanganate can be made easily available in villages in developing countries (generally used for cleaning water in wells) and is non-corrosive. Hence farmers can use this chemical at home for detanninification of tannin-rich feedstuffs. The use of organic solvents for extraction of tannins has an advantage over oxidising agents because the solvents can be largely recycled and the tannins can be recovered for the tanning of leather or for other industrial applications. The oxidising agents convert tannins to quinones, which are not capable of forming complexes with proteins under normal physiological conditions. However, the use of organic solvents is expected to be more expensive, unless the value of tannins recovered is higher than the cost of organic solvents used in the treatment.

The white-rot fungi (*Sporotricum pulverulentum, Ceriporiopsis subvermispora* and *Cyathus steroreus*) which degrade lignin, were also found to decrease tannin content by about 60% in 10 to 20 days of fermentation [47, 48]. This approach though presently in its infancy, may also find a place in industry in the future.

The above technologies for the detanninification of tree leaves on a small scale industrial level do not seem to be economically viable because large quantities of leaves are seldom available in one place (unlike agro-industrial by-products) and the cost of collecting them is high. There is a lack of information on the effects of judicious lopping of tree leaves and strict control, especially in developing countries, on the extent of lopping. Even if the information were available, its application would seems to be difficult, particularly when the interests of industries in detannifying or making protein supplements from trees or shrubs are at stake. However, the detannification technologies presently available may well set the stage for the utilization of agro-forestry by-products including tree pods.

3.1.2. Use of PEG by farmers and by the feed industry

Addition of a tannin-complexing agent, PEG to tannin-rich diets is another attractive option to enhance the feeding value of such diets. This approach can be used both by farmers and by the industry. Farmers can give PEG directly to animals through water, by mixing it with a small amount of concentrate, by spraying it on tannin-rich feedstuffs or better still as a part of nutrient blocks (see below). Industry can incorporate PEG in a pelleted diet composed of ingredients including tannin-rich by-product(s). Amongst various tannin-complexing agents investigated, PEG of molecular weight 6,000 was the most effective in binding to tannins at near neutral pH values [15]. Incorporation of PEG has been shown to have beneficial effects in monogastrics and both beneficial and adverse effects in ruminants. The incorporation of PEG had beneficial effects for feedstuffs such as *Quercus calliprinos, Pistacia lentiscus, Ceratonia siliqua* [49–51], *Zizyphus nummularia* [52], *Hedysarum coronarium* [53], *Acacia aneura* [54–56], *Lotus pendunculatus* [57–58], *Desmodium ovalifolium* and *Flamingia macrophylla* [59], and *Acacia saligna* [60] which are rich in tannins (condensed tannin content: 5–10%). Inactivation of tannins through PEG increased the availability of nutrients and decreased microbial inhibition, which in turn increased degradability of nutrients leading to better animal performance. On the other hand, for *Lotus corniculatus*, the condensed tannin content of which varied from 2 to 4%, addition of PEG decreased wool growth, weight gain [61–62] and milk yield [63]. These decreases were attributed to a substantially lower absorption of amino acids from the intestine resulting from increased digestion of proteins in the rumen [64]. Addition of PEG to a diet based on *L. corniculatus* which containing 1% condensed tannins did not have any effect [65], suggesting that 1% condensed tannin from *L. corniculatus* may be insufficient to protect feed protein from degradation in the rumen. The lower performance of ruminants whose diets are supplemented PEG could also be due to lower efficiency of microbial protein synthesis in the rumen ([32]; see above). Not only the concentration of tannins, but also their nature influences the response of animals to PEG incorporation. A diet containing 1.8% condensed tannins from *L. pedunculatus* caused significant reduction in the levels of rumen ammonia and SCFAs and reduced nitrogen digestibility, whereas the same level of condensed tannins (1.8%) from *L. corniculatus* had lesser effects [64]. The effect of PEG also depends on the level of proteins in the diet. Proteins mimic the effect of PEG — the higher the level of proteins, the lesser the effect of PEG [66]. It is evident from the literature that addition of PEG is advantageous when the tannin content of the feed is high and is deleterious when the tannin content is low. There is some evidence that activity of tannins from tropical plants (expressed as protein precipitation capacity per unit of tannins) is much greater than those from temperate plants [2], and therefore for tropical plants the addition of PEG might be beneficial even when the levels of condensed tannins are low. Characterising tannin-containing feeds in terms of parameter(s) representing the biological activity of tannins, such as protein precipitation capacity, will give a better insight into the role of tannins and will be

more useful in the development of approaches for their inactivation than simply measuring the condensed tannin contents alone.

PEG has been incorporated into the diets at a level from 3 to 120 g per day, with varying responses. Supplementing sheep and goats, fed *Ceratonia siliqua* leaves, with 25 g of PEG/day seems to be the optimal amount in terms of the cost-benefit response under Israeli conditions [49]. Although this technique is quite effective, the likelihood of its adoption will depend on the cost-benefit ratio.

The effect of the manner of application of PEG (MW 6000) on some fermentation parameters after 24 h of incubation of tannin-rich feeds in the *in vitro* rumen fermentation system (mentioned above) has also been studied. PEG was applied as a single dose (51 mg) or in split doses (7 doses of 7.3 mg at 2 h interval starting at 0 h) in incubations containing *Calliandra* leaves (protein precipitation capacity: 0.45 mg BSA precipitated/mg feed). The gas and SCFA production increased substantially on addition of PEG. Purine as a marker for microbial mass was similar for the control and single application of PEG but with the split application it was higher. Efficiency of microbial protein production was also higher with the split application of PEG [37]. The implications of these results are that PEG given to animals on tannin-rich diets in the form of a molasses-mineral block (leading to a slow consumption of PEG) would lead to better animal performance than the currently used approaches which are based on a single dose in the drinking water or in a small amount of concentrate feed. Ben Salem and Nefzaoui [68] have shown that sheep fed nutrient blocks containing PEG have a higher intake of *Acacia* leaves, higher nitrogen retention, and greater urinary excretion of allantoin (a marker for microbial protein supply to the intestine) than controls without PEG. But it would be interesting to compare these parameters when PEG is fed as a part of nutrient blocks or given as a single dose mixed in water or in a small amount of concentrate. Under the auspices of the IAEA, projects are now being planned for improving the utilization of tanniniferous forages by feeding PEG-molasses-multinutrient blocks. When the animals lick these blocks the PEG will be released slowly, and this is expected to supply higher microbial protein post-ruminally as a result of higher efficiency of microbial protein synthesis mediated probably via better synchronisation of ATP production and release of nutrients.

Addition of PEG to tannin-containing feeds increased *in vitro* gas and SCFA production, and *in vitro* degradation of nitrogen. Therefore, there appears to be a potential for improving the utilisation of tannin-containing feeds by the use of tannin-binding agent such as PEG without altering the genetic pool of tannin-containing plants. Inclusion of energy sources with the aim of synchronising nitrogen degradability and availability of energy increased the efficiency of microbial protein synthesis in the presence of PEG [25]. A rapid degradation of nitrogen not matched to the availability of energy could lead to high absorption of NH_3N from the rumen *in vivo*. *In vivo*, the NH_3N not captured in the rumen is absorbed into the blood and converted into urea in the liver which requires expenditure of energy; each mole of urea requiring four moles of ATP. The loss of nitrogen as urea in urine is energetically costly, is a loss of a valuable nutrient and causes environmental pollution.

4. CONCLUSIONS AND FUTURE RESEARCH

Research and development efforts are required to establish a feed library for non-conventional 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.

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, and
- the *in vivo* significance of the PF so obtained.

The results of the limited experiments conducted so far have shown that simple models employing gas kinetic parameters and the PF are capable of predicting the dry matter intake of roughages and level of emission of methane by ruminants. Experiments also need to be done to test whether, for any given feed, the microbial protein synthesis as derived from digestion kinetic parameters (including PF) *in vitro* is sufficient to explain the observed microbial protein supply to the small intestine *in vivo*. At present, the simplest way of determining the latter parameter is to calculate it from the level of urinary purine derivatives. This validation exercise should be conducted for a wide range of feed constituents and diets which should enable the above mentioned simple technique of measuring gas and microbial mass to be a routine and powerful tool for feed evaluation thus avoiding the need for time-consuming, laborious and expensive feeding studies. Lately, much emphasis has been given to the development of statistical or mathematical models, 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 by-products 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 by-products and the shortages of conventional feeds.

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