The contribution of chemical constituents of fodder tree and shrub leaves to gas produced during *in vitro* fermentation in nitrogen free and nitrogen rich media

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Abstract

Samples of twenty fodder tree and shrubs species were analysed for their chemical constituents and fermented in an *in vitro* gas production system with nitrogen rich (165 mg N/l) and nitrogen free media. The chemical composition was correlated to gas production. Rather than predict the gas production simply from the chemical components, the objective of this analysis was to identify which were the main chemical entities contributing to gas production and the time at which such contributions were more important. A first fermentation was carried out for 166 hours with nitrogen rich medium. Multiple regression equations were produced by stepwise regression. Results showed that, under nitrogen rich medium, the main components from the leaves affecting the fermentation were: soluble carbohydrates between 3 and 6 hours, soluble protein between 9 and 12 hours, starch both at early (0 to 12 hours) and late stages (from 70 hours onwards) and total phenols and condensed tannins from 28 to 39 hours. Insoluble protein did not affect the fermentation at any time. Results highlight the importance of the soluble components of the leaves during the first stages of the fermentation with nitrogen rich medium. No a single chemical component could predict the gas production from 45 to 60 and from 94 to 166 hours indicating that from 45 hours onwards the chemical composition of the substrate has little or none effect on the gas production. This also suggests that short term fermentations can be carried out confidently as incubating the substrate for more than 70 hours does not add anything to potential gas production. A second fermentation was conducted for 70 hours incubating the plant material with nitrogen rich and nitrogen free medium. The main components affecting the gas production under a nitrogen free medium were identified, after a lag phase of about 6 hours, as: reducing sugars at 9 hours, starch from 12 to 33 hours and at 45 and 60 hours, insoluble protein from 39 to 45 hours, condensed tannins from 33 to 52 and at 70 hours, total phenols at 3 and at 48 hours, NDF at 3, 9 and 60 hours and ADF from 0 to 33 hours. Insoluble protein, condensed tannins and total phenols appeared together as the main components involved in the gas production at 45 hours. In the case of nitrogen rich medium, the best prediction equations were found in the early stages whereas, with nitrogen free medium, they appeared towards the end of the fermentation.
Keywords: Fodder trees and shrubs, gas production, nitrogen, nutritive value.

1. Introduction

In the tropics, fodder trees and shrubs are a very important source of nutrients. In particular they often contain high levels of crude protein which can be lacking in many other tropical forages such as grasses and crop residues. Leaves can constitute the whole ration, given as a mixture or as single feed, or they can be offered as a supplement. Despite the fact that the list of trees and shrubs with potential use as fodder comprises more than 300 species, the emphasis has been placed on very few. Therefore, there is a need to evaluate further species for their potential as animal feeds.

Chemical composition has been widely used to characterise feedstuffs. However, to assess nutritive value, it is also important to define parameters describing how they might be digested by the animal. *In vitro* methods exist which predict digestibility (Tilley and Terry, 1963) as well as *in situ* methods which estimate degradability (Mehrez and Ørskov, 1977). Recently, alternative *in vitro* methods have been developed which attempt to describe the kinetics of fermentation, by measuring the gas produced when substrates are incubated with rumen fluid (Menke *et al.*, 1979; Theodorou *et al.*, 1994). These methods have the potential to replace *in situ* methods and to evaluate large numbers of samples in a short time with minimal use of surgically modified animals. Although these techniques have been originally developed from experimentation with temperate forages they are now being used widely as a method of feed evaluation. Attempts have been made to relate gas production parameters to those determined using the *in situ* method (Khazaal *et al.*, 1993) and to *in vivo* parameters (Blümmel and Ørskov, 1993). However, the method is still not fully understood and there have been few attempts (Nsahlai *et al.*, 1994) to identify which feed components are primarily responsible for the gas production observed.

The media for *in vitro* digestibility systems (Tilley and Terry, 1963; Van Soest, 1975; Czerkawski and Breckenridge, 1977; Menke *et al.*, 1979; Merry *et al.*, 1987) are based on the composition of the artificial saliva described by McDougall (1948). The original *in vitro* gas production method (Menke *et al.*, 1979) used a medium which was nitrogen free. In 1988, Menke and Steingass changed the original composition of the medium adding nitrogen to the medium. The resultant medium is similar to the so called basal medium D described by Theodorou and Brooks (1990) for the gas production method of Theodorou *et al.* (1994). This nitrogen rich medium is the most widely used for determining the fermentation characteristics of animal feeds. However, In the case of feed evaluation, the effect of N may be of primary importance and for fodder trees this may be particularly relevant. Fodder trees often contain high levels of tannins and other complex chemical components. Tannins can bind to proteins, thus altering their availability to the microbes and ultimately the animal itself. Evaluation of these fodders in a medium rich in nitrogen may mask these effects which are important to understand.

The present trial aimed to study the fermentation of a range of tropical fodder trees and shrubs by rumen microbes in both nitrogen rich and nitrogen free media, in order to identify the main chemical entities contributing to gas production and the time at which such contributions were most important. This knowledge is important for further developing of the method, and to understand the factors affecting the nutritive value of browse, the associative effects of feed mixtures and the effect of supplements.

2. Materials and Methods

2.1. Fodder tree and shrub samples

Samples of leaves from twenty different plant species were collected in the Cauca valley in the south west of Colombia. This fertile valley is 1000 metres above sea level, with an average temperature of 23°C and an annual rainfall between 600 to 1200 mm. Preliminary studies have shown that farmers in the valley region used over 32 different plant species a year to feed their

animals. A list of the 20 species with most potential was made, based on their use by the farmers, palatability and degradability studies (Rosales *et al.*, 1992; Vargas, 1994). The samples were taken from the following plant species: *Malvastrum* sp., *Canavalia ensiformis* (L.) DC., *Dioclea sericea* (Humb. Bonpl. & Kunth), *Sapindus saponaria* (L.), *Amaranthus dubius* Mart., *Urera baccifera* (L.) Gaud., *Heliconia* sp., *Tithonia diversifolia* (Hemsl.) Gray, *Simphytum peregrinum* Lebed., *Bidens pilosa* (L.), *Clitoria ternatea* (L.), *Inga* sp., *Enterolobium cyclocarpum* (Jacq.) Griseb., *Erythrina edulis* Triana ex Micheli, *Erythrina poeppigiana* (Walp.) O.F. Cook, *Pithecellobium dulce* (Roxb.) Benth, *Prosopis juliflora* (Swartz.) DC., *Leucaena leucocephala* (Lam.) de Wit, *Trichanthera gigantea* (Humb. et Bonpl.) Nees. and *Gliricidia sepium* (Jacq.) Steud.

Samples of approximately 0.5 kg were collected from individual plants by harvesting leaves (young and adult) from the top, bottom, sunny and shady sides of the tree or shrub. The collected leaves were pooled and a subsample of approximately 1 kg was taken. The subsamples were dried in a forced-draught oven at 60°-70°C for 48 hours, ground in a hammer mill with a 2 mm mesh, packed in plastic bags and taken to the United Kingdom. The samples were ground again to a uniform particle size for the chemical analyses. This time, a high speed mill (Tecator MPF 570-010V) fitted with a fine mesh screen of 1 mm was used. The ground samples were stored in glass jars fitted with air-tight lids. For all methods of chemical composition, samples were analysed in duplicate with a maximum acceptable error of ± 2%.

2.2. Chemical analysis

The samples were analysed for their chemical composition in terms of dry matter, crude protein, ether extract, ash and organic matter according to conventional methods (MAFF, 1986).

The solubility of the protein in water was measured according to the method proposed by Wohlt *et al.*, 1973. The nitrogen content was measured in the liquid fraction obtained after centrifugation (2000 rpm x 10 minutes) by the Kjeldahl analysis (AOAC, 1984) and not in the dry residue after filtration as described in the original method.

Soluble carbohydrates were determined according to the method proposed by Thomas (1977). The starch, reducing and total sugars were estimated by the spectrophotometric method according to AOAC (1984).

The crude lignin, cellulose and silica fractions of the plant material were estimated by measuring the acid detergent fibre (ADF) and the cell wall material (lignin, cellulose and hemicellulose) by measuring the neutral detergent fibre (NDF), as proposed by Van Soest (1975).

2.3. Extraction and analysis of phenols

Each sample was accurately weighed (500 ± 10 mg) into a glass 10 ml beaker and homogenised for 1 minute in 5 ml of 70% aqueous acetone using an Ultra Turrax blender on medium power. Then the mixture was centrifuged at 2,500 rpm for 10 minutes. The same extract was used to determine tannins, total phenols and protein precipitation activity.

The samples were analysed for the protein precipitation activity (PPA) by the method of Hagerman (1987) as modified by Wood *et al.* (1994), condensed tannins (CT) by the acid butanol method (Hagerman and Butler, 1989) and total phenols (TP) by the Prussian blue method (Price and Butler, 1977). The TP method was adapted by taking an aliquot of 10 ml of extract instead of 100 ml, as the acetone extract used was more concentrated than that proposed in the original method.

Choosing the appropriate standard for tannins is critical, especially if meaningful biological results are to be obtained from the analyses. Tannin compounds that may be appropriate standards

for chemical analysis may be inappropriate to estimate the biological activity. Suitable tannin standards to determine the absolute amount of tannin in a given plant could only be obtained by purifying the standard from the plant of interest (Hagerman and Butler, 1989). The extraction and purification of tannin is labourious and complex, and demands huge quantities of plant material (Terrill *et al.*, 1992). This is adequate for studying phenolic compounds on a single species but impractical with a wide range of plant species. Because the objectives of this work were not to compare with data from other sources it was decided to express the total phenols and condensed tannin results as the maximum absorbance (optical density/g of DM).

2.4. In vitro fermentation

The gas production method of Theodorou *et al.* (1994) as outlined by Prasad *et al.* (1994) was used. This method uses the accumulation of gas pressure and gas volume in the head-space of culture bottles containing dried and ground forages incubated at 39°C with an anaerobic medium inoculated with rumen liquid. A pressure transducer connected to a digital readout voltmeter and a gas-tight syringe assembly is used to measure and release the accumulated gas pressure from the incubating culture bottles. The modifications to the original method are explained below.

Two separate fermentations were conducted. In the first one, the plant material was fermented for 166 hours, as in the original method, with a nitrogen containing medium. After analysing the results of the first fermentation, the second one was carried out for 70 hours. In this run, the plant material was fermented with nitrogen free and nitrogen containing medium to characterise the response to nitrogen. For both fermentations the amount of sample was 1 g total substrate, weighed to tolerance of ± 0.002 g. Samples were fermented in triplicates. The gas readings were made at 3, 6, 9, 12, 16, 20, 24, 28, 33, 39, 45, 52, 60, 70, 80, 94, 106, 118, 142 and 166 hours for the first fermentation and followed the same time table up to 70 hours for the second one.

The nitrogen rich medium used here was similar to the so-called basal medium D, described by Theodorou and Brooks (1990). The only difference was that trypticase peptone was not included. The nitrogen-free Menke medium used in this work has the composition described by Menke *et al.*, (1979). In 1988, Menke and Steingass changed the original composition of the medium by changing the buffer solution from 39 g/l of $NaHCO_3$ to 35 g/l of $NaHCO_3$ plus 4 g/l of NH_4HCO_3. Basal medium D is similar to Menke (1979 version) but it is less concentrated as more water is added. Apart from nitrogen concentration (basal medium D has 165 mgN/l), the main differences are in the macromineral solution (as described above) and in the reducing agent. Basal medium D's reducing agent uses cysteine hydrochloric acid, $C_3H_7NO_2S.HCl$, but Menke's does not (to keep the medium free of nitrogen). Due to the absence of cysteine HCl, it takes longer for Menke's medium to be fully reduced. It is therefore necessary to add a greater amount of reducing agent to speed up the process.

2.5. Statistical analyses

The relationships between mean values for condensed tannins, total phenols and protein precipitation activity were established by linear regression analysis.

A data set was constructed to evaluate the relationship between the different chemical components and *in vitro* gas production in a nitrogen rich medium during 166 hours. The correlation between the chemical components was found by calculating a Pearson matrix to exclude highly correlated variables. An automatic selection process, stepwise regression, was used on the data set to establish a useful subset of predictors for the *in vitro* gas production at different times.

The final model for each time was tested by multiple regression analysis and possible interactions between the chemical components were tested by the General Linear Model (GLM) procedure.

The effect of the chemical components on the gas production under nitrogen free conditions during 70 hours was examined also by stepwise regression analysis and the final models tested by GLM. The chemical composition data set was the same as that used for the nitrogen rich medium but the gas values corresponded to the non-cumulative gas production of the plant material with Menke's medium.

All statistical analyses were carried out using the MINITAB statistical package Release 10.1 (1994). Gompertz equation was fitted *in vitro* gas production data to obtain the fermentation kinetic parameters using Genstat 5 Release 3.1 (1993).

3. Results and discussion

3.1. Chemical composition

The composition of the feeds is shown in Table 1. Large differences between the samples are not surprising due to the wide variability among plant species. The chemical composition of the samples was found similar to that reported in the literature for the same species (Devendra, 1992., FAO, 1993., Benavides, 1994., Norton, 1994). Features to note are as follows.

1. In the cases of *Canavalia ensiformis* and *Erythrina poeppigiana*, the values obtained for reducing sugars were higher than the values for total sugars. Both total and reducing sugars values from these species were considered erratic and therefore excluded from the data set.
2. In two cases, the value of ADF was higher than the value of NDF (*Simphytum peregrinum* and *Prosopis julifora*). According to Van Soest and Robertson (1985) these apparently erratic ADF values may indicate problems with a particular sample but not necessarily an error. The causes for this are: very badly heat-damaged samples (Maillard products may be partially soluble in neutral detergent), and plants with high biogenic silica and low hemicellulose (biogenic silica is recovered in ADF but largely dissolved in neutral detergent). In any case, the higher figure must be regarded as the true value.
3. Extracts of most of the forages studied precipitated protein (only three species showed no activity). All plants with condensed tannins showed high protein precipitation activity (PPA), but this was also the case in plants with moderate levels of total phenols. This may indicate that the capacity to precipitate protein iş not a property exclusive to condensed tannins or that there are other phenolic compounds that are not detected by the acid butanol method but which have the capacity to bind proteins. A multiple regression analysis was carried out to establish the effect of both condensed tannins (CT) and total phenols (TP) on the precipitation of protein (PPA). The regression model ($PPA = 22.4 + 0.377\ CT + 1.32\ TP$) was highly significant ($P<0.001$, $R^2=0.823$). This suggests that phenolic compounds detected by both methods have the capacity to precipitate protein as discussed above or in other words that the radial diffusion method is a good technique for estimating the activity of both type of compounds. The multiple regression model confirms that 82% of the protein reaction can be explained by these two analysis.

3.2. Chemical components affecting the fermentation under a nitrogen rich medium

A data set was prepared to examine the effects of the chemical components on the fermentation. Non-cumulative gas production values were used as the aim here was to know the effects at each particular time. A correlation matrix of the chemical composition was constructed to

find highly correlated variables. Soluble and crude protein values were closely related so they were divided into soluble and insoluble fractions by subtracting the values of the water soluble protein from those of crude protein. Other measurements that were highly correlated to each other were excluded, for example total sugars as they were well related to soluble carbohydrates and to reducing sugars. PPA values were not included in the data set as they are closely related to condensed tannins and total phenols and because protein precipitation is an effect and not a chemical entity. Organic matter values were excluded from the analysis due to collinearity. Ether extract and ash were excluded from the data set as, in initial attempts, they were shown to have little effect on the gas production and caused noise in the analysis. Ash is regarded as a measure of the minerals in a feedstuff. However, the medium used for the gas production is sufficient in minerals. Stepwise regression analysis was carried out in the final data set.

Results are shown in Table 2. The table shows the coefficients of the regression equations at different times. It was possible to fit significant regression equations from 3 to 39 hours. From 45 to 60 hours and at 94 and 166 hours, no single chemical component could significantly predict the gas production. After 60 hours, although the regressions were significant, they were contributing little to the gas produced. The best prediction equation for the gas produced from the chemical composition was from 0 to 3 hours and used three predictors. That was the maximum number of predictors used in any equation.

In general, it was not possible with this data set to predict gas production from the chemical composition from 6 hours onwards, except from 24 to 28 hours when ADF and condensed tannins decreased the production of gas. Despite the extensive chemical analyses of the leaves, it was not possible to predict the gas production accurately using more than three chemical components at a time. An attempt was made to increase the precision of the prediction by including all chemical components in the data set. The best regression equations were calculated by the stepwise regression analysis. The best prediction ($P<0.001$, $R^2 = 0.804$) was achieved for the gas produced between 0 and 3 hours by the equation:

(1) $Gas_{3h} = -4.52 + 0.0437\ SolProt + 0.373\ SolCho - 0.00788\ PPA + 0.0189\ ADF$

where SolProt=soluble protein, SolCho=soluble carbohydrates, PPA=protein precipitation activity, ADF: acid detergent fibre.

The second best prediction ($P<0.001$, $R^2=0.741$) was found for the gas produced between 12 and 16 hours by the following equation:

(2) $G_{16h} = 19.2 + 0.0939\ SolProt + 0.0700\ Starch - 0.0896\ TotSug - 0.0355\ ADF$

where SolProt: soluble protein, TotSug: total sugars, ADF: acid detergent fibre.

3.3. Chemical components affecting the fermentation under a nitrogen free medium.

The results of the multiple regression analysis are shown in Table 3. It was possible to fit significant regression equations to every period of time. The best prediction equation for the gas produced from the chemical composition was from 39 to 45 hours with four predictors. The number of predictors and the R^2 value are reduced from 60 to 70 hours. There were no significant interactions between chemical components at any time.

As for the nitrogen rich medium, an attempt to increase the precision of the prediction was made with the full data set. In this case, the best predictions were obtained in the later stages of the

fermentation. The best equation was found for the gas produced between 45 and 48 hours and included 5 predictors ($P<0.001$, $R^2= 0.822$).

(6) Gas_{48h}= -1.2 + 0.0191 NoSolPro -0.0333 NoRxSug - 0.0187 CT + 0.0393 TP + 0.00873 ADF

where NoSolPro= non-soluble protein, NoRxSug= non-reducing sugars, CT= condensed tannins TP = total phenols, ADF= acid detergent fibre.

The second best prediction equation was found for gas produced in the 52 to 60 hours period ($P<0.005$, $R^2= 0.621$).

(7) Gas_{60h}= 9.08 - 0.102 NoRxSug + 0.0508 TP - 0.0206 CT

where NoRxSug= non-reducing sugars, CT= condensed tannins, TP = total phenols.

4. Disscusion

4.1. Chemical components affecting the fermentation under a nitrogen rich medium

The importance of these results is not to highlight individual prediction equations but to have a better understanding of the gas production method. From Table 2, a pattern can be outlined and the main components affecting the fermentation can be identified. Soluble carbohydrates increase the fermentation during the first 6 hours whereas soluble protein has a significant effect from 9 to 12 hours. Starch appears to affect the fermentation both in the early (0 to 12 hours) and also the later stages (from 70 hours onwards). Total phenols probably affect the gas production throughout the fermentation period whereas the negative effect of tannins is most significant from 28 to 39 hours. This representation shows that ADF affects the gas production negatively from 16 to 28 hours. There is a positive effect of ADF at 3 hours. This highlights the fact that, when working with multiple regression analysis ('best subsets' or 'stepwise regression analysis'), some estimated coefficients may be difficult to explain from a biological point of view. Andrighetto *et al.*, (1992) found a positive effect of NDF when estimating the relationship between chemical composition and organic matter digestibility (OMD) that could not be explained. The authors pointed out that NDF was a partial coefficient simultaneously fitted along with other variables and that its contribution to the general equation was small. In this study, the positive effect of ADF at 3 hours may be a casual relationship as there is a more consistent negative effect between 16 and 28 hours. Insoluble protein did not affect gas production at any time, probably due to the level of nitrogen in the medium. The results highlight the importance of the soluble components of the leaves on the fermentation during the first few hours. This can be explained by the fact that, in the gas production method, the substrate is placed in the bottles with the buffer and left in an incubator at variable temperature (4 °C for 10 hours and 39 °C for 8 hours, to facilitate the management of hundreds of bottles) before adding the rumen inoculum the next day. This allows the soluble components of the feed to go into solution and be rapidly fermented by bacteria when the inoculum is added. In general, chemical components gave good biological explanations of what happened during the first 39 hours. Beyond this period of time, the relationship between the gas produced and the chemical components of the leaves was not as strong. This is not surprising since little gas is produced during the later stages of fermentation, and that which is produced probably results from the fermentation of residues from the early hours and not from the original chemical components. Possible interactions between chemical components at each time were tested by the GLM procedure. There were no significant interactions between the predictors at any time.

Multiple regression equations (1) and (2) show that, it is possible to predict gas production reasonably from the chemical composition in the early stages. It is in the first hours that all chemical components have most effect on the fermentation. However, a prediction equation for the early stages cannot itself predict the extent of the fermentation process.

4.2. Chemical components affecting the fermentation under a nitrogen free medium

The main components affecting the fermentation were identified from Table 3. Soluble protein reduced gas production from 3 to 6 hours. Insoluble protein positively affected gas production from 39 to 45 hours. The soluble carbohydrates increased the fermentation from 33 to 45 hours, whereas starch contributed largely to gas production from 12 to 33 hours and at 45 and 60 hours. Reducing sugars increased the fermentation from 6 to 9 hours. The effect of condensed tannins was a reduction in fermentation from 33 to 52 and at 70 hours, whereas the effect of phenolic compounds was an increase in gas production in the early stages and from 48 to 52 hours. NDF contributed to gas production at 3, 9 and 60 hours. The effect of ADF was a constant reduction in gas production from 0 to 33 hours. Although significant, it was not possible to give a biological interpretation of the negative effect of soluble protein from 3 to 6 hours. It may be a casual relationship due to the lag phase. The positive effect of phenolic compounds is not surprising since it has been found that phenols can be fermented by rumen bacteria. Polyphenols like quercetin, rutin and gallic acid, present in browse plants, have been shown to be degraded by rumen bacteria (Parrinder, *et al.*, 1991). A pattern can also be identified with Menke's medium. After the first 6 hours, where ADF is reducing the fermentation, the carbohydrate components: reducing sugars, starch and ADF, mainly affect the fermentation from 9 to 33 hours. After this time, other fractions become more important. This is the case with condensed tannins (from 39 to 52 hours). With Menke's medium, the insoluble protein from the forages becomes an important predictor of gas production at 45 hours.

Multiple regression equation (6) confirms that insoluble protein as important predictor in the nitrogen free medium and its coincidence with total phenols and condensed tannins at 48 hours.

Nsahlai *et al.*, 1994, evaluated the relationships between gas production by the Menke's method (1979) and chemical composition of 23 browses of the genus *Sesbania*. They established multiple regression equations between gas production parameters and chemical composition by a stepwise regression. They found that the main components affecting the extent of gas production were NDF, hemicellulose and lignin. They also identified a small and negative effect of proanthocyanidins. However these relationships were established on the total volume of gas produced (calculated by a non-linear equation) at the end of the fermentation (96 hours) and not along the incubation period as reported here.

In general, the predictors for the fermentation with both nitrogen rich and nitrogen free media were the same, but their effects were different. The importance of nitrogen is well illustrated by looking at the pattern presented by the regression matrices (Tables 2 and 3). This patterns are represented in Figures 1 and 2. With nitrogen rich medium (Figure 1), soluble carbohydrate and protein were more important during the first 12 hours, starch both at early (0 to 12 hours) and late stages (from 70 hours onwards) and total phenols and condensed tannins from 28 to 39 hours. Insoluble protein did not affect the fermentation at any time. This highlights the importance of the soluble components of the leaves during the first stages of the fermentation in the nitrogen rich medium. Chemical composition best predicted the gas production during the first few hours. This is biologically feasible as, in the basal medium D, the nitrogen may be used to ferment the soluble fractions. This would provide the initial nutrients to the bacteria. No a single chemical component could predict the gas production from 45 to 60 and from 94 to 166 hours indicating that from 45

hours onwards the chemical composition of the substrate has little or none effect on the gas production. In Menke's medium (Figure 2), soluble components are less important at the beginning. After a period of 6 hours (which may represent the lag phase) the fermentation was heavily influenced by the amount of carbohydrates, and ADF becomes the major constraint but other carbohydrates (reducing sugars and starch) are contributing to the gas production. After this period (from 33 hours onwards), other components become more important; insoluble protein from 39 to 45 hours, condensed tannins from 33 to 52 and at 70 hours, total phenols at 48 to 52 hours. An important observation is the coincidence of both protein and condensed tannins at 45 hours. In the case of nitrogen rich medium, the best prediction equations were found in the early stages whereas, with Menke's medium, they appeared towards the end of the fermentation.

5. Conclusions

The importance of these results is to highlight how the microbes appear to use different combinations of chemical components over time to produce a smooth curve of gas production. For the nitrogen rich medium, there is a clear division from 3 to 16 hours when the soluble components made a more significant contribution to gas production. From 20 to 33-39 hours the less soluble material had the largest effect on the fermentation. From 45 hours onwards, the chemical composition of the substrate had little or no effect on the production of gas. The small volume of gas produced at this stage is due partly to less easily fermentable material (such as starch) and partly to the residues of the chemical components fermented in the early hours. These results also suggest that an incubation period of about 33-39 hours appears to be a minimum for this type of substrate and incubating for more than 70 hours does not add anything to potential gas production. The main components affecting the fermentation in the nitrogen rich medium were identified as: soluble protein, soluble carbohydrates, starch, acid detergent fibre and phenolic compounds. Insoluble protein did not affect the fermentation. The reason for this may be the high nitrogen content of the medium. This medium may be more suitable to study the fermentation of cereals or fibrous agroindustrial residues.

The effect of the N-free medium was a change in the time at which the chemical components contributed to the fermentation. Except for the inclusion of insoluble protein as the main predictor of gas production with Menke's medium, the rest of the predictors were common to both media. Also with nitrogen rich medium the soluble components are more important than less soluble material during the first few hours. In Menke's medium, soluble components are less important and there is an immediate negative effect of ADF. After a period of 33 hours, other components become more important (insoluble protein among them). This may resemble more what happens in an *in vivo* situation. According to these results, Menke's medium is more suitable for the study of fodder trees and shrubs as sources of nitrogen.

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Table 1

Chemical composition of fodder trees and shrubs

Plants	Crude protein g/kg	Soluble protein g/kg	Water soluble carbohydrate g/kg§	Starch g/kg	Total sugars g/kg	Reducing sugars g/kg	NDF g/kg	ADF g/kg	Ether Extract g/kg	Organic Matter g/kg	Protein precipitation activity cm2/g	Condensed tannins OD/g*	Total phenols OD/g
Amaranthus dubius	189	37.3	9.1	209.1	31.7	19.7			3.9	811.9	82.9	0	10
Malvasrum sp.	124.9	13.5	13.6	269.2	93.1	64			17.9	934.9	256.9	254.1	122.6
Bidens pilosa	196.2	57.9	11	305.6	42.6	32.7			4.6	848.6	112.9	0	35
Dioclea sericea	123.3	13.5	10.1	194.2	53.2	39.1			18.8	941.2	155.9	222.5	68.8
Simphytum peregrinum	165.3	42.9	20.9	174.4	77.3	67.3			22	758.8	0	0	71
Urera baccifera	216.3	42.3	17.3	171.5	44.3	35.5			15.1	730.1	0	0	1.7
Canavalia ensiformis	227.7	45.2	7	125.3	15.2	30.6			24.3	817.1	71.6	0	23.3
Sapindus saponaria	237	88.4	22.3	170.6	66.5	37.2			10.1	865.8	72.7	0	29.6
Heliconia sp.	223.7	na	34.3	205.9	120	64.1			58	892.2	50.6	0	42.5
Tithonia diversifolia	242.7	40.2	7.6	172.7	39.8	35			14	785.9	0	0	12.3
Clitoria ternatea	294.1	74.7	15.4	254.3	91.7	75.6			13.4	913.6	80.3	0	39.7
Erythrina edulis	256.2	53.3	12.6	204.8	70.9	18.2			23.6	891.7	132.8	0	38.6
Enterolobium cyclocarpum	156.2	14.9	15.1	193.4	107.9	71.2			40.3	884.4	180.6	251.2	87.9
Pithecellobium dulce	178.3	37.6	10.7	223.2	81.6	48.1			9.1	898.2	114.5	182.3	67.3
Leucaena leucocephala	284.1	42	18.4	155.9	99.1	95.2			32.2	887.1	244.1	284.2	111.2
Trichanthera gigantea	178.2	35.4	43.2	248.2	170.1	91.6			31.2	804.1	323.5	0	208.8
Inga sp.	225.8	30.1	3.3	192.5	84.4	59.5			8.5	909.1	529	595.4	151.9
Erythrina poeppigiana	214.7	47	12.2	105.1	42.9	56.3			30.7	825.3	128.6	0	40.3
Gliricidia sepium	303.3	129.4	20.7	109.5	88.8	63.2			22.6	878.8	163.6	0	39.2
Prosopis juliflora	234.4	59.1	11	113.2	69.1	10.9			18.1	870.5	68.3	0	49.1

* Optical Density

Table 2
Coefficients for multiple regression equations for predicting non-cumulative gas production from the chemical composition at different incubation periods (nitrogen rich medium).

Non-cumulative gas production between times n (hours)																				
Predictors	**3**	**6**	**9**	**12**	**16**	**20**	**24**	**28**	**33**	**39**	**45**	**52**	**60**	**70**	**82**	**94**	**106**	**118**	**142**	**166**
Constant	-2.2	10.6	9.6	-3.3	30.2	26.9	19.5	14.2	9.8	7.6				2.64	3.15		2.54	1.63	1.02	
Soluble Protein			0.13	0.18																
Insoluble Protein																				
Soluble Carbohydrates	0.47	0.37																		
Starch				0.05										0.01	0.01		0.008	0.01	0.01	
Reducing Sugars																				
Condensed Tannins								-0.01	-0.01	-0.01										
Total Phenols	-0.03	-0.03						0.02	0.03	0.03					-0.01		-0.007	-0.009	-0.008	
NDF																				
ADF	0.01				-0.03	-0.03	-0.02	-0.01												
R2 (%)	75.4	52.1	33.1	52.2	34.8	45.6	49.2	68.5	48.0	38.6				23.3	36.2		38.2	48.9	49.9	
P	0.001	0.003	0.01	0.003	0.001	0.002	0.001	0.001	0.005	0.02				0.036	0.027		0.021	0.005	0.004	

Table 3
Coefficients for multiple regression equations for predicting non-cumulative gas production from the chemical composition at different incubation periods (nitrogen free medium).

Non-cumulative gas production between times n (hours)															
Predictors	**3**	**6**	**9**	**12**	**16**	**20**	**24**	**28**	**33**	**39**	**45**	**48**	**52**	**60**	**70**
Constant	1.51	10.8	4.33	7.93	8.66	8.14	6.33	7.7	12.1	7.28	-5.06	4.47	4.89	9.34	8.98
Soluble Protein		-0.050													
Insoluble Protein											0.0387				
Soluble Carbohydrates										0.194	0.166				
Starch				0.0212	0.040	0.034	0.0394	0.0436	0.0261		0.0308			0.0252	
Reducing Sugars			0.0556												
Condensed Tannins										-0.0106	-0.0113	-0.010	-0.010		-0.0106
Total Phenols	0.0161											0.021	0.0238		
NDF	0.0088		0.0172											-0.0148	
ADF	-0.0105	-0.011	-0.0248	-0.0138	-0.0203	-0.0169	-0.0169	-0.0164	-0.0213						
R2 (%)	43.8	30.5	51.2	50.2	47	38.4	38.3	37.1	49.2	49.1	68.1	52.2	45	34.7	25.6
P	0.03	0.05	0.01	0.004	0.00	0.02	0.02	0.02	0.004	0.00	0.002	0.003	0.00	0.03	0.03

Figure 1: Importance of the chemical constituents of tree leaves on the fermentation with a nitrogen rich medium (166 hours)

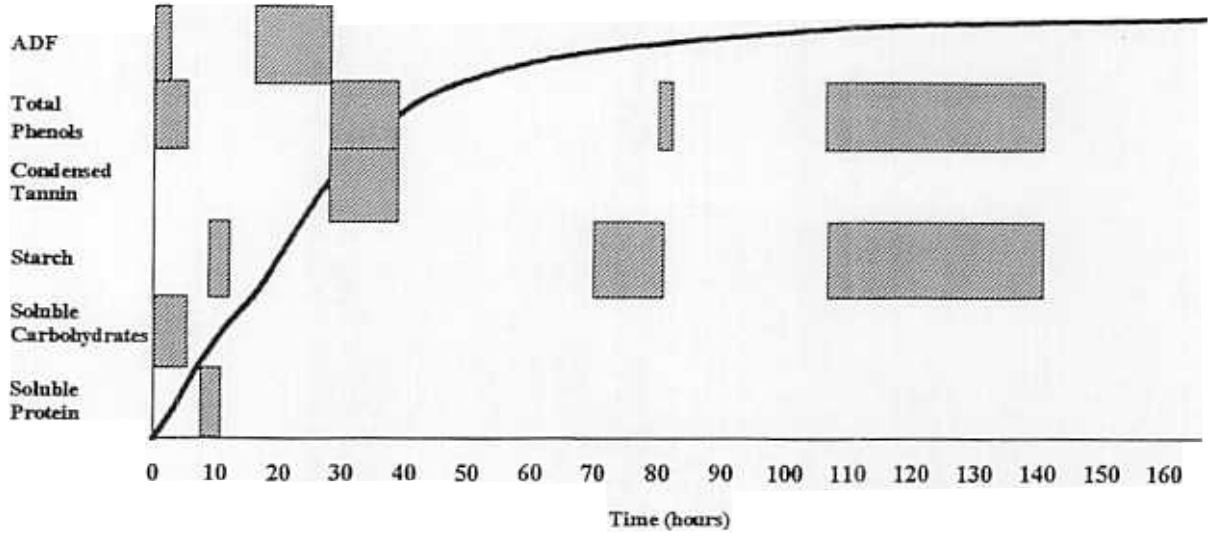

Figure 1
Graphical representation of the hourly importance of the chemical constituents of tree leaves on the fermentation with a nitrogen rich medium (166 hours).

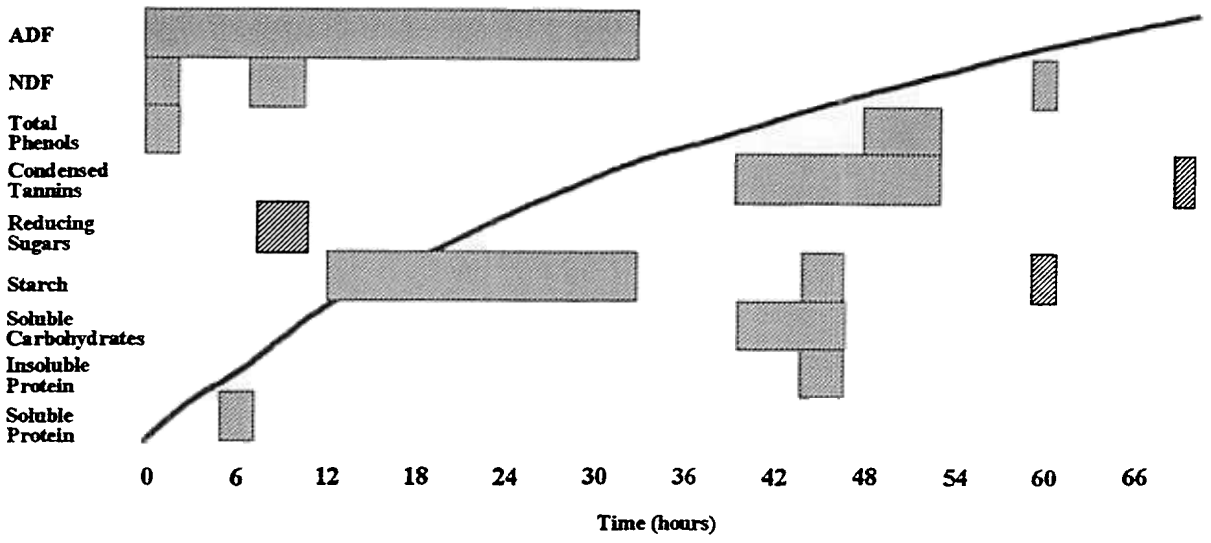

Figure 2
Graphical representation of the hourly importance of the chemical constituents of tree leaves on the fermentation with a nitrogen free medium (70 hours).