Development of the *in vitro* gas production technique to assess degradability of forages by ruminants

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by

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DECLARATION

I hereby declare that this thesis has been composed by myself and has not previously submitted for any other degree. The work described herein is my own and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the course of these studies.

Sameer A. Nagadi

ABSTRACT

The influence of microbial activity on the pattern of gas production and the possibility of developing a protocol for standardising the microbial activity of ruminal fluid were investigated (chapter 2). Firstly, ruminal fluid was diluted 1:2, 1:5, 1:9 and 1:20 (v/v) with buffer solution and the *in vitro* gas production from cellulose, glucose, hay and ryegrass studied. Gas production at 4, 24 and 120h were significantly decreased as the ratio of buffer solution to ruminal fluid increased. For each substrate, incubation with ruminal fluid diluted 1:2 (v/v) gave a higher ($P<0.05$) asymptotic value 'B', rate 'C 'of gas production and lower lag time than when incubated in the 1:5, 1:9 and 1:20 dilutions. Secondly, the effect of frequency of ovine ruminal sampling on microbial activity and substrate fermentation was investigated. The bacterial DM, bacterial absorbance and blank's gas volume were significantly affected by daily sampling of ruminal fluid. Daily sampling of rumen fluid did not significantly affect the gas production degradability parameters (B, C and Lag) until the bacterial DM fell below 0.09 g DM / 10 cm^3 strained rumen fluid. Bacterial DM was strongly related to the absorbance of ruminal fluid (R^2 = 0.99, $p<0.001$), suggesting that standardisation of the level of microbial activity between days and laboratory can be achieved by estimating the bacterial DM from the absorbance of the strained ruminal fluid.

The influence of donor diet on initial microbial concentration and gas production degradability was studied (chapter 3). Bacterial DM, bacterial absorbance and the blanks' gas volume increased significantly as the ratio of sheep pellet to hay increased. The gas production degradability parameters (B, C and Lag) were also affected by changing the donor diet ratio of sheep pellets and hay. NDF digestibility of

cellulose and hay was not significantly affected by donor diet. Bacterial DM was strongly related to the absorbance of ruminal fluid and the blanks' gas volume (R^2 = 0.99, p<0.001). These results suggest that changing the ratio of concentrate to hay reduced the initial bacterial concentration and affected the gas production degradability parameters. Estimation of bacterial DM either from bacterial absorbance or blanks' gas volume was not affected by changing the diet of donor animal.

The effect of physical form of feed sample incubated in vitro using gas production technique on the pattern of gas production was investigated (chapter 4) by comparing the gas production profiles of fresh and dry forages. The gas production degradability of ryegrass, red fescue and first stage of maturity of brachiaria decumbens (a, b, c and lag) were similar between fresh crushed using a pestle and mortar for one min (FC1), two min (FC2), three min (FC3) and dry fine ground (DFG) samples but it was lower on dry 1mm ground (D1mmG) samples than on the others. The rate of gas production (c) for the third stage of maturity of Brachiaria decumbens leaf was higher on DFG and D1mmG than on FC1, FC2 and FC3 whereas D1mmG had a similar rate to fresh treatments for the stem fraction. Also, the fermentation characteristics of fresh and dry B.brizantha over various stages of maturity were investigated. The gas production degradability parameters of the fresh and dry materials varied between stages of maturity.

The effect of harvesting ruminal micro-organisms to remove the fermentable nitrogen present in ruminal fluid on the gas production profiles and the effect of fermentable nitrogen availability on NDF gas production degradability using a harvested ruminal micro-organisms were studied (chapter 5). Use of diluted ruminal

fluid or harvested ruminal micro-organisms did not influence the gas production degradability parameters (A, B, C and Lag) of cellulose, oatfeed, ryegrass, cocksfoot, wet and dry season kikuyu grasses. The response of NDF degradability of cellulose, star, guinea, brachiaria decumbens and wet season kikuyu to nitrogen availability was broadly consistent with earlier in vitro and in situ studies, indicating that the ammonia concentration required to maximise the rate of NDF gas production varied according to the availability of fermentable NDF and that a minimum of about 100 mg N/l was required to achieve the maximum degradation of NDF. The effect of nitrogen availability on NDF gas production degradability appeared to be mainly, if not entirely, on the rate of gas production rather than its extent.

The suitability of using ovine faecal fluid (OFF), bovine faecal fluid (BFF) and equine faecal fluid (EFF) as source of inocula for the in vitro gas production technique to replace ruminal fluid was studied (chapter 6). Bacterial DM and Bacterial absorbance and blanks gas volume were significantly different between OFF, BFF, EFF and ovine ruminal fluid (ORF). Gas production degradability parameters and molar proportion of VFA of cellulose, glucose, ryegrass, wet season kikuyu grass and star grass incubated in buffered BFF or EFF were significantly different from those incubated in buffered ORF. Glucose, ryegrass and wet season kikuyu grass incubated in buffered OFF had a similar gas production degradability parameters and molar proportion of VFA to those incubated in buffered ORF. In contrast, cellulose and star grass incubated in OFF had a significant longer lag time, lower rate and total gas production, and different molar proportion of VFA compared to those incubated in buffered ORF. No significant differences in total VFA concentrations and NDFD of feed samples incubated either in OFF, BFF, EFF or ORF were observed.

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CHAPTER 1

GENERAL INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Agriculture is considered to be one of the oldest activities of mankind. It is not clear when people started agricultural cultivation and domestication of animal, but it is believed that it may have taken place gradually from 40, 000 to 10, 000 B.C. (Flannery, 1965). Before that time, people were principally food gatherers, but the time came when they settled and started to become food producers. Nowadays, food production is the world's most vital primary activity without which neither modern civilisation nor primitive cultures can survive.

Due to the huge expansion of the world population and increased demand for food in the last 100 years, it became important to maximise the output of ruminant production (meat, milk and wool) and yet minimise the cost of it. Feed is the major single source of ruminant production systems (De Boer and Bickle, 1988). Therefore, the practical importance of research into feed consumption, digestion and utilisation by ruminants is obvious.

In ruminants, the amount and type of nutrients available for absorption, and ultimately for production, generally differ largely from the profile of nutrients eaten. These differences result from the metabolic activities of rumen micro-organisms.

Microbial material and the end products of rumen fermentation, represent a major part of the total available nutrient supply.

The major source of stored energy and nutrients is plant cell wall constituents. Symbiotic relationships between animals and microbes allow microbial capability for digestion of this resource to be tapped by mammals. However, the rate of degradation of plant cell wall by rumen microbes is influenced by the supply of nutrients from ingested food or from endogenous recycling into the rumen (Wilson and Kennedy, 1996). The rate of degradation is also depressed if the supply of nutrients is suboptimal for microbial requirements for maintenance and growth. Due to the crystalline nature of cellulose and to the lignin association with carbohydrate which resists the degradation of plant fibre by rumen micro-organisms, the fibre fraction from the feed consisting of plant cell walls is not fully utilised by ruminants (Hoover 1986). Physical and chemical procedures such as grinding and additives (e.g. ammonia treatment) would enhance the availability of these feeds and subsequently results in an increase of the feeding value and enhance the intake (Preston and Leng, 1987; Van Soest, 1994). Therefore, digestion kinetics are important in order to determine the proportion of consumed nutrients that can be absorbed for the use by the animal (Mertens, 1993) or to predict the effect of treatments in the rumen digestion system.

The majority of past and current approaches to food characterisation are designed to meet the needs of rationing systems which essentially require information about the quantity of energy and protein needed for ruminant animals to

meet their requirements for maintenance and production (Givens and Gill, 1998). Thus, the energy value of ruminant feedstuffs is commonly predicted from the apparent digestibility of dry mater (DM) or organic matter (OM) through the entire digestive tract. This approach has led to development of several biological and chemical methods to predict *in vivo* digestibility such as the two stage ruminal fluid technique of Tilley and Terry, 1963 or the enzymatic method (Jones and Hayward, 1975). However, ruminant animal production faces numerous challenges and it seems that both economic and biological benefits will be derived from changing characterisation from simple energy and protein based approaches to those which assess nutrient supply in some detail (Givens and Gill, 1998).

In vitro and *in situ* methods are used to estimate the degradation characteristics of ruminant feedstuffs (Tilley and Terry, 1963; Jones and Hayward, 1975; Ørskov *et al.*, 1980). These methods involve the measurement of substrate disappearance during incubation in the rumen (*in situ*), in buffered rumen fluid or in cell-free fungal cellulase (*in vitro*) and are mostly endpoint measurements. The kinetics of degradation can be determined by measuring the differences in the residues after various incubation times (Goering and Van Soest 1970). However, this approach is expensive, laborious and the fermentation course of water-soluble components cannot be quantified (Pell and Schofield, 1993).

Alternatively, the kinetics of ruminant feedstuffs degradability can be determined from the fermentative gas production, which measures the amount of gas released directly as the end product of the fermentation and indirectly from buffered

rumen fluid (Menke *et al.*, 1979; Beuvink and Spoelstra, 1992; Pell and Schofield, 1993; Theodorou *et al.*, 1994; Cone *et al.*, 1996). The gas production kinetics are dependent on a sequence process. Upon incubation, substrates are partly solubilized. The water-soluble components are rapidly fermentable within four hours (this depends on microbial activity) of the incubation (Jessop and Herrero, 1996). Subsequently the fermentation of the insoluble parts occurs more slowly after the hydration and colonisation by rumen micro-organisms have taken place (Van Milgen *et al.*, 1993). The rate of these processes depend on the ability of the rumen micro-organisms present in the mixture of rumen fluid and buffer solutions to colonise, ferment and utilise the fermentation products for growth (Hidayat *et al.*, 1993). The main advantages of this technique are that:

1) It measures gas as a fermentation end product rather than disappearance of OM.
2) Only one feed sample is needed to measure its fermentation dynamics at different time points.
3) A small amount of feed sample (100 mg DM) can be used (Pell and Schofield, 1993).
4) Gas production reflects the amount of all carbohydrate fermented (soluble and insoluble) but unfermentable carbohydrate does not contribute to gas.
5) It is relatively easy to automate (Beuvink and Spoelstra, 1992; Cone *et al.*, 1996) and computerise (Pell and Schofield, 1993).
6) It is not laborious or expensive.

However, the *in vitro* gas production technique is liable to many sources of error, which makes it difficult to control and this will be discussed in detail later in this chapter. Further development of the *in vitro* gas production technique is needed in order to improve its accuracy to predict the fermentation kinetics of ruminant feedstuffs and to facilitate its application in feed evaluation system.

In this thesis, each experimental chapter is preceded by a specific review of literature pertinent to the experiment, therefore the following review is a general introduction to this thesis.

1.2 The degradation characteristics of forages

Since forages have a higher proportion of protein (CP) and carbohydrate (CHO) than lipid, the degradation of these substances is going to be explained in detail.

Description of forages in terms of their degradation characteristics would provide useful basic information for their evaluation, since the utilisation of forages is highly dependent on microbial degradation in the rumen (Leng, 1990). Moreover, if we want to understand processes that influence intake or if we want to be able to move towards systems that predict the supply of nutrients (Cornell system) rather than the supply of metabolisable energy (ME) we need to know more about the type of substrates degraded by rumen micro-organisms, their rate of degradation and their rate of passage.

The Metabolisibale Protein (MP) system (AFRC, 1992) has recognised the importance of distinguishing between the total dietary Metabolisibale Energy (ME) and the ME fermentable in the rumen (FME) and hence related to the energy available to rumen micro-organisms for growth. Although, this system recognises the need for describing the degradation of feed protein dynamically, the way in which energy is released for microbial concentration has not been taken into account. However, the Cornell Net Carbohydrate and Protein System (Sniffen *et al.*, 1992) has considered the need of describing the fermentation dynamics of energy and protein in ration systems.

The overall degradability of carbohydrate and protein within the rumen is shown in Figure 1.1. A simplified description of the degradability (P) of forages by ruminant, as described by AFRC (1993), Van Soest (1994) and according to the model of Ørskov and McDonald (1979) $P=a+b\ (1-e^{-ct})$, divides the dry matter of forages into degradable (a+b) and undegradable (1-a-b) fractions of CP or CHO.

The degradable fraction (a+b) is composed of a soluble or quickly degradable portion (a) of CP (QDP) or CHO (QDCHO) and an insoluble, slowly degradable portion (b) of CP (SDP) or CHO (SDCHO). The undegradable fraction (1-a-b) may be defined as that fraction which has not been degraded during its passage through the rumen, but which is sufficiently digestible to be absorbed in the lower intestines (AFRC, 1993).

Both QDP and SDP have the potential to be captured by rumen microbes. The amount of protein that can be captured and degraded by rumen micro-organisms

before material passes out of rumen is known as Effective Rumen Degradable Protein ERDP (AFRC, 1993). Also, part of QDCHO and SDCHO are assumed to be fermented by rumen micro-organisms (FME). The MP System assumed that the efficiency of QDP and SDP captured by rumen microbes are 0.8 and 1.0 respectively (AFRC, 1993). This assumption is inaccurate where the efficiency of capture of QD and SD materials are dependent on the rate of their degradation (c) and dilution rate (r). In other word, if the dilution rate (r) is high in comparison with the degradation rate (c), a large proportion of the SD fraction will be converted to the UD fraction and the rest will be converted to ERDP or FME and vice versa.

Forty to sixty percent of dry matter of the microbial cell is protein (Preston and Leng, 1987), which is provided by ERDP. The ATP which is produced by the conversion of CHO to VFA's is the major source of energy required by micro organisms to synthesise their own cells and to provide energy for their maintenance. Extreme variations in the supply of CHO and nitrogen (N) in the form of ammonia to the microbes, such as with grass silage diets (Beever and Reynolds, 1994) can have a an effect on fermentation and animal performance (Nocek and Russell, 1988), such that:

1) if the rate of protein degradation exceeds that of the rate of CHO fermentation, large quantities of N can be lost as ammonia through the rumen wall and excreted in urine, at a considerable energy cost to the animal and possible damage to the environment.

2) if the CHO fermentation exceeds the rate of protein degradation, microbial protein production may be reduced.

Therefore, degradation profiles of individual feeds are needed in order to match energy and protein supply to the rumen micro-organisms to improve utilisation of feed.

In theory, CHO and CP degradation in the rumen should proceed in synchrony, to supply ATP, hexose skeletons and ammonia respectively (Beever and Reynolds, 1994). Also, it has been suggested that the efficiency of microbial growth and microbial protein production might be improved by balancing the over-all ratio of ruminally available energy and protein in the diet (Nocek and Russell, 1988). Varying the source and degradability of non-structural carbohydrates (plant cell contents) and rumen degradable protein may increase microbial protein synthesis and efficiency of ruminal fermentation (Casper *et al.*, 1999). Taking this into account, several studies have investigated the importance of close synchrony of energy and protein in the rumen. Herra-Saldana *et al.*, (1990) observed an improvement in the efficiency of microbial growth and flow of microbial protein to the small intestine with better synchronised diets. Newbold and Rust (1992) also reported significantly increased bacterial population density from 5-8 h of incubation for the synchronised nutrient supply. Hoover and Stokes, (1991) found that decreasing the non-structural carbohydrates and rumen degradable protein ratio increased the quantity of microbial protein synthesised *in vitro* and *in vivo*. In contrast, Casper *et al.*, (1990 and 1999) observed no such responses to the synchronisation of energy and protein. Henning *et al.*, (1993) suggested that improving synchronisation of energy and protein supply does not increase microbial protein synthesis and that efforts should be directed at manipulating ruminal energy supply. However, further research is needed to investigate the interaction between energy and protein in the rumen.

Several techniques have been developed to study the fermentation dynamics of ruminant feedstuffs, but in this thesis only the *in vitro* ruminal fluid techniques for measuring ruminal digestibility and degradability of carbohydrate will be reviewed.

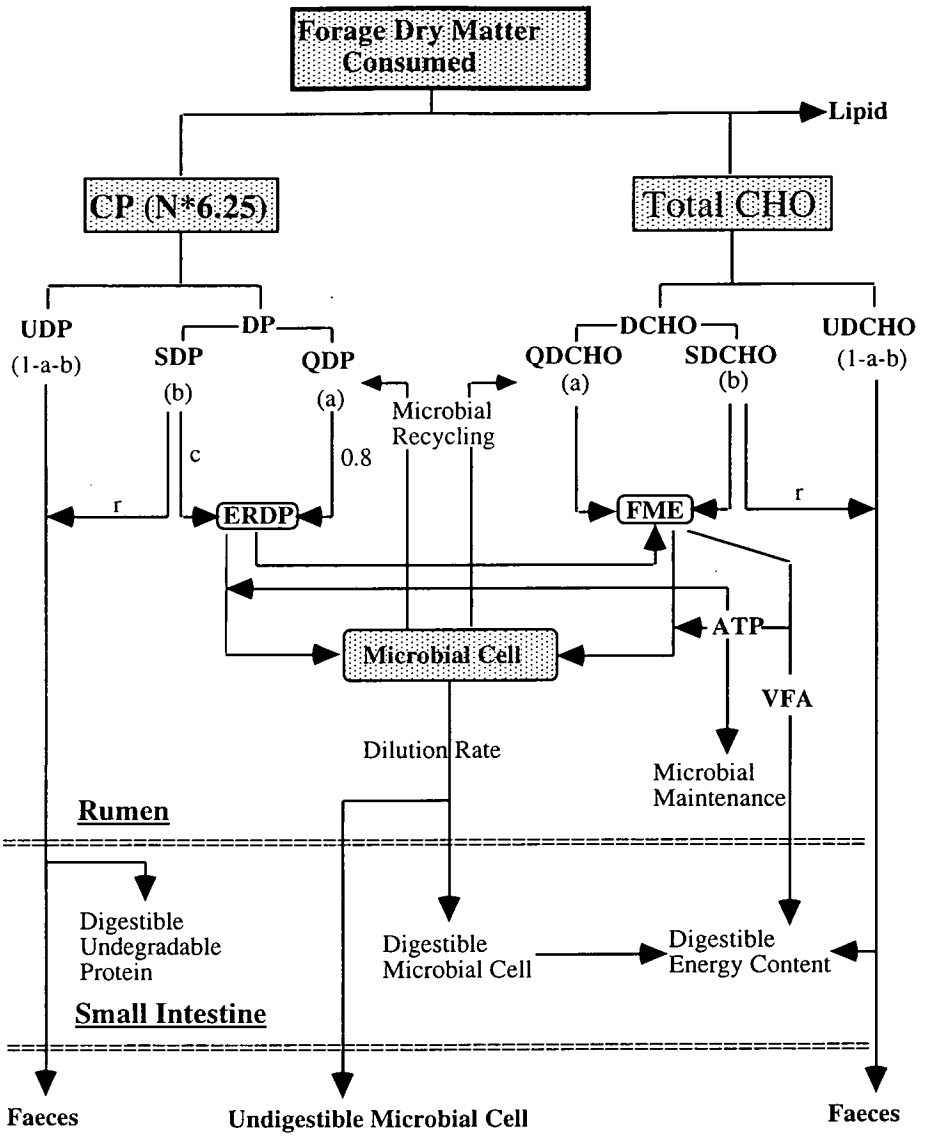

Figure 1.1 Interactions between protein and energy in the rumen (Nagadi 1996). For detail of abbreviations see section 1.2.

1.3 *In vitro* ruminal fluid techniques for measuring ruminal digestibility and degradability of carbohydrate

As the name indicates, these methods involve the use of living ruminal micro-organisms to digest a known amount of feed samples anaerobically in a test tube or gas-tight syringe under a controlled environment of temperature and pH for a specific period of time.

The disappearance of the incubated samples in the *in vitro* ruminal fluid method will be determined by microbial activity. This is in contrast to the chemical methods where the analyst analyses the plant cell wall in an attempt to identify a chemical functional group (s) which might be related to digestibility. Therefore, it is not surprising that the rumen liquor method will perform better in predicting *in vivo* digestibility of forages than the chemical methods. Van Soest (1982) emphasised that the superiority of the *in vitro* method over the chemical methods relies on its ability to reflect biological events.

1.3.1 The two-stage *in vitro* technique

The early use of these methods was reported as a one-stage process. Asplund *et al.* (1958) predicted *in vivo* DMD digestibility of 11 hays with an R^2 of 0.56 and RSD of 3.5%, when using an incubation time of 24 hours. Subsequently, Tilley *et al.* (1960) found that *in vivo* DMD of 20 cocksfoot samples could be predicted with an R^2 of 0.83 and RSD of 3.6%. When using two stages in which forages are subjected

to a 48 h incubation time with rumen fluid followed by 48 h of digestion acid pepsin, Tilley *et al* (1960) reported an improvement in *in vivo* DMD of 20 cocksfoot from an R^2 of 0.83 and RSD of 3.6%, using the one-stage process to R^2 of 0.96 and RSD of 2.0% using the two-stage process.

This method has been widely used and is known as the two stage *in vitro* technique and is found to be a good predictor of *in vivo* digestibility (Tilley and Terry, 1963).

In the first stage the rumen micro-organisms are used to measure the digestible fibrous fraction of the plant cell wall, whereas the acid pepsin in the second stage solubilises the microbial dry matter and nitrogen resulting from the first stage. Therefore, the complete system is a simulation of the digestive process that occurs inside the ruminant animal. Van Soest *et al.* (1966) replaced the acid pepsin stage by neutral detergent which solubilises bacterial cell walls and other indigenous materials. They reported these systems as estimating *in vitro* true digestibility rather than the apparent digestibility of Tilley and Terry (1963). The Van Soest system takes two days to complete, but it has not been found to be more accurate than the original method (Cottyn *et al.*, 1986).

Some modifications in the original procedure of Tilley and Terry (1963) were proposed. Alexander and McGowan (1966) noted that the centrifugation at the end of each stage made the technique unsuitable for routine use. For this reason they suggested that direct acidification at the end of the first stage should be followed by

immediate filtration at the end of the second stage and they were able to process 250-300 samples per week without loss of accuracy.

Despite the fact that the method of Tilley and Terry (1963) has been cited as the best and the most accurate technique for prediction of *in vivo* digestibility of forages over a variety of other techniques (Oh *et al.*, 1966; McLeod and Minson, 1978; Abrams *et al.*, 1981; Morgan and Stakelum, 1987; Coelho *et al.*, 1988; Wiess, 1994), the digestibility results of this technique have been shown to be affected by the diet of the donor animal. Nelson *et al.*, (1972) reported that when corn silage was the donor diet, the coefficient of variation of replicated analyses for a variety of substrates averaged 6.4%, but when perennial ryegrass was the donor diet, the coefficient of variation averaged 2.8%. Also, Horton *et al.*, (1980) reported that feeding a barley : alfalfa diet (1:1) to donor animals produced lower *in vitro* DM digestibility (IVDMD) values than when host animals were fed medium quality grass-legume hay. In addition, the screen size used to grind the sample may also affect digestibility (Cone *et al.*, 1989; Richards *et al.*, 1995). Samples ground to pass 2 mm and 1 mm screens had different starch digestibility. Cone *et al.*, (1991) reported increased rates of starch disappearance when particle size was decreased from 1 to 0.1mm.

Moreover, the digestibility results obtained from this technique have been observed to be influenced by the method of collection, the sampling and processing of the rumen liquor (Barnes, 1973; Cone *et al.*, 1989; Minson, 1998). Also, the activity of rumen liquor collected from cattle or sheep has also found to be different

(Van Dyne, 1962; Weiss, 1994). Other sources of variations like maintaining adequate pH, temperature, adequate anaerobiosis and length of incubation have been found to affect the results (Barnes, 1973; Therion *et al.*, 1982; Stern *et al.*, 1997).

Furthermore, this method does not provide information on the kinetics of forage digestion and is very labour intensive (Theodorou *et al.*, 1994). The first step of this method has been used to determine *in vitro* digestion by analysing the incubated residues after different time of incubation (Goering and Van Soest 1997) but this method is still an endpoint method that measures substrate loss which is laborious and requires a large amount of equipment and feed samples. Those reasons will undoubtedly limit the use of this method and will lead to the search for more simple methods which can provide information on the kinetics of feed degradation. In this respect, the gas production technique may offer an acceptable alternative.

1.3.2 *In vitro* gas production technique

(I) *Origin of gas*

Measurement of gas production can be used to study the fermentation kinetics of feedstuffs since the anaerobic fermentation of carbohydrate by ruminal micro-organisms generates directly volatile Fatty Acids (acetate, propionate and butyrate) and gases (CO_2 and CH_4) (Hungate, 1966). The gas produced in the gas production technique is the result of the gas produced directly from the fermentation of substrate incubated in buffered ruminal fluid, as indicated above and indirectly from CO_2 released from the reaction of bicarbonate (HCO^-_3) in buffer media with the VFA's

produced (Beuvink and Spoelstra, 1992). A positive relationship between gas production and total VFA formation *in vitro* has been reported by many researchers (O'Hara and Ohki, 1973; Naga and Harmeyer, 1975; McBurney *et al.*, 1990; Beuvink and Spoelstra, 1992; Blummel and Ørskov, 1993; Makkar *et al*; 1995). The total gas production is affected by the stoichiometry of VFA production. The fermentation of substrate to acetate and butyrate produces 4 and 3 moles gas per mole hexose fermented from both direct and indirect gases respectively whereas substrate fermented to propionate produces only 2 moles gas per mole hexose fermented from indirect gases (Beuvink and Spoelstra, 1992). Therefore, a higher concentration of propionate is associated with lower total gas production for the reason that an extra carbon atom in propionate would have appeared as CO_2 (Wolin, 1960). However, for forages about 50% of the total gas production is generated indirectly and the rest is evolved directly from the fermentation of feedstuff (Blummel and Ørskov, 1993) whereas about 60% of the total gas production is generated indirectly from buffering of VFAs in concentrate diets (high molar proportion of propionate) (Getachew *et al.*, 1998a). However, high correlations between gas production and NDF disappearance (R^2 =0.99) have been reported (Pell and Schofield 1993; Herrero and Jessop 1996) or between gas production and DM disappearance (R^2 =0.95) (Prasad *et al.*, 1994).

The contribution of gas produced from the fermentation of protein is small as compared to carbohydrate fermentation (wolin, 1960) as well as the contribution of fat to gas is relatively small and assumed to be negligible (Menke and Steingass

1988; Getachew *et al.*, 1998b). However, Moss *et al.*, (1998a) recommended fat extraction for concentrate foods with 100 g fat / kg DM content or more.

(II) *Historical development of the in vitro gas production technique*

The idea of measuring gas production from carbohydrate fermentation is not new. McBee (1953) introduced a manometric method permitting quantitative determination of gas produced from the fermentation of cellulose and hemicellulose for assaying microbial activity in the rumen. This method was applicable to a wide variety of problems involving the rumen fermentation such as the size of inoculum, feed sample and manometric vessels which have subsequently modified by Hungate *et al.*, (1955).

El-Shazly and Hungate (1965) described a manometric technique for measuring the rate of fermentation of rumen micro-organisms. The rate of gas production by the rumen contents was measured by inserting a needle of a 10-cm^3 water-lubricated syringe through a rubber bung into a 1-pint fruit jar that contained approximately 200 g of rumen contents and reading the volume of gas produced every five min for a period of one hour.

Czerkawski and Breckenridge (1969) described a manometric technique to study the fermentation of sugar-beet pulp and sucrose and to see the effect of linseed oil fatty acids on the fermentation. The apparatus used consisted of five fermentation units, each with a gas inlet, water bath, expansion vessel, gas sampling port, liquid sampling port, manometer and peristaltic pump. The large scale of this method

enabled only two multiple incubations during any one working week to be completed and analyse all the samples even with five units (Czerkawski and Breckenridge 1970). To overcome this problem and simplify the technique Czerkawski and Breckenridge (1970) developed a small scale syringe system consisting of ten units for studying rumen fermentation but even these ten units were still laborious and time consuming to use and relatively large amounts of sample were needed for each incubation.

A simple method for measuring the gas production of rumen micro-organisms using a pressure transducer to sense pressure buildup was developed by Wilkins (1974). This technique consisted of a pressure transducer and a pressure equaliser valve connected with the metal cap of a test tube. Gas pressure was recorded on a strip-chart recorder and the response curves of the gas pressure showed a lag, a rapid pressure buildup and a "leaving off" phase. In this study, the relationship between inoculum size and length of the lag period was demonstrated. Moreover, the effect of shaking on the rate of gas release was examined and the result showed that culture shaken at 200 oscillations / min lead to a remarkable increase the rate of gas release over stationary cultures. Although the application of this method was developed only for detecting coliforms and other gas-producing micro-organisms in clinical samples and for sterility testing of foods, it provided the basis for the development of the pressure transducer systems for feed evaluation (Pell and Schofield, 1993; Theodorou *et al.*, 1994; Cone *et al.*, 1996).

(A) *Hohenheim method (Menke's technique)*

To overcome these problems, Menke *et al.* (1979) suggested an alternative and simpler method to determine the fermentability of feeds by measuring the cumulative volume of gas produced. Using this indirect technique, a high correlation (R^2=0.98) was reported between gas production *in vitro* and apparent digestibility *in vivo*.

Fermentation is carried out in 100 cm^3 gas-tight glass syringes. The syringe nozzle is fitted with a silicon rubber tube of 4-5 cm length, closed by a plastic clip. The feed sample of 200 mg DM is placed in the bottom of the syringe. The microbial inoculum consisted of one part of strained ruminal fluid, and two parts of a nitrogen free buffer media. Thirty cm^3 were pipetted into each prewarmed (39°C) syringe through the silicon rubber tube. Gas bubbles were removed by pushing down the plunger, the plastic clip closed and the position of the piston read and recorded. The syringes were incubated in a rotor that contains two parallel disks with 60 holes in a horizontal location held in a ventilated drying oven at 39°C. As the substrate ferments, so gas is produced both directly from fermentation end products (CO_2 and CH_4) and indirectly from CO_2 released from the buffer by the VFA's produced. These gases cause the syringe plunger to rise and thus enabling the temporal pattern of gas evolution to be determined.

Menke and Steingass, (1988) modified the technique to include ammonium bicarbonate to the media. They also used of the volume of gas production at 24 h from the fermentation of 200 mg feed DM and the chemical constituents of feed (crude protein, fibre, fat and ash) to predict Metabolisable Energy (ME). This method was also modified by Blummel and Ørskov (1993) in that the syringes were placed in a thermostatically controlled water bath instead of a rotor in an incubator.

The method first described by Menke *et al.,* (1979) and later adapted by Menke and Steingass (1988) has been used by many researchers to determine fermentation kinetics by measuring the gas production at different time intervals (Krishnamoorty *et al.*, 1991; Beuvink and Spoelstra, 1992; Blummel and Ørskov 1993). Being a manual method, the accuracy of this system might be low due to the manual reading and recording gas volume. Moreover, this system requires a large amount of expensive glassware and manual periodic reading of the gas production which makes it laborious and time consuming. Taking this into account, Beuvink *et al.*, (1992) developed an automated liquid displacement system to measure gas production in 24 bottles.

(B) *Liquid displacement system*

Beuvink *et al.*,(1992) developed a closed system that automates the registration of gas production over time upon incubation of feedstuffs with buffered ruminal fluid. This system is operated by incubating a 400 mg feedstuff sample and 60 cm^3 of CO_2/HCO_3-buffered rumen fluid (2:1 v/v) anaerobically in a 100 cm^3 fermentation bottle placed in a shaking water bath (39^oC) and connected to the water

displacement system. The calculation of gas production in this system is based on the weight of fluid displaced by gas produced from fermentation of the feed, followed by conversion to gas volume and registration by a data logger. Prior to the start of actual measurement of gas, the system needs to be equilibrated. Although this system is fully automated, it is technically difficult to maintain (Cone *et al.*, 1996) and the preparation prior to the start of measurements is laborious and complicated (Getachew *et al.*, 1998).

(C) *Manometric system*

Waghorn and Stafford (1993) developed a manometric technique to study gas production and nitrogen digestion by rumen microbes from deer and sheep. The system consists of a 250 cm^3 fermentation flask placed in a shaking water bath (39^oC). Each flask is connected to a manometric measuring device that enabled gas volume to be determined at atmospheric pressure. Gas measurement is carried out by incubating a 1.25 g feed sample with 20 cm^3 ruminal fluid diluted by a factor of four with artificial saliva (McDougall, 1948). Gas production is recorded every 30 min for about 10 h. However, similar to other manometric methods described in section II, a large number of samples cannot be used or handled easily in this system. This makes these techniques unsuitable for use in routine feed evaluation.

(D) *Pressure transducer systems*

(1) *Cornell system*

Pell and Schofield (1993) developed a computerised gas production system to facilitate the study of the fermentation dynamics of forages using a pressure sensor to monitor the gaseous products from the fermentation process. This system requires a computer, pressure sensors, an interface card and appropriate software to record and monitor gas production continuously. Incubation is carried out in a 50 cm^3 serum bottle with 100 mg of feed sample wetted with 1 cm^3 of distilled water before addition of 7 cm^3 of the buffer medium recommended by Goering and Van Soest (1970). The bottles need to be equilibrated for five min in an incubator (39^{O}C) prior to the injection of ruminal fluid (2 ml), and inserting of a pressure sensor onto the bottle. After that, the sensor is connected to the computer, and reading is initiated. In this system light bulbs are used to control the incubator temperature. The interval between gas reading was 60 min and each reading is an average of the sensor voltage taken over a 20-s interval. The procedure of this method was revised by (Schofield and Pell 1995) and the revised procedure added a new step which consisted of recalibration of the pressure sensor by infusing a known volume of carbon dioxide every time they were used. One concern is that cultures are not vented in this technique and Theodorou *et al.*, (1994) have reported some perturbation of microbial growth when the pressure exceeded 7 p.s.i (48.3 kPa, 0.47 atm). However, Pell *et al.* (1998) suggested the use of larger fermentation vessels or decreasing the sample size in order to prevent perturbing the fermentation by pressures greater than 7.5 p.s.i (51.7 kPa). The weakness of this method is that it requires a pressure sensor for each

incubation bottle, which makes it more complex and more expensive, and that led Theodorou *et al.*, (1994) to develop a manual transducer system.

(2) *Manual pressure transducer technique*

Theodorou *et al.*, (1994) described a simple gas production technique using a manual pressure transducer similar to that of Wilkins (1974), to provide detailed information on the fermentation dynamics of ruminant feeds. Gas production was measured by incubating 500 mg DM of substrate and 90 cm^3 of reduced buffer medium in 125 cm^3 serum flask. The flask was sealed with a butyl rubber stopper plus aluminum crimp cap and injected with 10 cm^3 of strained ruminal fluid through the stopper. The bottle was placed in an incubator at 39^{o}C and the head-space gas pressure in it adjusted to ambient pressure prior to and just after inoculation with the microbial suspension. Gas production was measured by recording the volume of gas displaced into a syringe barrel on withdrawal of the syringe plunger until the head-space gas pressure returned to ambient pressure, indicated by a zero reading on a digital displaced unit. The main advantage of this method over that of Pell and Schofield (1993) is that it requires only one pressure transducer to measure the gas volume which minimises the method cost (around 500 Sterling pounds) and increases the analytical capacity (number of samples) per incubation series while in method of Pell and Schofield (1993) each incubation flask has its own pressure sensor. However, the disadvantage of this technique is that gas volume measurements have be read and entered manually which limit both the capacity and the accuracy of this system and makes it labour intensive and time consuming. Moreover, Lowman *et al.*, (1998b) studied the effect of head-space pressure on gas production profiles using the

method of Theodorou *et al.*, (1994) and noted a significant effect of reading interval on gas production. They attributed that to the increased pressure in the head-space that might lead to an increase in the volume of gas adsorbed by the culture medium.

(3) *Automated pressure transducer evaluation systems*

Davies *et al.*, (1995) developed an automated pressure evaluation system for determining the fermentation characteristics of ruminant feeds. This system consists of 50 gas-tight culture bottles (140 cm^3), each fitted with a pressure sensor and solenoid valve linked to a PC for continuous monitoring of all bottles. The incubation procedures (the size of substrate, buffer medium and ruminal fluid) are similar to the method of Theodorou *et al.*, (1994). During the fermentation of substrates, each of the in-line solenoid valves are activated at given gas pressure (0.9 psi) causing them to open and release the accumulated gas. To enable construction of gas accumulation profiles, the number of vents and time between each vent is automatically recorded for each bottle which is the main advantage of this technique. Similar to the disadvantage of the Cornell system (Pell and Schofield 1993) this method requires a pressure sensor for each incubation bottle which makes it more complex and more expensive.

Alternatively, Cone *et al.*, (1996) described a fully automated gas production apparatus using sensitive electronic pressures transducers in combination with electronic micro-valves to allow overpressure release during the incubation. Fermentation is conducted in 250 cm^3 serum bottles in which about 400 mg DM substrate is incubated in a mixture of 40 cm^3 buffer medium and 20 cm^3 of strained ruminal fluid. The bottles are then closed with screw caps on which electronic

pressure meters are mounted and connected to the electronic micro-valves, and placed in a shaking water bath at 39^{o}C. The pressure build up in the bottles is measured by the electronic pressure transducer until the pressure in the bottle is equal to a preset upper value (0.65 kPa). When the pressure reaches that preset upper limit, the electronic valve opens allowing the pressure to fall back until the preset minimum value (atmospheric pressure) is reached resulting in closing of the valve. The opening of each valve represents a known amount of gas production (set at approximately 0.7 ml of gas) and the number of valve openings are recorded in a data logger. Despite the obvious benefits of this fully automated system the high cost (approximately £8000 per unit consisting of 12 fermentation serum bottles), complexity and maintenance problems render this system inappropriate for many laboratories particularly in developing countries.

(4) *Semi-automated pressure transducer evaluation system*

Recently, Mauricio *et al.*, (1999) developed a semi-automated *in vitro* technique for ruminant feedstuff evaluation. This method is similar to the method of Theodorou *et al.*, (1994) and only differs in that the requirement to measure gas volume with a syringe was modified by predicting gas volume from accumulated head-space pressure using a pressure transducer connected to a visual display and interfaced with a PC to permit direct data capture. The main advantages of this technique are the simplicity and the low cost (approximately £650) which makes it attractive particularly for developing countries. However, this method still requires periodic reading of gas production which makes the measurements laborious and time consuming.

(III) *Mathematical Models describe gas production profiles*

The time course measurements of *in vitro* gas production of feedstuffs incubated with a mixture of buffer solutions and rumen fluid give data about the rate of feed fermentation. Therefore, a mathematical model is needed to translate these data in the form of curves.

Beuvenk and Kogut, 1993 distinguished three phases of a gas production curve. Firstly, the initial phase (the phase of slow or no gas production produced), which explains the lag time of hydration, attachment and colonisation of insoluble substrate by ruminal microbes. Secondly, the exponential phase (the phase of rapid gas production), which describes the fermentation of the quickly degradable part of the substrate. Thirdly, the asymptotic phase (the phase of slow gas production and finally reach zero), this phase corresponds to the fermentation of the slowly degradable part of the substrate. However, the interpretation of gas production data is not so simple and a number of different models have been proposed. These range from simple, single-pool exponential models to more complex, two-pool logistic ones

However, for the purpose of this review, the models most commonly used with the gas production technique (Table 1.1) will be explained.

Ørskov & McDonald (1979) introduced an exponential model which is widely used in ruminant feedstuff evaluation to describe degradation kinetics measured with

the nylon bag technique. In this technique the soluble material (A) is assumed to be the material lost from the bag by washing it with cold water. This assumption can not be valid in gas production technique where the soluble and insoluble materials are fermented in a sealed gas tight syringe. Therefore, the failure to predict the soluble fraction by the model of Ørskov & McDonald (1979) may be ascribed to the unsuitability of that model for the gas production technique.

Krishnamoorthy *et al.* (1991) derived a new model from the above one. This model has a major weakness when it is used to describe gas production profiles of forages. It underestimates the rapid gas produced from the degradation of soluble material and subsequently overestimates the potential gas produced from the degradation of insoluble material. However, based on the assumption that gas volume up to 4 hours incubation is the gas produced from the soluble material, Jessop and Herrero (1996) modified this model to enable fermentation of the fibrous fraction to be described in a way that over comes the above limitations. They suggested that gas production data should be corrected for the fermentation of soluble components (gas production up to 4 h) of the feed prior to the curve fitting using the model of Krishnamoorthy *et al.* (1991).

Beuvink and Kogut (1993) tested different curve fitting models (i.e., exponential, Gompertz, logistic and Richards) to describe the gas production of grass silages. They reported that none of the models described gas production satisfactorily. They also developed a modified Gompertz model that divided gas production into two fractions, one arising from rapidly fermentable feed components and the other from slowly fermentable feed components.

France *et al.* (1993) introduced a more complex model to describe gas production profiles by use of additional parameters. Generally these parameters result in a better fit to the data but are open to criticism on the grounds that the biological meaning of such parameters is unclear. After testing several mathematical models, Schofield *et al.*, (1994) developed a model with ability to study rate and extent of digestion of specific subfractions for a given ingredient. They concluded that a modified double logistic model with a single lag value was the best model to predict cellulose digestion using the *in vitro* gas production technique. Shortly after that, a method for using a double logistic model to account for the presence of soluble components in feed material was described by Schofield and Pell (1995). This method relies on a curve subtraction process in which the pattern of gas production from both the whole forage and NDF forage are obtained separately and the latter subtracted from the former to give the pattern of gas production and from the NDS fraction. Thus NDF has to be prepared and two separate runs performed for each sample which makes it laborious and time consuming.

Table 1.1 Mathematical description of the commonly used models with gas production technique.

Model	Equation*
Ørskov & McDonald (1979) **(Model 1)**	$Y = A + B\left(1 - e^{-C_B t}\right)$
Krishnamoorthy *et al.* (1991) **(Model 2)**	$Y = B\left(1 - e^{-C_B(t-Lag)}\right)$
France *et al.* (1993) **(Model 3)**	$Y = B\left(1 - e^{-C_B(t-Lag)-d\left(\sqrt{t}-\sqrt{Lag}\right)}\right)$
Beuvink and Kogut (1993) **(Model 4)**	$Y = Be^{\left[-\frac{\mu_r}{D_r}e^{-D_r t}-\frac{\mu_s}{D_s}e^{-D_s t}\right]}$
Schofield *et al.*, (1994) **(Model 5)**	$Y = \frac{A}{\left[1 + e^{\left(2+\frac{4\mu_r}{a}(Lag_r - t)\right)}\right]} + \frac{B}{\left[1 + e^{\left(2+\frac{4\mu_s}{b}(Lag_s - t)\right)}\right]}$
Groot *et al*, (1996) **(Model 6)**	$Y = K_n / (1+(F_n/t)^{Cn}$

* In models 1 and 5, A is the total volume of gas produced (cm^3) from fermentation of initially fermenting component, B is the total volume of gas produced (cm^3) from fermentation of later fermenting fraction. In models 2, 3, and 4, B is total volume of gas produced (cm^3) from fermentation of the substrate. In model 6, K is the maximal gas production (cm^3), F is the time (h) at which half of the maximal gas production (K) is reached. For all models, t is the time (h) from the start of the incubation, Lag is the time before the maximum absolute rate of fermentation of insoluble material occurs, C is the fractional rate of gas production from the fermentation of b fraction, D is a modifying rate ($h^{1/2}$), μ_r and μ_s are the maximum rates of gas production ($cm^3 .h^{-1}$).

Groot *et al.*, (1996) developed a multi-phasic model which differentiates soluble, insoluble but fermentable, and microbial turnover. Although this can provide a conceptually useful data, it requires a large number of data-points which needs an automated equipment.

(IV) *Ruminant nutrition applications of gas production technique*

Since the gas production technique was introduced by Menke *et al.*, (1979), several researchers applied it to ruminant nutrition. The prediction of ruminant performance on diets of different quality roughages by using simple, reliable and cheap techniques has become very important in animal nutrition. The *in vitro* gas production technique has been widely and successfully used to predict *in vivo* apparent digestibility (Menke *et al.*, 1979; Khazaal *et al.*, 1993; Prasad *et al.*, 1994; Khazaal *et al.*, 1995; Adesogan *et al.*, 1998; Fernandaze, 1998; Macheboeuf *et al.*, 1998; Romney *et al.*, 1998) and to predict Metabolisable Energy (ME) content of ruminant feedstuffs (Menke and Steingass 1988; Krishnamoorty *et al.*, 1995).

Moreover, the prediction of ruminant voluntary intake is important in animal nutrition. Taking this into account, several researchers have used the *in vitro* gas production technique to try to predict ruminant food intake. Blümmel and Ørskov (1993) compared the accuracy of the gas production and nylon bag technique in predicting the intake in cattle when they adopted the gas test to describe the kinetics of fermentation for 10 cereal straws based on the exponential model $P=a+b\ (1-e^{-ct})$ (Ørskov and McDonald, 1979). They reported that the total gas production (a+b)

from 10 cereal straws correlated in a multiple regression model with DM intake (R^2=0.88), digestible DM intake of the straws (R^2=0.93) and growth rate (R^2=0.95) of the steers. Similarly, Khazaal *et al.* (1993) compared the accuracy of gas production during incubation with rumen liquor *in vitro* and nylon bag degradability as predictors of the voluntary intake of 10 hays. They concluded that both the gas production and nylon bag technique were capable of predicting intake (R^2=0.63). Other workers have also reported significant correlation between *in vitro* gas production and DM intake (Khazaal *et al.*, 1995; Blummel and Becker 1997; Blummel *et al.*1997; Fernandaze, 1998; Romney *et al.*, 1998).

Furthermore, the *in vitro* gas production technique has been used to evaluate the nutritional value of several browse species (Kibon and Ørskov 1993; Siaw *et al.*, 1993; Nsahlai *et al.*, 1994; Tolera *et al.*, 1997) or to study the fermentation dynamics of legumes (Herrero and Jessop, 1997; Abreu and Bruno-Soares, 1998). The gas production technique has been also employed for evaluation of the interaction between basal and supplementary diets (Manyuchi *et al.*, 1992; Castro *et al.*, 1993; Sampath *et al.*, 1995; Bonsi *et al.*, 1996; Wood and Manyuchi *et al.*, 1997).

In addition to that, the *in vitro* gas production technique has been used to study the effects of anti-nutritional factors such as tannins, saponins and alkaloid on rumen fermentation dynamics (Khazaal *et al.*, 1994; Longland *et al.*, 1995; Makkar *et al.*, 1995a; Makkar *et al.*, 1995b). The gas production method has also been used to assess the minimum ammonium concentration needed to optimise the fermentation rate of carbohydrate (Dryhurt and Wood 1998) but in this study the nitrogen

concentration indicated may underestimate the true concentration of nitrogen available to the microbes because the contributions of feedstuff and inoculum nitrogen were not taken into account.

(V) *Weaknesses of gas production technique*

Being a biological system, the *in vitro* gas production method is liable to many sources of error, which makes this technique difficult to control. The single greatest source of uncontrolled variation in the *in vitro* gas production system is the use of ruminal fluid. Variation in microbial activity of ruminal fluid has been shown to vary between batches of rumen fluid taken at different days and resulted in a changing in the gas production profiles (Krishnamoorthy *et al.,* 1991; Cone *et al.,* 1996). Beuvink *et al.,* (1992) noted a significant difference in the total gas production from glucose, cellulose and rice starch incubated in ruminal fluid taken from different days. Nagadi (1996) observed that the curves extrapolated from the gas production data were influenced significantly by the variation in ruminal microbial activity taken from different days. Several factors were found to influence the rumen liquor activity. Recently, Rymer *et al.,* (1999) also reported a significant differences in gas production profiles of temperate dried grass incubated in ruminal fluid collected in different days. Although the above authors stated this variation in microbial activity of ruminal liquor between days, little attention has been paid to study the factors that might cause this uncertainty of ruminal microbial activity.

Furthermore, the diet of the donor animal could affect the microbial activity of ruminal fluid and subsequently influence the gas production profiles. Several

studies have indicated that changing the diet of the host animal influences the total gas production (Trei *et al*, 1970; Menke and Steingass, 1988; Huntington *et al.*, 1998; Mertens *et al.*, 1998) or the rate of gas production (Bonsi *et al.*, 1995; Cone *et al.*, 1996; Das and Singh 1998). But it is still unknown if there is a relationship between the quality of donor diet and the microbial concentration of ruminal fluid, and what the minimum microbial concentration is needed from the donor diet to supply sufficient micro-organisms to optimise the *in vitro* gas production profile.

A consequence of the development of different gas production apparatus (section II) is variation in inoculation buffer and dilution of the ruminal fluid with buffer. This makes comparison of results between laboratories difficult. While Menke and Steingass, (1988); Beuvink *et al.*, (1992) and Cone *et al.*, (1996) used one part of rumen fluid in two parts of buffer, Theodorou *et al.*, (1994) diluted rumen liquor by a factor of 10. The two main fates of degraded carbohydrate are either fermentation to VFA and gases or incorporation into new microbial matter (Jessop and Herrero, 1998). The balance between the two will depend on the rate of ATP production relative to microbial maintenance requirement (Jessop and Herrero, 1998). Differences in dilution of rumen fluid will alter the microbial mass added to each incubation. As microbial mass added decreases, so potential for degraded carbohydrate to be incorporated into new microbial matter and hence not into gasses increases. Pell and Schofield, (1993) studied the effect of increasing the size of the ruminal fluid inoculum on gas production of alfalfa and suggested that a 20% (v/v) ruminal fluid inoculum was sufficient to ensure the maximum rate of fibre digestion whereas Rymer *et al.* (1999) reported that increasing rumen fluid concentration in the

inoculant from 5 to 30% (v/v) resulted in an increase in the total volume and the rate of gas produced and a decrease in the lag time of high temperature dried grass.

In addition to the above mentioned factors, microbial activity of ruminal fluid has been noted to vary both between different species of animal (Goncalces and Borba 1996; Calabro and Williams 1998) and the time at which it is collected (Menke and Steingass, 1988; Pell and Schofield, 1993; Cone *et al.*, 1996).

The above uncertainties about ruminal fluid microbial activity emphasise the need for standardisation of the level of microbial concentration used in the *in vitro* gas production technique in order to minimise variation in microbial activity between days and to facilitate comparison of results between different laboratories. Rymer *et al.*, (1998a) emphasised the importance of this standardisation after the reproducibility of gas production technique between laboratories was to be poor in a ring test that was done by them. This has not, as yet been addressed.

Besides the uncontrolled variation mentioned above, the objection to the use of fistulated animals by animal welfare organisations is strong. Alternatively, several studies have indicated that a faecal inoculum has potential to replace ruminal fluid and hence reduced the dependence of the *in vitro* gas production method on ruminally cannulated animal as inoculum donor (Apile *et al.*, 1992; Harris *et al.*, 1995; Nsahlai and Umunna 1996; Altaf *et al.*, 1998; El-Meadaway *et al.*, 1998; Macheboeuf *et al.*, 1998; Mauriccio *et al.*, 1998a&b; Lowman *et al.*, 1999). There were discrepancies between those studies in the gas production profiles between

ruminal and faecal inoculum. While Apile *et al.*, (1992), Altaf *et al.*, (1998), El-Meadaway *et al.*, (1998), and Macheboeuf *et al.*, (1998) reported a similar total gas production between ruminal and faecal inoculum, Mauriccio *et al.*, (1998a) noted significant higher gas production when a feed sample was incubated in ruminal than in faecal fluid whereas Harris *et al.*, (1995) observed greater total gas from faecal rather than from ruminal fluid. All of them agreed that faeces usually give a longer lag phase and lower rate of gas production except Harris *et al.*, (1995) who reported the converse. It seems from these studies that the longer lag and lower rate of gas production associated with faecal fluid were probably due to a lower microbial activity in faecal fluid in comparison with ruminal fluid. However, in order to make a proper comparison between ruminal and faecal inoculum, the above uncertainties about ruminal microbial activity should be addressed.

Due to the need to use feed samples that are homogenous and representative, the *in vitro* gas production technique is based on the use of feed material which has been dried and ground through a 1mm screen. However, animals consume fresh or ensiled forages and drying the feed samples might influence the pattern of gas production. Sanderson *et al.*, (1997) emphasised the importance of the physical form of the feed sample incubated *in vitro* using the gas production technique when they noted a significant differences in the gas production profiles between fresh and dry silages. Givens and Gill (1998) also stressed the importance of the physical structure of feed samples incubated *in vitro* after Deaville (1995) observed that milling wheat grain samples prior to the measurement of gas resulted in a higher fermentation rate than the same wheat treated with sodium hydroxide at different moisture contents.

Cone (1998) investigated the difference in gas production profiles between fresh grass silage cut in small pieces and freeze dried and milled grass silage. He reported that the fresh sample had a higher initial gas production than the dry sample but after 3 to 4 hours the rate of gas production of dried sample was higher than the fresh. However, more research is needed in this area and mimicking animal mastication of feed samples should also be taken into account.

Gas production profiles have also been noted to be affected by other factors such as a method of inoculation (Huntington and Givens 1997), the use of different gas production apparatus (Rymer and Givens 1997), pre-soaking substrate with medium (Rymer *et al.*, 1999) and extensive gassing the buffer medium with carbon dioxide (Rymer *et al* 1998b).

1.4 The aims and objectives of this thesis

1 To study the influence of microbial activity on the pattern of gas production and test the possibility of developing a protocol for standardising the microbial activity of ruminal fluid by examining:

A) The effect of varying the dilution of ruminal fluid on the pattern of gas production and NDF digestibility

B) The effect of frequency of sampling of ruminal fluid on microbial activity and subsequent fermentation

C) The influence of donor diet on initial microbial concentration and gas production degradability.

2 To investigate the effect of physical form of feed sample incubated *in vitro* on the pattern of gas production by comparing the gas production profiles of fresh and dry forages.

3 To examine the potential of different sources of faecal fluid used as the inoculum for the *in vitro* gas production method to replace ruminal fluid.

4 To examine the suitability of using the *in vitro* gas production technique to study protein-energy interactions by studying the effect of fermentable nitrogen availability on the gas production degradability of NDF.

Chapter 2

The influence of microbial activity on the pattern of gas production

2.1 ABSTRACT

The microbial activity of the inoculant used in the *in vitro* gas production technique was varied and the consequences for the pattern of gas production investigated. In experiment 1, ruminal fluid was diluted 1:2, 1:5, 1:9 and 1:20 (v/v) with buffer solution and the *in vitro* gas production from cellulose, glucose, hay and ryegrass studied. Gas production at 4, 24 and 120h were significantly decreased as the ratio of buffer solution to ruminal fluid increased. The gas production degradability parameters (B, C and Lag) obtained from fitting the data to the model Gas = B ($1\text{-}exp^{-C(t\text{-}Lag)}$) were also affected by extensive dilution of ruminal fluid with buffer solution. For each substrate, incubation with ruminal fluid diluted 1:2 (v/v) gave a higher ($P<0.05$) asymptotic value 'B', rate 'C 'of gas production and lower lag time than when incubated in the 1:5, 1:9 and 1:20 dilutions. In experiment 2, the effect of frequency of ovine ruminal sampling on microbial activity and substrate fermentation was investigated. The bacterial DM, bacterial absorbance and blank's gas volume were significantly affected by daily sampling of ruminal fluid. Daily sampling of rumen fluid did not significantly affect the gas production degradability parameters (B, C and Lag) until the bacterial DM fell below 0.09 g DM / 10 cm^3 strained rumen fluid). Bacterial DM was strongly related to the absorbance of ruminal fluid ($R^2 = 0.99$, $p<0.001$), suggesting that standardisation of the level of microbial activity between days and laboratory can be achieved by estimating the bacterial DM from the absorbance of the strained ruminal fluid. A strong relationship between bacterial DM and blank gas volumes ($R^2 = 0.99$, $p<0.001$) was observed, suggesting that comparison of initial microbial concentration can be achieved by estimating the initial bacterial DM from the blank's gas volume.

2.2 INTRODUCTION

Food eaten by a ruminant firstly undergoes microbial fermentation within the rumen. Nutritionally important characteristics of the food are the rate and extent of fermentation of its carbohydrate fraction, which can be measured by *in situe* method (Mehrez and Ørskov, 1977) or *in vitro* techniques (Tilley and Terry, 1963; Goering and Van Soest, 1970; Cone *et al.*, 1991). These methods estimate the kinetics of degradation by analyzing the incubated residues after different time of incubations. However, those methods measure feed loss which may not equate to use of feed.

Menke *et al.* (1979) described the principles of the *in vitro* gas production technique to overcome this limitation by measuring the products of fermentation (e.g. CO_2 and CH_4) rather than the disappearance of feed. Since then, several attempts have been made to develop the technique to measure the kinetics of gas production (Beuvink *et al.*, 1992; Pell and Schofield, 1993; Theodorou *et al.*, 1994; Cone *et al.*, 1996).

The *in vitro* gas production technique measures the degradation characteristics of feed carbohydrate. As feed carbohydrate is fermented by micro-organisms, so gas is produced both directly as CO_2 and CH_4 and indirectly from reaction of VFA with bicarbonate in the buffer. However, the potential exists for degraded carbohydrate to be incorporated into microbial matter and thus not to result in gas production. If the technique is to be used to measure the potential rate of forage degradation (which is a characteristic of the forage material), then

incorporation of degraded carbohydrate into new microbial matter should be minimised since it will cause underestimation of this rate. Partition of degraded carbohydrate between pathways of fermentation and incorporation are likely to be influenced by the rate of microbial ATP production relative to microbial maintenance requirement. As this ratio approaches unity so the potential for microbial growth will be reduced (Jessop and Herrero, 1998).

The *in vitro* gas production technique is used by many different groups. One factor which varies between these groups is the extent to which ruminal fluid is diluted by buffer solution. Menke and Steingass, (1988); Beuvink *et al.* (1992) and Cone *et al.* (1996) use one part of rumen fluid to two parts of buffer, Pell and Schofield, (1993) recommended the use of one part of rumen fluid in four parts of buffer, whilst Theodorou *et al.* (1994) diluted rumen liquor by a factor of 10. This will result in differences in the quantity of microbial cells used as an inoculate and hence affect the microbial maintenance requirement and potential for microbial growth.

In addition to variation in the extent of dilution of ruminal fluid, variation in microbial activity of ruminal fluid has been shown to vary between batches of rumen fluid taken at different days (Krishnamoorthy *et al.*, 1991; Beuvink *et al.*, 1992; Cone *et al.*, 1996; Nagadi, 1996; Rymer *et al.*, 1999). But little attention has been paid to study the factors that might lower microbial concentration and cause the variation in microbial activity of ruminal fluid.

The aim of this study are 1) to investigate the effect of varying the dilution of ruminal fluid on the pattern of gas production and NDF digestibility, 2) to determine whether the frequency of sampling of ruminal fluid affected the microbial activity and subsequent fermentation and 3) to develop a protocol for standardrising the microbial activity of ruminal fluid.

2.3 MATERIAL AND METHODS

Animals and diet

Two ruminally fistulated Suffolk sheep fed 750g sheep pellets (NDF and CP 338 and 175 g/kg DM) and 250g grass nut (NDF and CP 345 and 150 g/kg DM) twice a day at 8.00 and 17.00 h were used. Water was available to the sheep *ad libitum.*

Inoculum preparation

A sample of rumen contents (according to the design of each experiment) containing both solid and liquid material was taken before morning feeding and collected in a prewarmed vacuum flask. In the laboratory ruminal fluid was filtered through two layers of muslin cloth, the solid material was squeezed lightly. The strained ruminal fluid was mixed with anaerobic medium described by Menke and Steingass, (1988). Once prepared, the suspension of micro-organisms was kept at 39 °C with CO_2 bubbling through for approximately 20 min before addition to syringes.

Experimental treatments

Experiment 1

The aim of this experiment was to study the effect of varying the dilution of ruminal fluid on the pattern of gas production and NDF digestibility. Samples of strained ruminal fluid were taken and mixed with the anaerobic medium to produce four different dilutions (ruminal fluid : buffer solution) 1:2, 1:5, 1:9 and 1:20 (v/v). The initial pH of each dilution was measured. For each dilution gas production was measured in the absence of substrate and from the incubation of 200mg DM α-cellulose (Sigma, Chemical Co. Ltd. Poole, Dorset, UK), hay , ryegrass and 50mg DM D-glucose (Merck Ltd, Merck House, Poole, Dorset, UK) in 100 cm^3 glass syringes (Fortuna, Germany). Each combination of dilution and substrate was replicated four times. NDF residues were collected at the end of the incubations for the estimation of NDF digestibility (NDFD) using a modified micro technique (Pell and Schofield, 1993) by emptying the contents of each syringes into 100 cm^3 medical flat bottles. Each syringe was washed three times with 5 cm^3 of NDF solution, the washings being added to the appropriate bottle. Forage samples (hay and ryegrass) were analysed for neutral detergent fibre (NDF) using the above technique, crude protein (CP) using standard Kjeldahl nitrogen determinations and for ash (Table 2.1).

Experiment 2

The aim of this experiment was to study the effect of frequency of sampling of ruminal fluid on microbial activity and subsequent fermentation. In a replicated block design 1.5L of ruminal fluid was taken from each of two fistulated sheep (and combined) every 24h for five days (Period 1) and every 72h (Period 2). The animals had two weeks rest before and between successive periods. The ruminal fluid was prepared as described above and mixed (1:2 v/v) with the anaerobic medium. Gas production was measured without added substrate (blank) and from the incubation of 200mg DM α- cellulose, 50mg DM D-glucose. Bacterial DM was measured directly by centrifuging 30 ml of the buffered rumen fluid at 1000 X G (to remove protozoa and fungi and particulate matter) for 5 min at 30 °C. The pellet was discarded and the supernatant was recentrifuged at 26000 X G for 15 min at 4 °C. The supernatant was discarded whilst the bacterial pellet was resuspended in NaCl (9g/l) solution and centrifuged again. The supernatant was discarded and the bacterial pellet was dried at 80 °C to constant weight for determination of bacterial DM (Henning *et al.*1991). Bacterial DM was also measured indirectly by reading the absorbance at 600 nm (Brown *et al*, 1989; Wells and Russell, 1996) of ruminal fluid that had been diluted fifty fold with the buffer.

Gas production measurements and analytical procedures

The *in vitro* gas production was performed as described by Jessop and Herrero (1996). The cumulative gas production for each syringe was recorded at 1,2,3,4,5,6,8 h; thereafter every 4 h until 60 h, and then 72, 84, 96 and 120 h and fitted to the model Gas = $B(1-exp^{-C(t-Lag)})$ (Krishnamoorthy *et. al.*, 1991) using Marquart algorithm as implemented by GraFit (Leatherbarrow, 1992). B is the asymptotic gas production from the fermentation of carbohydrate (cm^3), C is the fractional rate of gas production (/h), t is time (h) and Lag is the lag phase before the fermentation of carbohydrate begins (h).

Statistical analysis

Analysis of variance using MINITAB statistical package (1993) was used to compare both gas volume and estimated parameters. Least significant differences were calculated from the standard error of the differences between means. Regression was used to study the relationship between bacterial DM, absorbance and blank's gas volume.

Table 2.1 The chemical compositions of Hay and Ryegrass.

Feed sample	NDF (g/kg DM)	CP (g/kg DM)	Ash (g/kg DM)
Hay	612 ± 24.3	146 ± 6.7	103 ± 4.9
Ryegrass	476 ± 16.1	193 ± 4.4	82 ± 7.2

2.4 RESULTS

The effect of variation in the dilution of ruminal fluid on the pattern of gas production and NDF digestibility

The pattern of gas production

Figure 2.1 shows the effect of diluting ruminal fluid on the pattern of the gas production from the incubation of cellulose, glucose, hay and ryegrass. Both the total quantity of gas and the rate at which it was produced differed. This can be seen by comparing gas volume at 4, 24 and 120 h (Table 2.2). In all cases the pattern of gas production appeared to be sigmoidal at a dilution of 1:20.

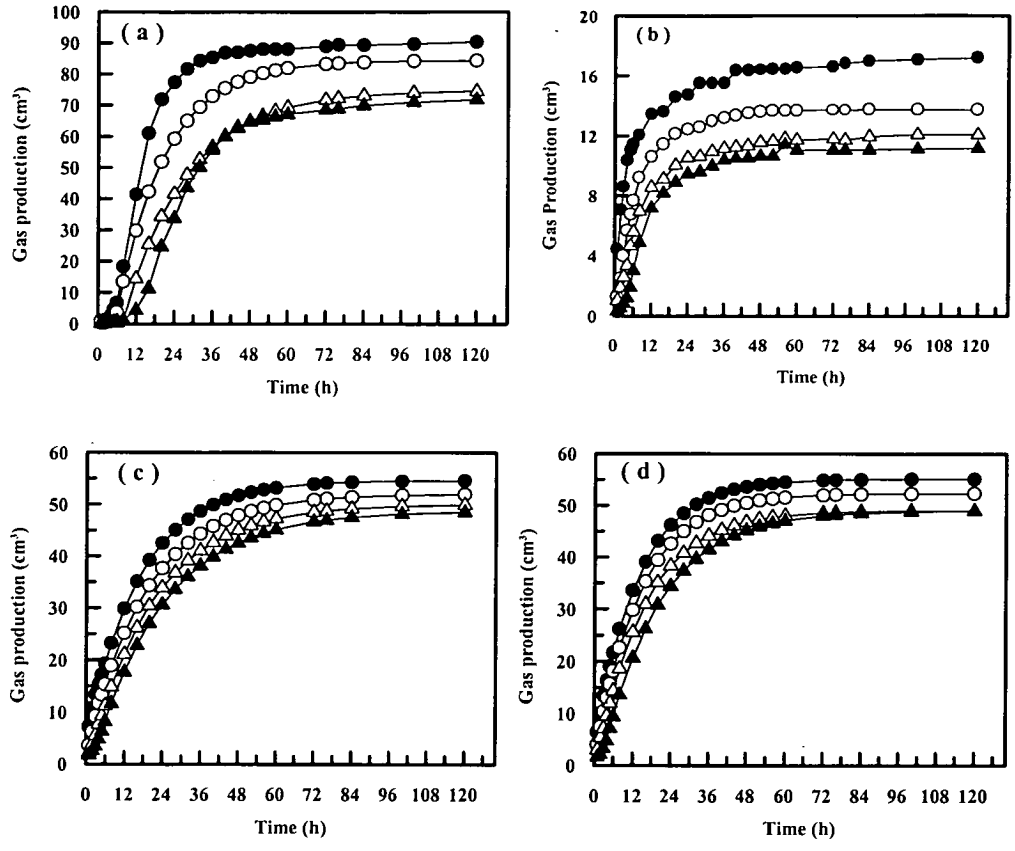

Figure 2.1 The pattern of gas produced from the incubation of cellulose (a), glucose (b), hay (c) and ryegrass (d) in rumen fluid diluted with a different ratio of buffer solution (1:2 (●), 1:5 (○), 1:9 (Δ) and 1:20 (▲) (v/v)).

Gas production at 4, 24 and 120 h.

The effect of diluting ruminal fluid with different volumes of buffer solution on the gas produced at 4, 24 and 120 h from the incubation of cellulose, glucose, hay and rye are presented in Table 2.2.

After 4 h incubation, the gas produced from each substrate varied depending on the microbial activity added in the inoculant. The greater the activity added, the more gas produced. The only exception to this was that there was no difference between gas produced at 1:9 and 1:20 dilution for cellulose.

A similar pattern was seen in the net gas produced after 24 h incubation. Again for all substrates, the higher the microbial activity added, the higher the volume of gas produced. The only exception to this was for glucose where there were no differences between gas produced at 1:9 and 1:20 dilution.

After 120 h incubation, the total net volume of gas produced for each substrate was greater for 1:2 dilution. The 1:5 dilution produced more gas than the 1:9 and 1:20 dilutions which did not differ for glucose, hay and ryegrass. For cellulose there was more gas produced by 1:9 when compared to 1:20. The differences in gas production between 1:2 and 1:20 dilutions were 20, 35, 10 and 11% for cellulose, glucose, hay and rye grass respectively.

Table 2.2 Gas production at 4, 24 and 120 h from the incubation of cellulose, glucose, hay and rye in a different ratio of ruminal fluid : buffer solution (*n=4*). Means with different superscripts within the same row are significantly different (*P<0.05*).

Time (h)	Feed samples	Gas produced (cm^3) (rumen fluid : buffer solution v/v) 1:2	1:5	1:9	1:20	SED
4 h	Cellulose	2.6^a	1.5^b	0.3^c	0.2^c	0.59
4 h	Glucose	10.3^a	5.6^b	3.3^c	1.2^d	0.76
4 h	Hay	15.3^a	11.7^b	7.7^c	4.8^d	0.71
4 h	Rye	16.3^a	13^b	9.5^c	4.8^d	0.79
24 h	Cellulose	77.5^a	59.3^b	41.7^c	33.8^d	1.07
24 h	Glucose	14.8^a	12.4^b	10.5^c	9.5^c	0.56
24 h	Hay	42.5^a	37.6^b	33.9^c	30.6^d	1.31
24 h	Rye	46.2^a	42.6^b	38.3^c	34.4^d	1.57
120 h	Cellulose	90.1^a	84.4^b	74.8^c	72.0^d	0.49
120 h	Glucose	17.1^a	13.7^b	12.0^c	11.1^c	0.87
120 h	Hay	54.6^a	51.9^b	49.5^c	48.7^c	1.04
120 h	Rye	55.1^a	52.3^b	49.1^c	49.0^c	1.08

Gas production degradability dynamics.

Table 2.3 shows the effect of dilution of ruminal fluid on the gas production parameters of cellulose, glucose, hay and rye grass obtained from the model of Krishnamoorthy *et al.* (1991).

The effect of diluting rumen fluid with a different ratio of buffer solution on the asymptotic gas production (B) from the fermentation of cellulose, glucose, hay

and rye grass was similar to their pattern of gas production at 120 h which has already been described in page 48.

The fractional rate of gas production of cellulose, glucose, hay and ryegrass decreased as the dilution of ruminal fluid increased. Cellulose, glucose, hay and rye grass incubated in 1:2 had the highest ($P<0.05$) rate which were respectively about 41, 53, 27 and 24% higher than those incubated in 1:20.

The estimated lag for each substrate was increased ($P<0.05$) as the dilution of ruminal fluid increased.

Table 2.3 *In vitro* gas production dynamics of cellulose, glucose, hay and rye incubated in a different ratio of ruminal fluid : buffer solution ($n=4$). Means with different superscripts within the same row are significantly different ($P<0.05$).

Feed samples	Fitted parameter	The ratio of diluting rumen fluid (rumen fluid : buffer solution v/v) 1:2	1:5	1:9	1:20	SED
Cellulose	B (cm^3)	89.2^a	84.4^b	74.8^c	70.9^d	0.61
	C (/h)	0.106^a	0.065^b	0.051^b	0.063^b	0.0081
	Lag (h)	4.5^a	5.7^b	7.7^c	13.6^d	0.43
Glucose	B (cm^3)	16.4^a	13.5^b	11.7^c	10.9^c	0.77
	C (/h)	0.206^a	0.135^b	0.102^c	0.097^c	0.0068
	Lag (h)	-1.5^a	-1.1^b	-0.7^c	2.3^d	0.15
Hay	B (cm^3)	54.7^a	52.1^b	49.3^c	48.9^c	1.07
	C (/h)	0.059^a	0.050^b	0.047^b	0.043^b	0.0036
	Lag (h)	-1.8^a	-1.3^b	-0.3^c	1.6^d	0.17
Rye	B (cm^3)	55.3^a	52.4^b	49.2^c	49.2^c	1.14
	C (/h)	0.076^a	0.066^b	0.063^b	0.058^b	0.0043
	Lag (h)	-1.6^a	-1.1^b	-0.6^c	1.8^d	0.12

NDF Digestibility, total blanks volume and initial pH

The influence of diluting ruminal fluid to differing extents (1:2, 1:5, 1:9 and 1:20) on the NDF digestibility of cellulose, hay and ryegrass, the gas produced after 120 h in the absence of substrate (blanks gas volume) and the initial pH of each dilution are shown in Table 2.4.

NDF digestibility was not influenced by dilution of ruminal fluid for any substrate. Total gas produced from blanks was significantly ($P<0.05$) decreased as extents of dilution of ruminal increased. It was about 56, 79 and 93% higher on 1:2 than on 1:5, 1:9 and 1:20 dilutions respectively. 1:20 dilution had the highest ($P<0.05$) initial pH value, followed by 1:9, 1:5 and 1:20 respectively. However, there were no significant differences in the initial pH between 1:5 and 1:2.

Table 2.4 NDF Digestibility of cellulose, hay and rye incubated in a different ratio of rumen fluid : buffer solution ($n=4$), total gas produced from the blanks ($n=3$) at 120h and the initial pH ($n=3$). of each dilution (1:2, 1:5, 1:9 and 1:20). Means with the different superscripts within the same column are significantly different ($P<0.05$).

Rumen fluid : Buffer solution (v/v)	NDF Digestibility (g/kg DM)			Blanks volume (cm^3)	Initial pH
	Cellulose	Hay	Rye		
1:2	1000^a	686^a	862^a	20.5^a	6.93^a
1:5	1000^a	670^a	860^a	9.1^b	6.94^a
1:9	1000^a	664^a	857^a	4.3^c	6.98^b
1:20	997^a	660^a	848^a	1.5^d	7.01^c
SED	2.5	16.1	12.8	1.03	0.01

Effect of frequency of ovine ruminal sampling on microbial activity and substrate fermentation

Bacterial DM, bacterial absorbance, blanks' gas volume and in vitro gas production degradability parameters

Effect of frequency of ovine ruminal sampling on bacterial DM, bacterial absorbance, blanks' gas volume and gas production degradability parameters of cellulose and glucose are presented in Table 2.5.

Daily sampling of ruminal fluid reduced ($P<0.05$) bacterial DM, bacterial absorbance and blank gas volume by 32.1 (mg/10 cm^3 of strained rumen fluid), 0.036 (A) and 5.6 cm^3 a day respectively.

The gas production degradability parameters (B, C and lag) for cellulose and glucose were not significantly affected by daily sampling of ruminal fluid until bacterial DM, bacterial absorbance and blank gas volume were lower than 93.7 (mg), 0.111(A) and 15.5(cm^3) respectively. Below that the fractional rate of gas production (C) and the asymptotic gas production from fermentation of cellulose and glucose (B) were reduced ($P<0.05$) and the lag for cellulose fermentability was increased ($P<0.05$).

There were no significant differences in the bacterial DM, bacterial absorbance, blanks' volume and the gas production degradability parameters (B, C and lag) when animals were sampled at 72 h intervals.

Table 2.5 Effect of different sampling frequencies of ruminal fluid on microbial activity and the *in vitro* gas production dynamics.

Period	Sampling Frequency (h)	Bacterial DM (mg/10cm^3)	Bacterial Absorbance (A)	Blanks' Gas Volume (cm^3)	Gas production degradability parameters					
					Cellulose			Glucose		
					B (cm^3)	C (/h)	Lag (h)	B (cm^3)	C (/h)	Lag (h)
1	**0**	166.2^a	0.191^a	29.0^a	93.3 ^{a}	0.109^a	4.4^a	18.2^a	0.210^a	0
	24	120.2^b	0.134^b	20.0^b	90.6 ^{a}	0.103^a	4.5^a	17.0^a	0.200^a	0
	48	93.7^c	0.111^c	15.5^c	89.4^a	0.096^a	4.7^a	16.7^a	0.198^a	0
	72	60.0^d	0.071^d	10.0^d	84.1^b	0.068^b	5.6^b	14.3^b	0.13^b	0
	96	36.0^e	0.042^e	6.0^e	76.9^c	0.054^b	7.5^c	12.7^c	0.111^c	0
2	**0**	158.0^a	0.183^a	27^a	91.9^a	0.104^a	4.5^a	17.7^a	0.203^a	0
	72	157.0^a	0.182^a	27^a	91.7^a	0.097^a	4.5^a	17.7^a	0.202^a	0
	SED	6.60	0.0053	0.92	2.09	0.0076	0.20	0.78	0.008	---

*Means with different superscripts within the same column differ significantly ($P<0.05$).

Relationship between bacterial DM and bacterial absorbance

The relationship between bacterial DM and bacterial absorbance are shown in Figure 2.2. Bacterial DM was linearly related (R^2 = 0.99, p<0.001) to bacterial absorbance such that:

Bacterial DM (mg / 10 cm^3 of strained rumen fluid) =

868 (SE ± 3.9)* Bacterial Absorbance (A)

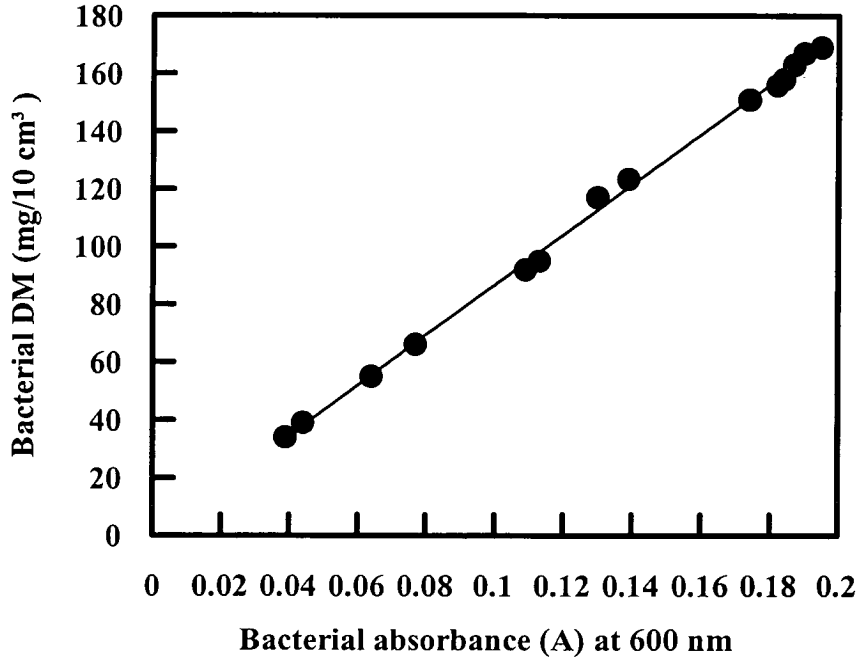

Figure 2.2 The relationship between bacterial DM mg / 10 cm^3 of strained rumen fluid and bacterial absorbance A (*n=14*).

Relationship between bacterial DM and blanks gas volume

Figure 2.3 shows the relationship between bacterial DM and blanks gas volume. There was a linear relationship ($R^2 = 0.99$, $p<0.001$) between bacterial DM and blanks gas volume such that:

Bacterial DM (mg / 10 cm^3 of strained rumen fluid) =

5.89 (SE ±0.043)*Blanks' Volume (cm^3).

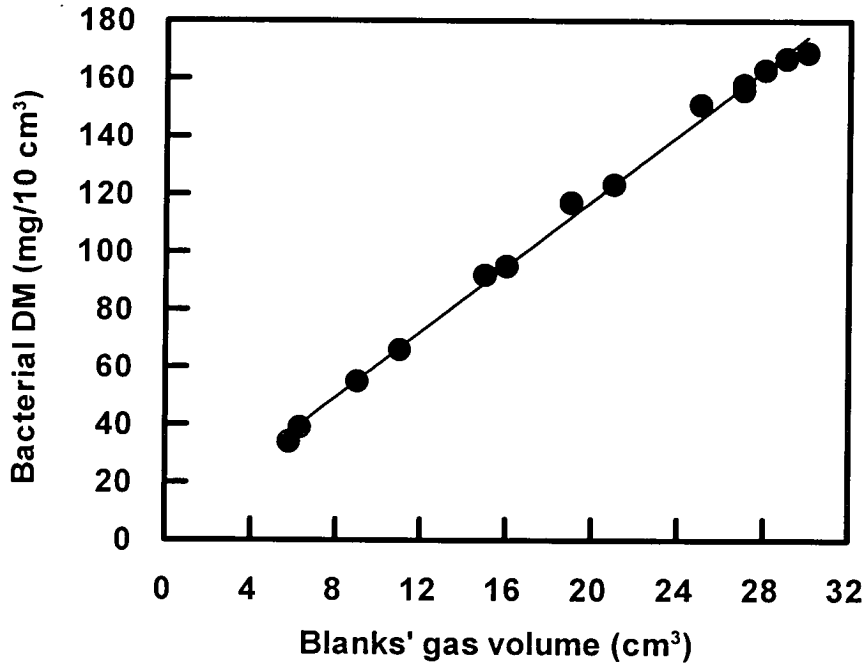

Figure 2.3 The relationship between bacterial DM mg / 10 cm^3 of strained rumen fluid and blanks gas volume (*n=14*).

2.5 DISCUSSION

The effect of diluting ruminal fluid with a different ratio of buffer solution on the pattern of gas production and on the NDF digestibility

The results of the present study indicate that lowering the concentration of ruminal fluid by increasing the amount of buffer solution reduced the volume of gas produced from fermentation of substrate without altering NDF digestibility. This suggests that as the initial microbial mass was reduced (i.e. more diluted ruminal fluid) less gas was produced per unit of substrate fermented. Stoichiometric relationships have shown that around 0.47 cm^3 of gas should be produced per mg of NDF fermented (Wolin, 1960; Herrero and Jessop, 1996). Taking cellulose as pure NDF, it can be observed that at a 1:2 dilution, 0.45 cm^3 gas were produced per mg NDF degraded which fell to 0.36 cm^3 / mg NDF degraded at a dilution of 1:20.

Jessop and Herrero (1998) argued that initial microbial concentrations of less than 0.09g DM per 10 cm^3 of strained ruminal fluid for sample size of 200 mg DM tended to partition degraded carbohydrate towards microbial growth rather than fermentation and hence gas production, thus explaining the results of the present study. This is in agreement with Lowman *et al*, (1998a), who found that independently of the gas production technique used, naked oats incubated with ruminal fluid diluted 1:2 produced higher total gas production than on a 1:9 ratio. Rymer *et al.* (1999) also studied the effect of increasing rumen fluid concentration in the inoculant from 5 to 30% (v/v) on the gas profile of high temperature dried grass and they noted that increasing rumen fluid concentration resulted in an increase in the

total volume and the rate of gas produced and a decrease in the lag time. Pell and Schofield, (1993) studied the effect of increasing the size of the ruminal fluid inoculum on gas production of alfalfa and suggested that a 1:4 dilution factor is sufficient to ensure the maximum rate of fibre digestion and enough to obtain the asymptote fermentation of alfalfa, while we found that a lower dilution was required. The discrepancy between studies is probably related to differences in the initial microbial concentration of the rumen fluid which has been shown to vary between days (Beuvink *et al*, 1992; Cone *et al*, 1996; Nagadi, 1996; Rymer *et al*, 1999) and also the different initial sample size used in both studies. Pell and Schofield, (1993) use 100 mg of feed DM while we use 200mg DM.

In terms of the kinetics of gas production, the present study has shown the rate of gas production (C) and the lag time were substantially affected by the extent of dilution of the ruminal fluid. Lower rates of gas production and longer lag times were observed when a more diluted inoculum was used, in agreement with the work of Rymer *et al* (1999). Similar results also were observed by Hidayat *et al* (1993), who found higher rates of gas production as the initial microbial density was increased. The results also suggest that at high dilutions of ruminal fluid, the rate of gas production followed second order kinetics i.e. gas production depended, not on substrate mass, but on microbial mass as well (Schofield and Pell 1995). As stated before, microbial growth occurs in highly diluted inocula, and should be prevented for an adequate description of the feed.

The aim of the gas production technique is to describe the potential degradation kinetics of feeds. For this to be achieved, it is important to reduce all possible artifacts caused by external factors.

Effect of frequency of ovine ruminal sampling on microbial activity and substrate fermentation

Bacterial DM, bacterial absorbance, blanks' gas volume and in vitro gas production degradability parameters

The primary objective of this study was to investigate the effect of frequency of ovine ruminal sampling on microbial activity and substrate fermentation. The results show that the microbial activity is extremely sensitive to the frequency of ruminal sampling. Bacterial DM, bacterial absorbance and blanks gas volume significantly decreased as the number of consecutive daily samplings increased (Table 2.5). A possible explanation could be that sampling a large volume of ruminal fluid every day increased the empty space in the rumen and resulted in more oxygen entering the rumen and mixing with the food. This may have prevented the anaerobic conditions in the rumen being maintained, thus preventing microbial growth. Another possible explanation could be due to that removal of microbial biomass and substrate (food) without allowing compensation by letting the animal eat more food. Allowing an interval of two days between samplings was enough to enable the rumen microbial levels to recover. Table 2.5 indicates that there were no significant differences in bacterial DM, bacterial absorbance blanks gas volume and gas production degradability parameters when the animals had two days rest from the first sampling.

The *in vitro* gas production degradability parameters (B, C and lag) of cellulose and glucose were not significantly affected by daily sampling of rumen fluid until bacterial DM, bacterial absorbance and blank gas volume were lower than 93.7 mg/ 10 cm^3 of strained rumen fluid, 0.111 A and 15.5 cm^3 respectively. This suggests that there is a minimum threshold initial concentration of bacterial DM for adequately describing feed using the gas production technique. This study shows such a threshold to be around 0.09 g bacterial DM / 10 cm^3 in agreement with Jessop and Herrero (1998) who proposed the initial microbial activity should be about 0.09 g DM for 200 mg DM sample size.

Relationship of bacterial DM with bacterial absorbance and blanks gas volume

The present studies have shown that a major source of variation in *in vitro* gas production studies is the initial level of microbial activity, which is reflected in blank gas volume and in the dilution of the ruminal fluid. Therefore, methodological developments that help to standardise the microbial activity of the inoculant would improve the reproducibility and reliability of the technique within and between laboratories.

The strong linear relationships between bacterial DM, bacterial absorbance and blank gas volumes shown in these studies (see Figure 2.2 & 2.3) provide the required methodological steps for controlling this source of variation. In order to maintain a constant microbial activity, the dilution of rumen fluid with the buffer should not be constant, but should be related to the quantity of bacterial DM in strained ruminal fluid. From a practical standpoint, this can be done easily by

measuring the absorbance of ruminal fluid (see material and methods) before the incubation and estimating the bacterial DM indirectly using the regression equation derived above (Figure 2.2).

For most of the work reported in this thesis, ruminal fluid was diluted until the bacterial absorbance of a 50 fold dilution reaches any value between 0.111 and 0.134 in order to ensure a minimum bacterial DM of 0.09 g (see Figure 2.4). However, to avoid exhaustion of the buffer (Beuvink and Spoelstra 1992) care should be taken to ensure that at least two parts of buffer are used per part of strained ruminal fluid (the ruminal fluid should not be used for gas production incubation if the bacterial absorbance of a 50 fold dilution was below 0.111). In addition the size of feed sample incubated in an *in vitro* gas production technique must be set in relation to the volume of buffered rumen fluid medium (Getachew *et al*, 1998a).

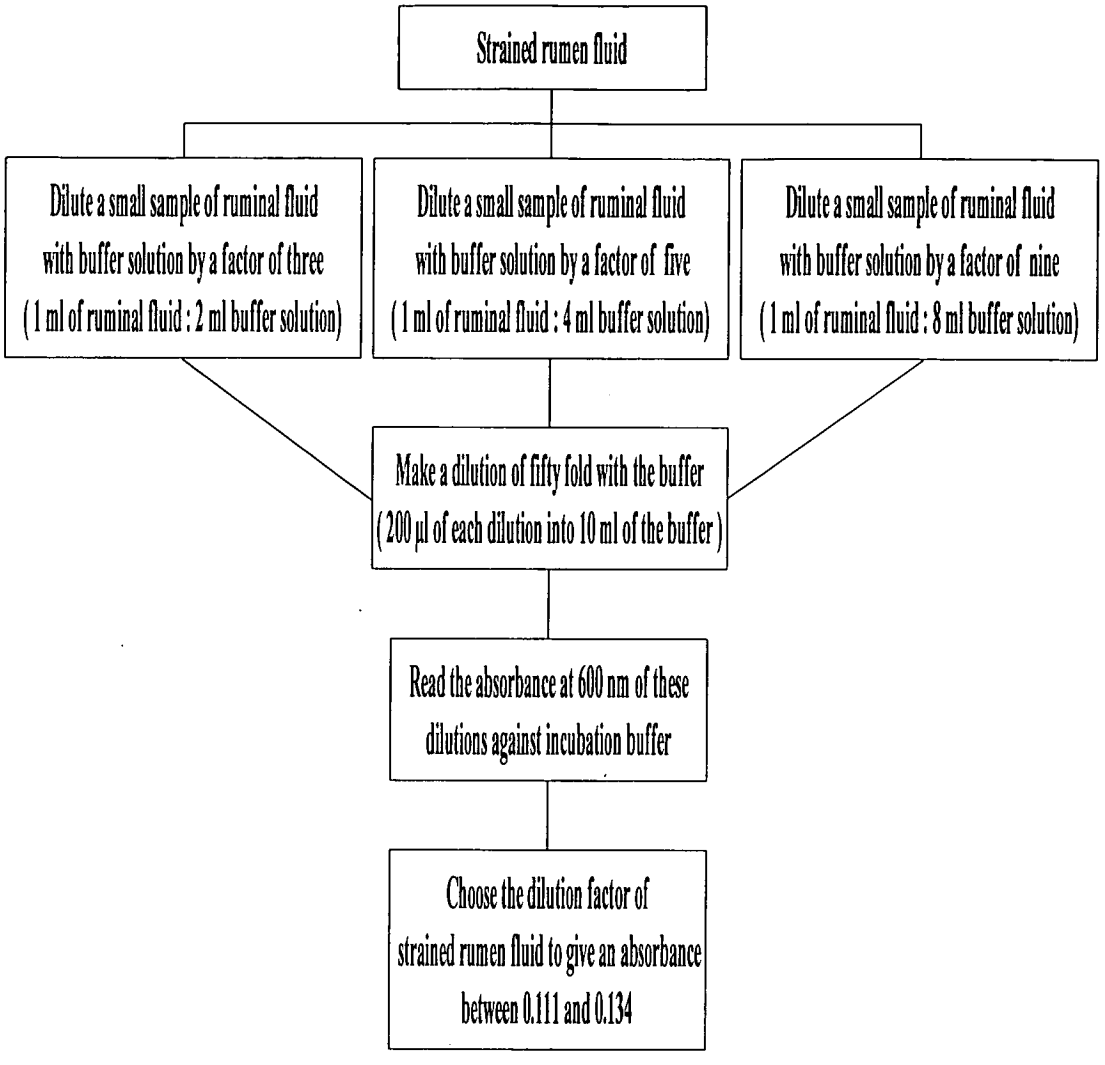

Figure 2.4 The guidelines for standardising the initial microbial concentration.

2.6 CONCLUSIONS

1)- Extensive dilution of the rumen fluid with buffer solution leads to an increase in the partitioning of degraded carbohydrate into microbial matter resulting in an underestimation of the pattern of gas production and subsequently the gas production degradability parameters (B, C and Lag).

2)- Frequent sampling of rumen fluid significantly decreased the bacterial DM and subsequently affected the descriptions of the feed obtained using the *in vitro* gas production technique.

3)- Standardising the level of microbial activity between days can be achieved by estimating the bacterial DM from the bacterial absorbence of the rumen fluid.

4)- The main aim of using the *in vitro* gas production technique is to estimate the potential fermentation dynamics of ruminant feed. This could be achieved by starting with a sufficient initial microbial concentration in order to minimize the partitioning factor of fermented carbohydrate into microbial growth and increasing the demand of ATP generated from the fermentation of carbohydrate to microbial maintenance. A minimal bacterial DM of 0.09 g/10cm^3 is recommended, higher than that used by many research groups.

Chapter 3

The influence of diet of the donor animal on the initial bacterial concentration of ruminal fluid and gas production degradability

3.1 Abstract

Six sheep were fed twice a day on a different ratio of sheep pellets and hay (20:80 (diet 1), 40:60 (diet 2) and 80:20 (diet 3)) in a replicated latin square design to study the effect of the host diet on the bacterial concentration of ruminal liquor and *in vitro* gas production degradability parameters of cellulose, glucose and hay. Bacterial DM, bacterial absorbance and the blanks' gas volume increased significantly as the ratio of sheep pellet to hay increased. The gas production degradability parameters (B, C and Lag) obtained from fitting the data to the model Gas = B $(1\text{-}exp^{-C(t\text{-}Lag)})$ were also affected by changing the donor diet ratio of sheep pellets and hay. For each substrate, incubation with ruminal fluid taken from sheep fed on diet 2 or 3 gave higher ($P<0.05$) asymptotic values 'B'(except hay) , rates 'C 'of gas production and lower lag times (cellulose and hay only) than when incubated in the ruminal fluid taken from sheep fed on diet 1. NDF digestibility of cellulose and hay was not significantly affected by donor diet. Bacterial DM was strongly related to the absorbance of ruminal fluid and the blanks' gas volume ($R^2 = 0.99$, $p<0.001$). These results suggest that changing the ratio of concentrate to hay reduces the initial bacterial concentration and affects the gas production degradability parameters but the estimation of bacterial DM either from bacterial absorbance or blanks' gas volume was not affected by changing the diet of donor animal.

3.2 Introduction

The *in vitro* gas production technique has the ability to characterise feeds not only by the quantity of digestible carbohydrate they provide but also by the rate at which these nutrients are released. Such characteristics are of central importance to understanding the dynamics of rumen fermentation. For this system to be useful for routine evaluation of forages, it must produce results with high precision and repeatability. One factor which can influence the *in vitro* gas production profiles, is the microbial activity of the inoculum (Jessop and Herrero, 1998). This could be affected by the frequent sampling of ruminal liquor (chapter 2), the time of collection (Menke and Steingass, 1988; Pell and Schofield, 1993; Cone et al., 1996) and the extent dilution with buffer (Pell and Schofield, 1993; Rymer *et al,* 1999; chapter 2).

The diet of the host animal influences the chemical environment within the rumen and subsequently the microbial population of ruminal fluid (Weiss, 1994). Several studies have indicated that the diet of donor animal influences both the total gas production (Trei *et al,* 1970; Menke and Steingass, 1988) and the gas production profiles (Bonsi *et al,* 1995; Cone *et al,* 1996; Huntington *et al,* 1998; Das and Singh 1998).

The diet of donor animal differs between research groups that use the *in vitro* gas production technique. Pell and Schofield, (1993), Theodorou et al., 1994 and Blummel and Becker, (1997) use ruminal fluid taken from donor animals fed on hay only, others use ruminal fluid taken from host animals fed on a different ratio of hay and concentrate (Menke and Steingass, 1988; Beuvink *et al.,* 1992; Cone *et al.,*

1996) although the composition of the concentrate is rarely specified. These differences in the diet of donor animals might be one of the factors that affects microbial activity or concentration and subsequently the gas production profiles and causes the differences in gas production data between laboratories (Moss *et al,* 1998b). In chapter 2, the results showed how the relationship between bacterial DM and bacterial absorbance could be used to predict the microbial activity of the inoculant. However, it is not known if this relationship is affected by the diet of the host animal.

The aims of this study were to examine :

1) The effect of varying the ratio of hay to concentrate in the diet of the host animal on bacterial concentration and gas production degradability parameters.
2) Whether the relationship between bacterial absorbance at 600nm and bacterial DM varies across diets.

3.3 Material and Method

Animals and diet

The experiment was conducted as two (complimentary) 3 x 3 Latin squares using six ruminally fistulated Suffolk sheep in three periods. In each period sheep were fed twice a day (at 8.00 and 17.00 h) on one of each of the following diets:

1- **Diet 1:** 200 g sheep pellets (NDF and CP 338 and 175 g/kg DM) and 800 g hay (NDF and CP 631 and 77 g/kg DM)

2- **Diet 2:** 400 g sheep pellets and 600 g hay

3- **Diet 3:** 800 g sheep pellets and 200 g hay

The sheep were given three weeks to adapt to their diet before and between successive periods. Water was available to the sheep *ad libitum.*

Inoculum preparation

A sample of rumen contents containing both solid and liquid material was taken from each sheep before morning feeding and collected in prewarmed vacuum flasks. In the laboratory ruminal fluid was filtered through two layers of muslin cloth and the solid material was squeezed lightly. The strained ruminal fluid was mixed (1:2 v/v) with anaerobic medium described by Menke and Steingass, (1988). Once prepared, the suspension of micro-organisms was kept at 39 °C with CO_2 bubbling through for approximately 20 min before addition to syringes.

Measurement of Bacterial DM

Bacterial DM was measured directly by centrifuging 30 ml of the buffered rumen fluid at 1000 X G (to remove protozoa and fungi and particulate matter) for 5 min at 30 °C. The pellet was discarded and the supernatant was recentrifuged at 26000 X G for 15 min at 4 °C. The supernatant was discarded whilst the bacterial pellet was resuspended in NaCl (9g/l) solution and centrifuged again. The supernatant was discarded and the bacterial pellet was dried at 80 °C to constant weight for determination of bacterial DM (Henning *et al.*, 1991). Bacterial DM was also measured indirectly by reading the absorbance at 600 nm (Brown *et al.*, 1989; Wells and Russell, 1996) of ruminal fluid that had been diluted fifty fold with buffer.

Gas production measurements and analytical procedures

The *in vitro* gas production was performed as described by Jessop and Herrero (1996). Gas production was measured in triplicate without added substrate (blank) and from the incubation of 50mg DM D-glucose, 200mg DM α- cellulose, and hay (same as fed to the sheep). The cumulative gas production for each syringe was recorded at 1,2,3,4,5,6,8 h; thereafter every 4 h until 60 h, and then 72, 84, 96 and 120 h and corrected for fermentation of soluble material (A) (for hay sample only) by subtracting the gas produced up to 4 h (Herrero and Jessop, 1996). Data were then fitted to the model Gas = $B(1-\exp^{-C(t-Lag)})$ (Krishnamoorthy *et. al.*, 1991) using Marquart algorithm as implemented by GraFit (Leatherbarrow, 1992) where B is the asymptotic gas production from the fermentation of carbohydrate (cm^3), C is the fractional rate of gas production (/h), t is time (h) and Lag is the lag phase before the fermentation of carbohydrate begins (h). NDF residues were collected at the end

of the incubations for the estimation of NDF digestibility (NDFD) using a modified micro technique (Pell and Schofield, 1993) by emptying the contents of each syringes into 100 cm^3 medical flat bottles. Each syringe was washed three times with 5 cm^3 of NDF solution, the washings being added to the appropriate bottle.

Statistical analysis

Analysis of variance using Genstat statistical package (1997) was used to compare bacterial DM, bacterial absorbance, blanks' gas volume and estimated gas production degradability parameters between different diets. Least significant differences were calculated from the standard error of the differences between means. Regression was used to study the relationship between bacterial DM, absorbance and blanks gas volume.

3.4 Results

The effect of sheep diet on the bacterial DM content of ruminal fluid, bacterial absorbance and the blanks' gas volume are summarised in Table 3.1. Ruminal fluid from sheep fed on diet **3** had the highest ($P<0.05$) mean values of bacterial DM, bacterial absorbance and the blanks gas volume followed by ruminal fluid obtained from sheep fed on diet **2** and **1** respectively.

Table 3.1 The effect of donor diet on bacterial DM, bacterial absorbance and the blanks gas volume

Donor diet	**Bacterial DM (mg/10 cm^3)**	**Bacterial absorbance (A)**	**Blanks gas volume (cm^3)**
1	73.9^a	0.085^a	11.5^a
2	119.0^b	0.137^b	18.7^b
3	155.0^c	0.179^c	24.5^c
SED	5.35	0.007	0.98

^{a,b,c} Means with different superscripts in the same column are significantly different (*$P<0.05$*).

The effect of sheep diet on gas production degradability parameters, NDF digestibility and final pH of glucose, cellulose and hay are presented on Table 3.2. It can be seen that ruminal fluid taken from sheep fed on diets 2 or 3 resulted in an increased ($P<0.05$) asymptotic gas production for cellulose and glucose (B), total gas production from the fermentation of hay (A+B) and fractional rate of gas production (C) from the fermentation of glucose, cellulose and hay when compared with the ruminal fluid taken from sheep fed on diet **1**. The lag phase before the fermentation of carbohydrate begins (Lag) for cellulose and hay was significantly shorter when incubated in ruminal fluid taken from sheep fed on diet **2** and **3** than that incubated in diet **1**. The gas produced from the fermentation of soluble material (A) for hay was significantly higher when incubated in ruminal fluid obtained from sheep fed on diet **2** and **3** than that incubated in diet **1** whereas the asymptotic gas production from the fermentation of NDF (B) was not effected by the diet of donor animal. The NDF digestibility (NDFD) of cellulose and hay was not significantly effected by source of ruminal fluid wither taken from sheep fed on diet **1**, **2** or **3**. Cellulose, glucose and hay incubated in ruminal fluid taken from sheep fed on diet 3 had the highest ($P<0.05$) final culture pH followed by those incubated in diet 2 and 1 respectively.

Table 3.2 The effect of donor diet on gas production degradability parameters, NDF digestibility and final pH.

Feed sample	Gas production Degradability parameters	Donor diet 1	2	3	SED
glucose	B (cm^3)	14.6^a	17.4^b	17.7^b	0.51
	C (/h)	0.143^a	0.204^b	0.206^b	0.0038
	LAG (h)	0	0	0	---
	Final pH	6.56^a	6.59^b	6.61^c	0.002
cellulose	B (cm^3)	84.7^a	90.7^b	91.1^b	0.49
	C (/h)	0.073^a	0.101^b	0.102^b	0.0029
	LAG (h)	5.2^a	4.5^b	4.4^b	0.10
	NDFD (g/kg DM)	999	1000	998	1.1
	Final pH	6.53^a	6.57^b	6.59^c	0.003
Hay	A (cm^3)	4.7^a	7.1^b	7.0^b	0.20
	B (cm^3)	30.0	31.5	31.9	1.7
	C (/h)	0.028^a	0.037^b	0.040^b	0.0017
	LAG (h)	5.9^a	5.3^b	5.2^b	0.08
	A+B (cm^3)	34.7^a	38.6^b	39.0^b	0.89
	NDFD (g/kg DM)	531	538	541	6.9
	Final pH	6.61^a	6.64^b	6.70^c	0.006

[a,b,c] Means with different superscripts in the same row are significantly different ($P<0.05$).

The relationship between bacterial DM and bacterial absorbance is shown in Figure 3.3.1. Bacterial DM was linearly related (R^2 = 0.99, p<0.001) to bacterial absorbance such that :

Bacterial DM (mg / 10 cm^3 of strained rumen fluid) =

863 (SE ± 4.5)* Bacterial Absorbance (A)

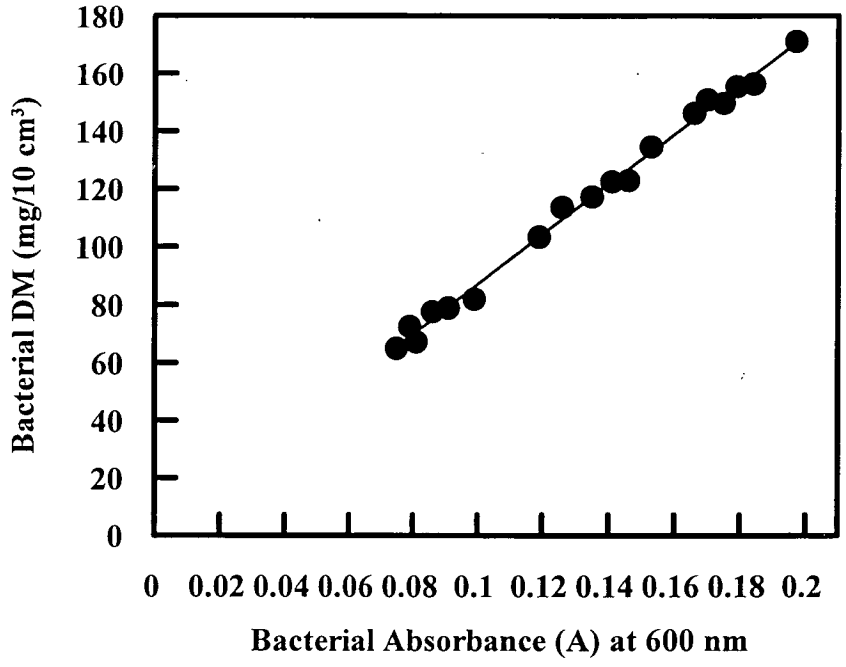

Figure 3.1 The relationship between bacterial DM (mg / 10 cm^3 of strained rumen fluid) and bacterial absorbance A (*n=18*).

Figure 3.2 shows the relationship between bacterial DM and blanks gas volume. There was a linear relationship (R^2 = 0.99, p<0.001) between bacterial DM and blanks gas volume such that :

Bacterial DM (mg / 10 cm^3 of strained rumen fluid) =

6.34 (SE ±0.085)*Blanks' Volume (cm^3).

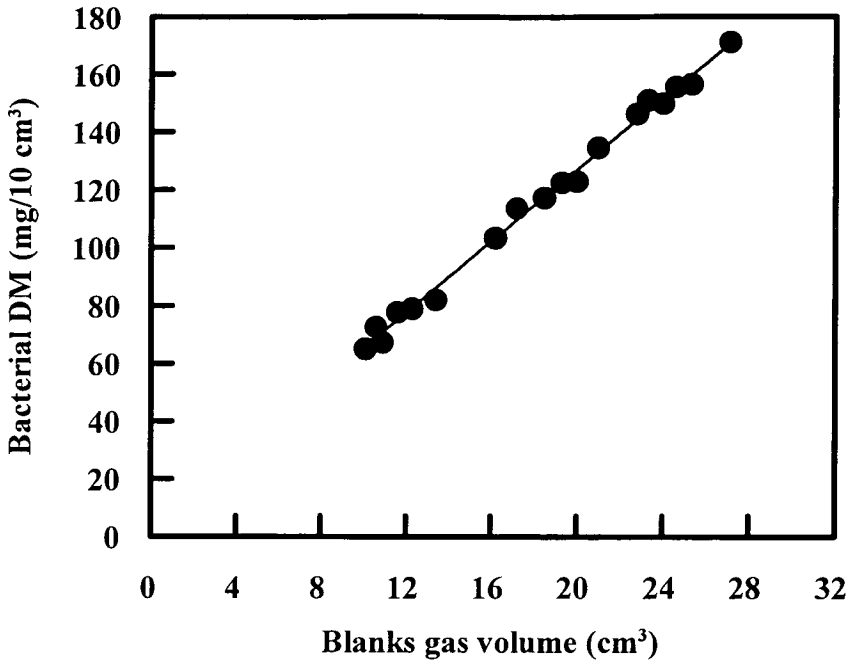

Figure 3.2 The relationship between bacterial DM (mg / 10 cm^3 of strained rumen fluid) and blanks gas volume (*n=18*).

3.5 Discussion

The primary objective of this work was to study the influence of donor diet on initial microbial concentration and gas production degradability. The results clearly showed that the bacterial DM, bacterial absorbance and the blanks gas volume were significantly increased as the ratio of concentrate (sheep pellet) to hay increased in the donor animals' diets from 20% (diet 1) to 40% (diet 2) and then to 80% in diet 3. However, there were no significant differences in the gas production degradability parameters (B, C and Lag) of cellulose, glucose and hay when incubated in ruminal fluid taken from sheep fed either on diet 2 or diet 3. But cellulose, glucose and hay incubated in ruminal fluid taken from sheep fed on 80% hay to 20% concentrates (diet 1) had a significantly lower gas production degradability parameters as compared with those incubated in ruminal fluid taken from sheep fed on diet 2 and 3. These differences might be attributed to the high ratio of low quality hay to low ratio of concentrate in diet 1 of the donor animal which was not enough to optimise the microbiological population and chemical environment within the rumen of the donor animal (Weiss, 1994). This resulted in a low initial microbial concentration (Table 3.1), lower than those recommended by Jessop and Herrero (1998) and in chapter 2. These results are in agreement with Menke and Steingass, (1988) who recommended the diet of the host animal to be 50 to 60% hay and 40 to 50% concentrates since they observed a 25% reduction in the total gas production when rumen fluid was taken from sheep fed only straw and no concentrate. They referred to the low level of microbial activity in the ruminal fluid of the donor animals fed on straw since the blanks gas volume (regarded as a measure of microbial activity) in that case dropped from 12-16 cm^3 to about 3 cm^3.

The results of this study also indicate that the host diet affects the final pH but the actual differences were small. However, the differences in gas production degradability (B, C and Lag) and final pH observed in this study as a result of changing the diet of the host animal are in agreement with some earlier works. Trei *et al,* (1970) studied the influence of inoculum source on *in vitro* gas production and found that the total gas production was greater when the inoculum was obtained from grain fed steers as compared to that from hay fed steers. Bonsi *et al,* (1995) studied the effect of rumen ecology on *in vitro* gas production of four sun-dried fodder tree incubated in ruminal fluid taken from sheep fed on teff straw or supplemented with either *Sesbania sesban* or *leucaena leucocephala*. They reported that the total and the rate of gas production of fodder tree were lower when incubated in ruminal fluid taken from sheep fed on teff straw as compared with that taken from sheep fed on either *Sesbania sesban* or *leucaena leucocephala*. Cone *et al,* (1996) tested the influence of the source of ruminal fluid on the rate of gas production and the found that the rate of gas produced from the incubation of maize cob mix in ruminal fluid taken from sheep fed on 800 g hay and 200 g concentrate was higher than that incubated from sheep fed on hay only.

Huntington *et al,* (1998) studied the effect of host diet on the gas production profiles of grass hay and high temperature dried grass. They found that grass hay and high temperature dried grass incubated in the ruminal fluid taken from the silage-based diet increased the asymptotic value and the time dependent rate and final pH but the lag time, total VFA and OMD were not affected by the diet of donor animal.

Recently, Das and Singh (1998) studied the effect of varying the level of berseem supplementation in donor animal diet on *in vitro* gas production of wheat straw. They noted that both the total and the rate of gas production were increased significantly with increasing the level of berseem in donor diet up to 30%.

Jessop and Herrero (1998) proposed that if microbial activity was low, this would become a limiting factor and a significant proportion of degraded carbohydrate would be incorporated into new microbial matter rather than be fermented to products that gave rise gas production. The reduced asymptotic gas production (B) but similar NDFD observed in this study when the concentration of bacterial DM and the blanks' gas volume of ruminal fluid taken from sheep fed on diet 1 were below the minimum values of microbial activity recommended in chapter 2 (90 mg /cm^3 and 15 cm^3 respectively) would support this assumption.

The strong relationships between bacterial DM and bacterial absorbance (Figure 3.1), bacterial DM and the blanks gas volume support the finding of chapter 2. The slopes of bacterial absorbance (863 s.e. ± 4.5) and blanks gas volume (6.34 s.e. ± 0.085) obtained in this study were very close to those (bacterial absorbance (868 s.e. ± 3·9) and blanks gas volume (5·89 s.e. ± 0.043)) observed by in chapter 2 indicating that the estimation of bacterial DM by reading the absorbance at 600 nm of fifty fold diluted ruminal fluid or from the blanks' gas volume is not affected by changing the diet of donor animal. Moreover, the above relationship between bacterial DM and bacterial absorbance could be used for monitoring the effect of

donor diet and its ability in providing enough microbial matter for the *in vitro* gas production technique.

3.6 Conclusion

1. The diet of the host animal can significantly affect the ruminal fluid microbial concentration and subsequently the gas production profile.

2. The ratio of 60% hay and 40% concentrates in donor animal diet is recommended to be a minimum ratio that should be offered to the donor animal in order to obtain sufficient microbial matter for gas production technique.

3. The diet of donor animal does not affect the relationships between bacterial DM, bacterial absorbance, bacterial DM and the blanks' gas volume.

4. The relationship between bacterial DM and bacterial absorbance can be used to determine whether the diet of the host animal allows the attainment of a sufficient microbial concentration in ruminal fluid or not.

Chapter 4

A comparison of the gas production profiles of fresh and dry forage

4.1 Abstract

The gas production profiles of fresh and dry forage were compared. In experiment 1, fresh leaves and stems of red fescue (*Festuca rubra*) and leaves of perennial ryegrass (*Lolium perenne*) were collected in June 1997 from the Pentland area in Scotland. In addition, leaves and stems of Brachiaria decumbens *(B.decumbens)* were collected at different stages of maturity (after 28 (first stage) and 84 days third stage)) from plants grown at 25°C in a green house. Each sample was divided into two sub samples. The first was dried at 65°C for 20 h, and then ground to either less than 0.4 mm (DFG) using a coffee grinder, 1mm (D1mmG) and 2mm (D2mmG) (experiment 2 only) using a hammer mill. Samples (220mg) of each of the above were incubated in 100 cm^3 gas syringes. Portions of the second sub sample of fresh material were crushed in a mortar and pestle for 1 (FC1) or 2 (FC2) min or chopped to 0.5 cm lengths (FCH) or left intact (FC0). Samples (1g) of each of these were incubated in 100 cm^3 gas syringes. Measurements of gas production were take at 1,2,3,4,5,6,8 h; thereafter every 4 h until 60 h, and then at 72,78,84,96,100, and120 h. Cumulative gas volumes were corrected for fermentation of soluble material **A** (gas produced up to 4 h) and then fitted to the model $GAS = B(1-exp^{-C(t-Lag)})$, where B is the asymptotic gas production from the fermentation of NDF (cm^3), C is the fractional rate of gas production (/h), and Lag is the Lag phase before the fermentation of NDF begins (h). An exponential model could not describe the pattern of gas production from FC0 and FCH. The degradability parameters, (A, B, C and Lag) of ryegrass, red fescue and first stage of maturity of *B.decumbens* were similar between FC1, FC2, FC3 and DFG but were lower for D1mmG. The rate of gas production (C) for the third stage of maturity of *B.decumbens* leaf was higher for

DFG and D1mmG than for FC1, FC2 and FC3 whereas D1mmG had a similar rate to fresh treatments for the stem fraction. In experiment 2, Leaves and stems of Brachiaria brizantha (*B.brizantha*) were grown in a green house at 35°C and collected every 28 days for three stages of maturity. Samples of fresh and dry material were treated as described above. The gas production degradability parameters (A, B, C and Lag) of the fresh and dry materials were varied between stage of maturities. For the first stage of maturity, degradability parameters for D1mmG were closer to those of the fresh but the D2mmG was closer to fresh treatments (FC1, FC2 and FC3) in the second stage and to FC3 in the third stage of maturity. The results also showed that DFG overestimated degradability of the fresh material. Therefore, caution should be taken in processing of the forage sample if the *in vitro* gas production technique is to be used to study the fermentation dynamics or to evaluate the nutritional value of forages particularly for grazing animals.

4.1 Introduction

The aim of using any *in vitro* method is to obtain a good description of the nutritional value of forages as they are fed, which in many cases is on a fresh basis. The *in vitro* gas production technique is inexpensive, simple and reasonably reproducible. This technique has the potential to characterise fermentation pattern of the carbohydrate fractions of forages, and has been used extensively with forage material that has been dried and ground through 1mm screen (Menke and Steingass, 1988). For grazing animals, the forage is eaten when fresh, not dried. It has been found that drying the forage samples leads to chemical and enzymatic changes of cell wall components, proteins and carbohydrates, and that these reactions may interfere in the quantification of the fibrous fraction (Burritt, *et al*, 1988; Deaville and Givens, 1993), nitrogen compounds (Abdalla, *et al*, 1988) and the quantity of non-structural carbohydrates (Acosta and Kothmann, 1978: Piccaglia and Galleti, 1987). Also, based on comparisons between fresh and dry forages, several reports have concluded that the *in situ* forage degradability (Ould-Bah and Michalet-Doreau, 1988; Peyaraud, 1990; Hristov, 1992; Chamberlain and Endalew, 1993; Lopez *et al*, 1995; Hristov, 1998; Valentin *et al*, 1999) and *in vitro* OM digestibility of forages (Kaumon and Thewis, 1990; Broesder *et al* 1992) can be affected by drying method.

Givens and Gill (1998) noted the importance of the physical structure of a feed samples incubated *in vitro* after Deaville (1995) observed that grinding wheat grain samples prior to the measurement of gas gave a higher fermentation rate than the same wheat treated with sodium hydroxide at different moisture contents. Despite this, little attention has been paid to comparison of the fermentation dynamics of

fresh and dry forage using the *in vitro* gas production technique. Bonsi *et al*, (1995) studied the effect of varying rumen environments on the *in vitro* gas production degradability of fresh and sun-dried foliages of four fodder trees. They found that the dry material had a lower total gas production and higher rate of gas production than did fresh foliages. Sanderson *et al.*, (1997) reported significant differences in the gas production profiles between fresh and dry silages. Cone (1998) also found that the fresh grass silage cut in small pieces had a higher initial gas production than freeze dried and milled grass silage but after 3 to 4 hours the rate of gas production of the dried sample was higher than that of the fresh. However, in the above studies fresh material was incubated intact or cut into small pieces without any attempting of mimicking the structural damage caused by the animal chewing.

The aims of this study were (1) to compare the gas production profiles of fresh (treated in different ways in order to mimic animal chewing) and dry forage, and (2) to investigate the fermentation characteristics of fresh and dry *B.brizantha* over various stages of maturity.

4.3 Material and Method

Forage samples

Experiment1 The aim of this experiment was to compare gas production dynamics between fresh and dry forage. Fresh leaves and stems of red fescue (*Festuca rubra*) and leaves of perennial ryegrass (*Lolium perenne*) were collected in June 1997 from the Pentland area in Scotland. Later, leaves and stems of Brachiaria decumbens *(B.decumbens)* were collected from plants grown at 25°C (fertilised with 200 kg nitrogen /hectare) in a green house at different stages of maturity (after 28 (first stage) and 84 days third stage)).

Experiment 2 The aim of this study was to investigate the gas production degradability characteristics of fresh and dry forage over various stages of maturity. Leaves and stems of Brachiaria brizantha (*B.brizantha*) were collected from plants grown in a green house (at 35°C and fertilised with 200 kg nitrogen /hectare) and collected every 28 days at three stages of maturity. The chemical composition of each stage of maturity of *B.brizantha* is presented in Table 4.1.

Table 4.1 Chemical composition of *B.brizantha* determined over various stages of maturity (*n=3*).

Forage part	Stage of maturity	DM (g/kg)	CP (g/kg DM)	NDF (g/kg DM)	Ash (g/kg DM)
Leaves	1	202 ± 7.9	138 ± 5.8	466 ± 19.6	72 ± 1.7
	2	243 ± 5.6	89 ± 6.3	516 ± 23.3	79 ± 3.4
	3	286 ± 11.4	61 ± 5.1	582 ± 17.6	86 ± 1.0
Stems	1	195 ± 3.3	63 ± 2.9	564 ± 13.7	78 ± 2.6
	2	261 ± 7.8	49 ± 2.2	642 ± 17.2	56 ± 2.2
	3	306 ± 14.6	28 ± 1.4	719 ± 26.3	50 ± 4.7

**se* is the standard error

The preparation of forage samples:

All of the above forage samples were stored in plastic bags on ice immediately after harvesting. Each sample was divided into two sub samples, the first was dried at 65°C for 20 h, and then ground to either less than 0.4 mm (DFG) (except *B.brizantha* stems) using a coffee grinder, 1mm (D1mmG) and 2mm (D2mmG) (experiment 2 only) using a hammer mill. Samples (220mg) of each of the above (*n=5* experiment 1 and *n=4* experiment 2) were incubated in 100 cm^3 gas syringes. For the second sub sample, portions (10g) of fresh material were crushed in a mortar and pestle for 1 (FC1), 2 (FC2) or 3 (FC3) min or chopped to 0.5 cm lengths (FCH) or left intact (FC0). Samples (1g) of each of these (*n=5* experiment 1 and *n=4* experiment 2) were added to gas syringes. Samples (1g) of each treatment (FC1, FC2, and FCH) were taken for DM determination. Fresh and dry samples from each harvest were incubated with the same microbial inoculant. Therefore, syringes

containing fresh samples were sealed with plastic film and kept at 4°C for 20 h until dried samples were ready.

All samples were analysed for CP using standard Kjeldahl nitrogen determinations, for NDF using a modified micro-technique (Pell and Schofield, 1993), and for Ash.

Animals and diet

Two ruminally fistulated *Suffolk* sheep fed 750g sheep pellets (NDF and CP 338 and 175 g/kg DM) and 250g-grass nut (NDF and CP 345 and 150 g/kg DM) twice a day at 8.00 and 17.00 h were used. Water was available to the sheep *ad libitum*.

Inoculum preparation

A sample of rumen contents containing both solid and liquid material was taken before morning feeding and collected in a prewarmed vacuum flask. In the laboratory rumen fluid was strained through two layers of muslin cloth, solid material was squeezed lightly. Incubation of each grass species were conducted on separate days. The microbial activity was standardised between different days by measuring the bacterial DM indirectly before the gas production incubation of each run (Table 4.2) as described in chapter 2. Based on the bacterial DM the strained ruminal fluid was mixed with the anaerobic medium described by Menke and Steingass, (1988). Once prepared, the suspension of micro-organisms was kept at 39 °C with CO_2 bubbling through for approximately 20 min before addition to syringes.

Table 4.2 Indirect estimates of the initial bacterial concentration of ruminal fluid collected at different days.

Incubated forage sample	Stage of maturity	Observed bacterial absorbance (A)	Estimated bacterial DM (mg/ 10 cm^3)	Observed blanks gas volume (cm^3)
Rye	**1**	0.121	105	18.0
Red fescue	**1**	0.113	98	16.3
B.decumbens	**1**	0.140	122	20.2
B.decumbens	**3**	0.127	110	18.2
B.brizantha	**1**	0.139	121	20.5
B.brizantha	**2**	0.116	101	17.0
B.brizantha	**3**	0.118	102	16.5

In vitro studies for the measurement of rate and extent of gas production

Incubations were performed as described by Jessop and Herrero, (1996). Measurements of gas production were take at 1,2,3,4,5,6,8 h; thereafter every 4 h until 60 h, and then at 72,78,84,96,100, and 120 h. Cumulative gas volumes were corrected to fermentation of 200mg DM and for fermentation of soluble material (A) by subtracting the gas produced up to 4 h (Herrero and Jessop, 1996). Data were then fitted to the model GAS = $B(1-exp^{-C(t-Lag)})$ (Krishnamoorty *et al.*, 1991) using Marquart algorithm as implemented by GraFit (Leatherbarrow, 1992), where B is the asymptotic gas production from the fermentation of NDF (cm^3), C is the fractional rate of gas production (/h), and Lag is the lag phase before the fermentation of NDF begins (h).

Statistical analysis

Analysis of variance was used to compare the degradation parameters of samples between treatments and MINITAB Statistical Package (1993) was used for this purpose. One way analysis of variance was performed to analyse data from experiment 1. A two way analysis of variance, using a General Linear Model was used to analyse data from experiment 2. Least significant differences were calculated from the standard error of the difference between means.

4.4 Results.

The gas production profiles of fresh and dry forages (red fescue and ryegrass and B.decumbens)

The chemical compositions of red fescue (leaves and stems), ryegrass (leaf), first and second stage of maturity of *B.decumbens* (leaves and stems) are presented in Table 4.3. It can be seen that there were no significant differences ($P>0.05$) between the treatments across all measured constituents except for NDF where FC0 and FCH for all forage samples and FC1, FC2 and FC3 for the 3rd stage of maturity of *B.decumbens* were significantly higher ($P<0.05$) than the other treatments.

The gas production profiles of ryegrass, red fescue, first and third stage of maturity of *B.decumbens* grasses for all treatments are given in Figure 4.1. It can be seen that the gases produced from FC0 and FCH treatments did not follow an exponential pattern. Therefore, those treatments were excluded from the estimation of the parameter values for the *in vitro* gas production dynamics.

Table 4.3 Chemical composition of the red fescue (leaves & stems), ryegrass (leaves), 1st and 3rd stage of maturity of *B.decumbens* (leaves & stems). Each value is the mean of four replicates ($n=4$). For each forage sample, means with different superscripts within the same column are significantly different ($P<0.05$).

Sample id	Treatment	DM (g/kg)	CP (g/kg DM)	NDF (g/kg DM)	ASH (g/kg DM)
	FC0	283	189	698 a	81
	FC1	293	177	540b	78
	FC2	298	172	551 b	78
Red fescue	FC3	291	179	543 b	79
Leaves	FCH	296	184	663 a	81
	DFG	287	186	535 b	80
	D1mmG	287	183	538 b	81
	Pooled SED	15.8	20.1	16.2	3.7
	FC0	279	81	713 a	67
	FC1	295	86	572 b	64
	FC2	291	84	564 b	64
Red fescue	FC3	294	80	577 b	66
Stem	FCH	283	83	687 a	66
	DFG	277	91	556 b	66
	D1mmG	277	89	561 b	65
	Pooled SED	17.3	11.5	21.7	2.3
	FC0	267	202	364 a	87
	FC1	255	207	290 b	86
	FC2	257	210	293 b	87
Ryegrass	FC3	253	207	284 b	87
Leaves	FCH	261	200	354 a	86
	DFG	258	203	285 b	86
	D1mmG	258	202	288 b	87
	Pooled SED	13.8	9.6	9.4	1.9
	FC0	172	151	487 a	44
	FC1	177	149	431 b	41
first stage	FC2	185	158	418 b	44
B.decumbens	FC3	181	155	422 b	40
Leaves	FCH	189	153	479 a	42
	DFG	176	156	420 b	44
	D1mmG	176	151	427 b	45
	Pooled SED	23.1	17.7	21.4	6.4
	FC0	200	79	730 a	49
	FC1	190	80	658 b	47
first stage	FC2	193	80	663 b	48
B.decumbens	FC3	195	77	652 b	46
Stems	FCH	198	79	703 c	49
	DFG	196	81	654 b	47
	D1mmG	196	77	657 b	48
	Pooled SED	11.9	8.8	13.5	4.4
	FC0	336	54	691 a	98
	FC1	326	54	653 b	96
third stage	FC2	329	57	649 b	96
B.decumbens	FC3	341	53	641 b,c	99
Leaves	FCH	337	55	676 a	95
	DFG	323	51	626 c	101
	D1mmG	323	51	630 c	102
	Pooled SED	19.2	9.7	15.9	7.8
	FC0	401	29	836 a	105
	FC1	410	25	784 b	109
third stage	FC2	408	28	781 b	111
B.decumbens	FC3	403	28	772 b	106
Stems	FCH	403	24	812 a	109
	DFG	401	26	725 c	107
	D1mmG	401	24	731 c	107
	Pooled SED	12.5	8.8	14.2	11.4

*For details of substrate treatments and procedures, see the preparation of forage

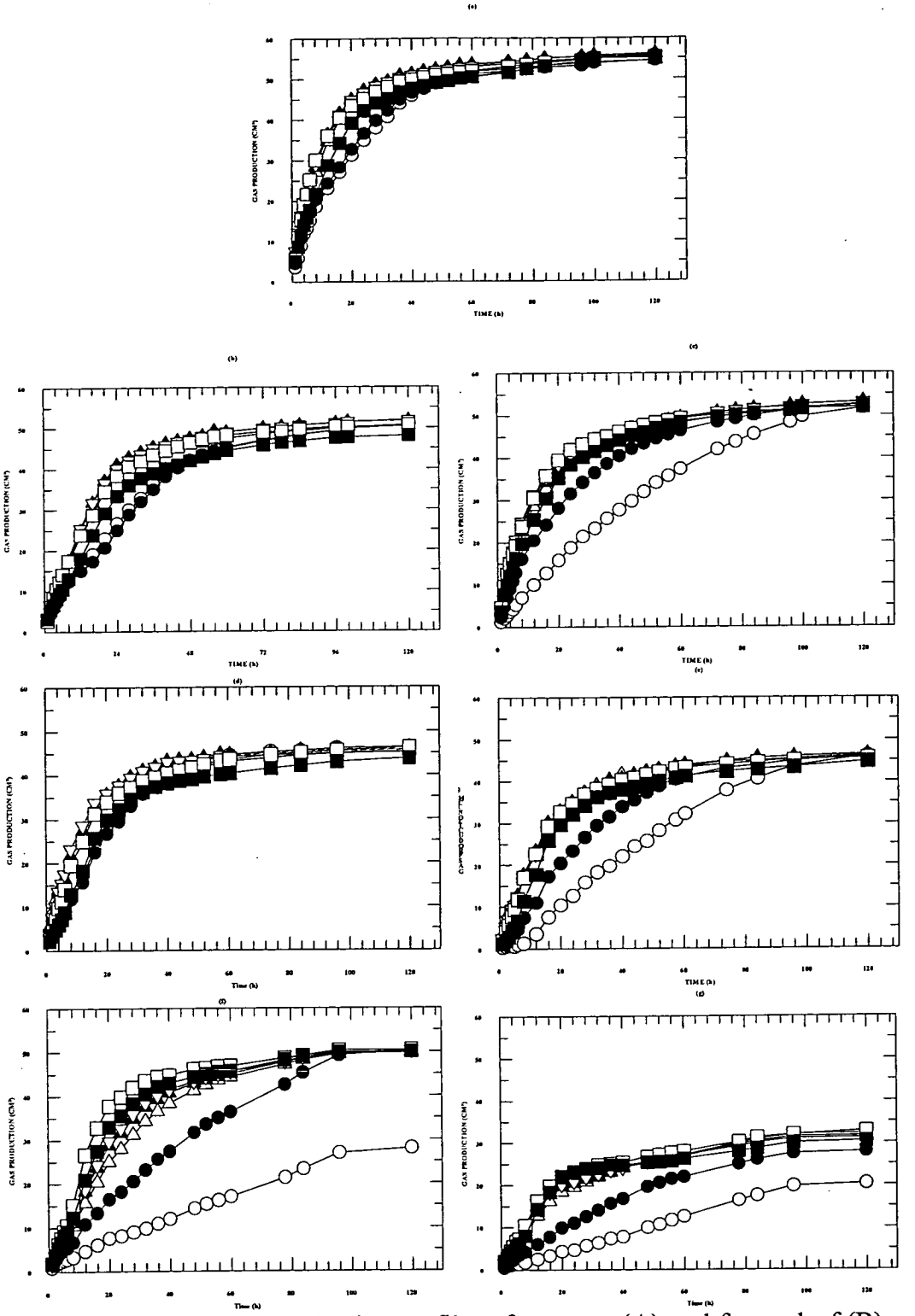

Figure 4.1 The gas production profiles of ryegrass (A), red fescue leaf (B) and stem (C), first stage of maturity leaf (d) and stem (e), and third stage of maturity of *B.decumbens* leaf (f) and stem (g) for FC0 (○), FCH (●), FC1 (Δ), FC2 (▲), FC3 (▽) DFG ( ) and D1mmG (■) treatments.

The estimated parameter values for *in vitro* gas production dynamics of fresh and dry ryegrass leaf are summarised in Table 4.4. These results show that the same total gas production (A+B) was reached at the end of incubation for all treatments. The rapid gas production (A) of DFG was similar to that of FC1, FC2 and FC3 whereas that of D1mmG was approximately 24% lower ($P<0.05$) than FC1, FC2 and FC3. The asymptotic gas production from the fermentation of NDF (B) was approximately 10% higher ($P<0.05$) for D1mmG than for all of the other treatments. The rate of gas production for the three fresh treatments was approximately 7 and 30% lower ($P<0.05$) for DFG and D1mmG respectively. The Lag phase before the fermentation of NDF begins (Lag) was significantly lower ($P<0.05$) on DFG than on the fresh treatments.

Table 4.4 *In vitro* gas production dynamics of fresh (F_) or dry (D_) ryegrass (leaf). The fresh leaf were incubated either crushed by pestle and mortar for 1 minute (_C1), 2 minute (_C2), or 3 minute (_C3). The dry leaf were incubated either in a fine ground (_FG), or 1mm ground (_1mmG). Each value is the mean of five replicates ($n=5$). Means with the different superscripts within the same column are significantly different ($P<0.05$).

Plant part	Treatment	Parameters				
		A (cm^3)	B (cm^3)	C (/h)	Lag (h)	A+B (cm^3)
(Leaf)	FC1	18.1^a	34.9^a	0.078^a	4.0^a	53.0
	FC2	18.0^a	35.7^a	0.080^a	4.1^a	53.7
	FC3	19.8^a	34.1^a	0.079^a	4.0^a	53.9
	DFG	19.2^a	34.4^a	0.072^b	3.4^b	53.6
	D1mmG	13.9^b	38.1^b	0.055^c	4.0^a	52.0
	Pooled SED	1.14	1.66	0.0033	0.07	1.79

In Table 4.5 the estimated parameter values for *in vitro* gas production dynamics of fresh and dry Red fescue leaf and stem grass are presented. As was observed earlier for ryegrass, all treatments for red fescue leaf or stem reached the same total gas production (A+B) and the rapid gas production (A) was about 26% and 31% higher ($P<0.05$) on the fresh treatments (FC1, FC2 and FC3) and DFG for both leaf and stem fractions than for the dry 1mm sieve ground (D1mmG). In contrast the asymptotic gas production from the fermentation of NDF (B) was about 7.5 and 11% higher ($P<0.05$) for D1mmG leaf and stem than on the fresh (FC1, FC2 and FC3) treatments. However, for red fescue leaf and stem there were no significant differences ($P>0.05$) in the asymptote gas production from the fermentation of NDF (B) between the dry fine ground (DFG) and the fresh (FC1, FC2 and FC3) treatments.

The rate of gas production (C) for red fescue leaf increased with maceration in the fresh samples and also increased with fineness of grind in the dried samples. The higher rate was observed with fresh samples macerated for two to three minutes and this was about 25% higher ($P<0.05$) than measured for D1mmG. For red fescue stem the highest rate was observed for DFG and the lowest for D1mmG with the fresh samples being intermediate. However, the differences were much less for the leaf samples.

Table 4.5 *In vitro* gas production dynamics of fresh (F_) or dry (D_) red fescue (leaf & stem). The fresh leaf or stems were incubated either crushed by pestle and mortar for 1 minute (_C1), 2 minute (_C2), or 3 minute (_C3). The dry leaf or stems were incubated either in a fine ground (_FG), or 1mm ground (_1mmG). Each value is the mean of five replicates ($n=5$). Means with the different superscripts within the same column are significantly different ($P<0.05$).

Plant part	Treatment	Parameters				
		A (cm^3)	B (cm^3)	C (/h)	Lag (h)	A+B (cm^3)
Leaf	FC1	10.8^a	38.8^a	0.060^a	4.6	49.6
	FC2	11.0^a	39.7^a	0.065^b	4.8	50.7
	FC3	11.0^a	39.7^a	0.063^b	4.5	50.7
	DFG	10.5^a	40.2^a	0.057^a	4.4	50.7
	D1mmG	8.1^b	42.6^b	0.049^c	4.9	50.7
	Pooled SED	0.56	1.36	0.0019	0.32	1.49
Stem	FC1	14.4^a	36.5^a	0.055^a	3.6	50.9
	FC2	14.0^a	37.4^a	0.057^a	3.6	51.4
	FC3	15.0^a	35.8^a	0.056^a	3.5	50.8
	DFG	14.9^a	35.5^a	0.062^b	3.8	50.4
	D1mmG	10.0^b	40.9^b	0.050^c	3.8	50.9
	Pooled SED	0.83	1.60	0.0021	0.27	1.84

For the first stage of *B.decumbens*, the overall picture was similar to that observed for rye and red fescue grasses (Table 4.6) where the gas production dynamics of DFG were closer to those of FC1, FC2 and FC3 than D1mmG and the total gas production (A+B) was similar between treatments. For D1mmG, the rapid gas production (A) and the rate of gas production (C) were lower and the asymptote gas production from the fermentation of NDF (B) was higher than for fresh or DFG

treatments for both leaf and stem materials. For stem, Lags were increased in D1mmG when compared to other treatments.

Table 4.6 *In vitro* gas production dynamics of fresh (F_) or dry (D_) the first stage of maturity of *B.decumbens* (leaf & stem). The fresh leaf or stems were incubated either crushed by pestle and mortar for 1 minute (_C1), 2 minute (_C2), or 3 minute (_C3). The dry leaf or stems were incubated either in a fine ground (_FG), or 1mm ground (_1mmG). Each value is the mean of five replicates ($n=5$). Means with the different superscripts within the same column are significantly different ($P<0.05$).

Plant part	Treatment	Parameters				
		A (cm^3)	B (cm^3)	C (/h)	Lag (h)	A+B (cm^3)
Leaf	FC1	13.6^{a}	30.7^{a}	$0.077^{a,b}$	4.6^{a}	44.3
	FC2	13.1^{a}	31.1^{a}	$0.075^{a,b}$	4.2^{b}	44.2
	FC3	13.4^{a}	31.1^{a}	0.079^{a}	4.0^{b}	44.5
	DFG	12.0^{a}	32.3^{a}	$0.069^{b,c}$	3.9^{b}	44.3
	D1mmG	6.0^{c}	37.2^{b}	0.065^{c}	4.6^{a}	43.2
	Pooled SED	0.86	1.01	0.0048	0.14	0.73
Stem	FC1	9.7^{a}	34.5^{a}	0.064^{a}	3.7^{a}	44.2
	FC2	10.0^{a}	34.7^{a}	0.063^{a}	3.0^{b}	44.7
	FC3	9.0^{a}	35.1^{a}	0.063^{a}	3.1^{b}	44.2
	DFG	8.8^{a}	36.0^{a}	0.065^{a}	3.7^{a}	44.8
	D1mmG	4.8^{b}	39.5^{b}	0.050^{b}	4.7^{c}	44.3
	Pooled SED	1.02	1.20	0.0038	0.27	1.18

For the 3rd stage of maturity of *B.decumbens* leaf and stem (Table 4.7), DFG gave rise to fermentation patterns that differed from the fresh material. The values for the rapid gas production (A) and the rate of gas production (C) were higher and those for the asymptote gas production from the fermentation of NDF (B) were lower

than fresh treatments although values for Lags and total gas production (A+B) were similar. For this forage, the D1mmG treatment gave values closest to those of the fresh although for the leaf, the rate of gas production (C) was still higher. Interestingly, the length of maceration increased the rate of gas production (C) in the fresh material.

Table 4.7 *In vitro* gas production dynamics of fresh (F_) or dry (D_) 3rd stage of maturity *B.decumbens* (leaves & stem). The fresh leaves or stems were incubated either crushed by pestle and mortar for 1 minute (_C1), 2 minute (_C2), or 3 minute (_C3). The dry leaves or stems were incubated either in a fine ground (_FG), or 1mm ground (_1mmG). Each value is the mean of five replicates ($n=5$). Means with the different superscripts within the same column are significantly different ($P<0.05$).

Plant part	Treatment	Parameters				
		A (cm^3)	B (cm^3)	C (/h)	Lag (h)	A+B (cm^3)
Leaf	FC1	6.3^a	43.2^a	0.045^a	4.7	49.5
	FC2	6.2^a	43.3^a	0.055^b	4.7	49.5
	FC3	6.4^a	42.7^a	0.059^c	4.6	49.3
	DFG	8.9^b	39.7^b	0.067^d	4.4	48.6
	D1mmG	6.7^a	42.1^a	0.064^e	4.8	48.8
	Pooled SED	0.34	1.05	0.0016	0.27	1.28
Stem	FC1	4.9^a	25.7^a	0.039^a	4.4^a	30.6
	FC2	5.1^a	25.6^a	0.041^a	4.4^a	30.7
	FC3	5.6^a	25.4^a	0.040^a	4.1^b	31.0
	DFG	7.5^b	22.9^b	0.054^b	4.1^b	30.4
	D1mmG	4.9^a	24.6^a	0.041^a	4.6^a	29.5
	Pooled SED	0.49	0.80	0.0025	0.19	1.36

A comparison of the gas production profiles of fresh and dry *B.brizantha* over various stages of maturity.

B.brizantha leaves

Gas production degradability parameters of *B.brizantha* leaves over various stages of maturity are shown in Table 4.8. It can be seen that the total gas production of each stage of maturity did not differ between treatments, but did decrease as stage of maturity increased. No significant interaction was found in the total gas production (A+B) between treatments and stages of maturity.

For fresh samples, increasing the period of maceration resulted in an increase in the rapid gas production (A) and the rate of gas production (C) (of 8 and 11% respectively), and a reduction in Lag by about 7%. For dry samples increasing the fineness of grind increased the rapid gas production (A) and the rate of gas production (C) approximately by 16 and 13% respectively and decreased the asymptote gas production from the fermentation of NDF (B) and the Lag time by 5 and 7% respectively. The D2mmG treatment gave a pattern of fermentation that was closest to those of the fresh treatments.

Similar effect of maceration time and fineness of grind were observed with the second and the third stages of maturity although the responses observed at the third stage of maturity were not nearly as marked. For example, the rate of gas production (C) increased by 6 and 9% for fresh and dried materials respectively, much less than the increases for first and second stages.

However, there was a significant ($P<0.05$) effect of maturity on gas production degradability parameters. While the total (A+B) and the asymptote gas production from the fermentation of NDF (B) were higher in the second stage followed respectively by first and third stages of maturity, the rapid (A) and the rate (C) of gas production decreased as the stage of maturity increased. It was also observed that the Lag time increased as the stage of maturity increased.

Table 4.8 *In vitro* gas production dynamics of fresh (F_) or dry (D_) *B.brizantha* leaves harvested over various stages of maturity. The fresh leaves were incubated either crushed by pestle and mortar for 1 minute (_C1), 2 minute (_C2), or 3 minute (_C3). The dry leaves were incubated either in a fine ground (_FG), 1mm ground (_1mmG) or 2mm ground (_2mmG). Each value is the mean of four replicates ($n=4$). Means with the different superscripts within the same column are significantly different ($P<0.05$).

Stage of maturity	Treatment	Gas production degradability parameters				
		A (cm^3)	B (cm^3)	C (/h)	Lag (h)	A+B (cm^3)
1	FC1	$8.7^{a,e}$	36.5^{a}	0.061^{a}	$4.6^{a,e}$	45.2^{a}
	FC2	$9.7^{a,b}$	35.6^{a}	0.075^{b}	4.2^{b}	45.3^{a}
	FC3	10.2^{b}	35.3^{a}	0.076^{b}	$4.0^{b,d}$	45.5^{a}
	DFG	12.1^{c}	32.8^{b}	0.087^{c}	3.4^{c}	44.9^{a}
	D1mmG	$9.7^{a,b}$	35.4^{a}	0.076^{b}	3.9^{d}	45.1^{a}
	D2mmG	$8.8^{a,e}$	36.0^{a}	0.067^{d}	3.9^{d}	44.8^{a}
2	FC1	$7.2^{d,g}$	41.9^{c}	0.048^{e}	4.5^{a}	49.1^{b}
	FC2	8.4^{e}	41.8^{c}	$0.059^{a,g}$	4.5^{a}	50.2^{b}
	FC3	$8.9^{a,e}$	40.5^{d}	0.065^{d}	3.9^{d}	49.4^{b}
	DFG	10.4^{b}	39.1^{e}	0.080^{b}	3.6^{c}	49.5^{b}
	D1mmG	$8.7^{a,e}$	$41.1^{c,d}$	0.072^{b}	3.9^{d}	49.8^{b}
	D2mmG	$7.9^{e,d}$	$41.0^{c,d}$	0.060^{a}	$4.0^{b,d}$	48.9^{b}
3	FC1	5.2^{f}	33.3^{b}	0.042^{f}	4.8^{e}	38.5^{c}
	FC2	$7.0^{d,g}$	32.5^{b}	0.043^{f}	4.6	39.5^{c}
	FC3	$6.1^{g,f}$	32.6^{b}	0.047^{e}	4.2^{b}	38.7^{c}
	DFG	$8.0^{e,d}$	31.2^{f}	0.060^{a}	3.6^{c}	39.2^{c}
	D1mmG	6.4^{g}	32.5^{b}	0.055^{g}	$4.6^{a,e}$	38.9^{c}
	D2mmG	5.3^{f}	33.6^{b}	0.050^{e}	$4.7^{a,e}$	38.9^{c}
S.E.D.		0.51	0.60	0.0021	0.12	0.71
Treatment		*	*	*	*	ns
Maturity		*	*	*	*	*
Treatment x Maturity		ns	ns	*	*	ns

*$P<0.05$
ns $P>0.05$

B.brizantha stem

The gas production degradability parameters of fresh and dry *B.brizantha* over various stages of maturity are shown in Table 4.9. The total gas production (A+B) of each stage of maturity was similar between treatments but decreased ($P<0.05$) as stage of maturity increased.

Increasing the period of maceration increased the rapid gas production (A) for the first two stages of maturity, increased the rate of gas production (C) and decreased the Lag time. Again as noted for leaf material, increases were much reduced for third stage of maturity when compared to the other two. Increasing fineness of grind increased the rapid gas production (A) for first stage only and increased the rate of gas production (C) for both the second and third stages of maturity but not for the first. D2mmG treatment gave a pattern of fermentation that most closely approximated to that of the fresh material.

Similar to what was observed for leaf fraction, there was a significant ($P<0.05$) effect of maturity on gas production degradability parameters of *B.brizantha* stem. The total (A+B), the asymptote gas produced from the fermentation of NDF (B), the rapid (A) and the rate (C) of gas production were decreased as the stage of maturity increased whereas the Lag time was increased as the stage of maturity increased.

Table 4.9 *In vitro* gas production dynamics of fresh (F_) or dry (D_) *B.brizantha* stems harvested over various stages of maturity. The fresh stems were incubated either crushed by pestle and mortar for 1 minute (_C1), 2 minute (_C2), or 3 minute (_C3). The dry stems were incubated either in 1mm ground (_1mmG) or 2mm ground (_2mmG). Each value is the mean of four replicates ($n=4$). Means with the different superscripts within the same column are significantly different ($P<0.05$).

Stage of maturity	Treatment	Gas production degradability parameters				
		A (cm^3)	B (cm^3)	C (/h)	Lag (h)	A+B (cm^3)
1	FC1	$6.3^{a,d}$	36.9^{a}	0.063^{a}	3.6^{a}	43.2^{a}
	FC2	6.8^{a}	36.4^{a}	$0.065^{a,b}$	$3.5^{a,b}$	43.2^{a}
	FC3	8.4^{b}	36.0^{a}	$0.068^{b,c}$	$3.3^{b,c}$	44.4^{a}
	D1mmG	7.6^{c}	36.3^{a}	0.070^{c}	3.1^{c}	43.9^{a}
	D2mmG	$6.5^{a,d}$	36.6^{a}	$0.066^{a,b,c}$	$3.3^{b,c}$	43.1^{a}
2	FC1	5.9^{d}	32.6^{b}	$0.042^{d,g}$	4.0^{d}	38.5^{b}
	FC2	7.5^{c}	32.5^{b}	$0.048^{e,g}$	3.9^{d}	40.0^{b}
	FC3	$6.9^{a,c}$	32.3^{b}	0.056^{f}	3.6^{a}	39.1^{b}
	D1mmG	$6.4^{a,d}$	32.7^{b}	$0.067^{b,c}$	$3.4^{a,b}$	39.1^{b}
	D2mmG	$6.4^{a,d}$	32.5^{b}	0.057^{f}	3.6^{a}	38.8^{b}
3	FC1	$4.9^{e,f}$	30.9^{c}	0.039^{d}	4.9^{e}	35.8^{c}
	FC2	$5.2^{e,d,f}$	30.1^{c}	0.043^{d}	4.7^{e}	35.3^{c}
	FC3	$5.4^{e,d}$	30.7^{c}	0.045^{g}	4.7^{e}	36.1^{c}
	D1mmG	4.6^{f}	31.0^{c}	0.056^{f}	4.3^{f}	35.6^{c}
	D2mmG	4.5^{f}	31.0^{c}	$0.048^{e,g}$	4.4^{f}	35.5^{c}
S.E.D.		0.38	0.48	0.0024	0.13	0.77
Treatment		*	ns	*	*	ns
Maturity		*	*	*	*	*
Treatment x Maturity		ns	ns	ns	ns	ns

*$P<0.05$
ns $P>0.05$

4.5 Discussion

The results of the chemical analysis (Table 4.3) shows that the DM, CP, ash, and NDF contents were similar between treatments. This indicates that the composition of the material was not affected by pre-treatment (for example by loss of cell contents during maceration). However, it was observed that FC0, FCH (for all forage samples), FC1, FC2, and FC3 (for 3rd stage of maturity for *B.decumbens* samples) had higher NDF values compared with the other treatments. These differences are probably due to the physical structure of those samples in which the degree of disruption was not sufficient to allow complete solubilisation of non-NDF material, causing an overestimation of NDF values.

The results of this study showed that the total pool of fermentable material (A+B) was not changed by treatments but the way in which it is apportioned between the rapid gas production (A) and the gas production from the NDF (B) differed (Table 4.4, 4.5, 4.6, 4.7, 4.8 and 4.9). A possible explanation to differences in (A) and (B) values could be attributed to the estimation of these values using the model of Jessop and Herrero, (1996). This model assumed that the gas production up to 4 hours represents the degradation of soluble material and their assumption was based on fermentation of mixtures of glucose and cellulose. This assumption was not valid in this study when fresh or dry fine ground (Table 4.4, 4.5 and 4.6) were used where the results showed that the gas production released from the fermentation of soluble material of these samples took less time than that assumed by the above model and hence the gas production from the fermentation of insoluble material of these sample start earlier than that assumption (before 4 h). Also, two exponential model

introduced by (Jessop and Herero 1996) was unable to distinguish between the rapid gas production (A) and the gas production from the NDF (B) when fitted to fresh temperate grass and DFG samples. This could be due to the fast initial gas production that associated with fresh and dry fine ground treatments and, insufficient number of data points that requires to distinguish between the rapid gas production (A) and the gas production from the NDF (B). However, Cone (1998) observed that fresh material incubated *in vitro* using gas production method had a higher initial gas production than the dry material.

Differences in estimating the amount of soluble (A) and insoluble (B) material that degraded between fresh and dry forage was observed by some *in sacco* studies. Lopez *et al.*, (1995) compared the degradation characteristics between fresh and dry forages by the nylon bag technique and they found that the DM disappearances of both fresh chopped and macerated ryegrass had a significantly lower A and higher B values compared to the oven dried but the potential degradability were similar. Conversely, they noted that both fresh chopped and macerated grass silage had a significantly higher (A) and lower (B) values compared to the oven dried but the potential degradabilities (A+B) were similar. Recently, Valentin *et al.*, (1999) compared the *in sacco* DM disappearance between fresh and oven dried maize silage and found that the quickly degradable material (A) was significantly higher on dry 3mm ground than on the 5mm chopped fresh maize silage and the insoluble degradable fraction (B) was lower on dry 3mm ground than on the 5mm chopped fresh maize silage but the potential degradability (A+B) was not significantly different between them.

On reviewing the data it can be seen that the rates of gas produced (C) from the fresh temperate (ryegrass and red fiscue) and first stage of maturity of *B.decumbens* (Table 4.4, 5 and 6) were higher than the dried 1mm ground and very close to the finely ground one. This appeared to be related to the effect of the preparation method (grinding) on the rate at which materials are available for degradation by rumen micro-organisms. In other words, this might be due to extensive grinding which may increase the surface area for microbial attachment subsequently increase the rate of gas produce from the insoluble fractions. Also, increasing the fineness of the grind will increase the extent of release of cell contents and hence increase gas production at 4 h. However, this would not affect the asymptotes (A+B) but it might slow down or increase the degradation processes, hence the rate of degradation and the relative portions of A and B, would be changed as was observed here when the D1mmG is compared with DFG. This result in agrees with those of other authors who observed a decline in DM degradability of the dried ground forages when compared with the fresh one *in vitro* (Kaumon and Thewis, 1990; Broesder *et al.*, 1992;) or *in situ* (Hirstove, 1992; Lopez *et al.*, 1995; Valentin *et al.*, 1999).

The Lag time was decreased with a decrease in particle size. This could be attributed to the changes in the percentage of fine particles, which are immediately soluble or rapidly degradable.

In term of the stage of maturity of *B.brizantha* (Table 4.8 and 4.9) the results showed that the gas production degradability parameters of the fresh and dry

materials vary between stage of maturaties. In the first stage of maturity, the gas production degradability parameters of D1mmG was closer to those of the fresh but the D2mmG was closer to fresh treatments (FC1, FC2 and FC3) in the second stage and FC3 in the third stage of maturity. The results also showed that the dry fine ground (DFG) overestimated the gas production degradability parameters of the fresh material. These differences in the gas production degradability parameters between fresh and dry samples in the three stages of maturity could be related to the changes in the physical structure of the plant. It was observed by Salimei *et al.*, (1994) that the biological and physical factors of forage growth environment, as well as stage of maturity and forage preservation influence cell wall composition and the physicochemical properties of fibre and the fermentation kinetics of forages. This could explain why crushing the fresh material for 1, 2 and 3 min was not enough to maximise the rate of gas production for tropical grasses whereas for temperate grasses the results showed that crushing the fresh sample of 2 min was enough to obtain the maximum rate of fermentation.

The differences in the plant anatomy between tropical and temperate grasses may play an essential role in estimating the rate of fermentation. Since it has been demonstrated by (Hatch and Slack, 1970; Laetsch, 1974) that most of tropical grasses have a C_4 pathway of photosynthesis and are characterised by a specialised leaf anatomy such as a radial arrangement of bundle sheath around the vascular bundles, size and structural dimorphism of chloroplasts in the bundle sheath and surrounding mesophyll cells and the large mitochondria presence in the bundle sheath whereas temperate grasses have C_3 pathway of photosynthesis and have not got the above

features. The bundle sheath cells have no intercellular air space and have thick suberised outer walls which make leaves resistant to the mechanical breakdown (Norton, 1981). These bundle sheath tissues reduce accessibility of plant cells to microbial digestion in the rumen (Hanna *et al.*, 1973). Therefore, grinding might destroy the physical properties of the tropical grasses which increases the material that became available for degradation by increasing the area accessible for microbial attachment (surface area). This would result in an overestimation of the fermentation dynamics for the tropical grasses and explain the differences in the rate of gas production between temperate and tropical grasses where using the DFG sample was mimicking the gas production degradability of fresh temperate grasses and overestimating that for the tropical grasses. This is in agreement with the *in vitro* gas production work by Bonsi *et al.*, (1995) who observed that the rate of gas production of dry foliages was higher than fresh ones but the dry material produced less gas than the fresh. Also, Sanderson *et al* (1997) reported that freeze-dried and milled silage produced a higher rate and total gas production than the chopped or unchopped fresh silage. The discrepancy in the total gas production between studies could be attributed to the differences in the ways of calculating the accumulative gas production where in this study the gas produced from the fresh and dry forages was corrected on the basis of the DM (200 mg DM) in order to justify the comparison between them whereas in that study the gas produced from 800 mg fresh material was not corrected. However, Kabuga and Darko, (1993) studied the *in sacco* degradability of dry matter in a fresh and oven dried tropical grasses and they concluded that the dry matter of oven dried and fresh samples were similar for all bag incubation times.

The differences in the results between first stage of *B.decumbens* and *B.brizantha* could be related to the differences in the temperature between them where the *B.decumbens* was grown under 25° C whereas the *B.brizantha* was grown under 35° C. Temperature has a high influence on the *in vitro* digestibility (Buxton and Fales 1994). Several studies have demonstrated a decline in the *in vitro* DM digestibility as the temperature increased (Dirven and Deinum 1977; Minson 1980; Buxton and marten 1989; Thorvaldson, 1990).

Drying, milling, pulverizing or chopping forages as substrates for laboratory methods may not effectively mimic mastication by the animal (Bailey, 1962; Playne *et al.*, 1978; Olubobkun *et al.*, 1990). Ruminants masticate food to decrease particle size and to expose more surface area and more area of degradable tissue within a given particle size and that causes the feed to be more crushed and cracked (Pond *et al.*, 1984). The results of this study showed that the use of dry fine, 1and 2 mm ground as sample preparation for the *in vitro* gas production technique might result in over or under estimation of forage degradability. Fresh crushed samples prior to the *in vitro* gas production incubation is probably the sample preparation which best reproduces chewing. However, the use of fresh sample necessitates location of the technique close to the site at which the forage is grown; this is often impractical.

4.6 Conclusion

The results of this study have demonstrated that caution should be taken in the processing of the forage sample if the *in vitro* gas production technique is to be used for the purpose of studying the fermentation dynamics or to evaluate the nutritional value of the ruminant forages particularly for grazing animals. Future research in this area could be directed at a better understanding of the way in which the animal masticates feed and improving the method of mimicking that mastication.

Chapter 5

The effect of fermentable nitrogen availability on the gas production degradability of NDF

5.1 ABSTRACT

The effects of harvesting ruminal micro-organisms on patterns of gas production were investigated in experiment 1 by incubating 200 mg DM of pure cellulose, oatfeed, ryegrass, cocksfoot, wet and dry season kikuyu grasses with either diluted ruminal fluid or harvested ruminal bacteria. The gas production degradability parameters (A, B, C and Lag) obtained from fitting the data to the model Gas = B $(1\text{-}exp^{-C(t\text{-}Lag)})$ after correcting gas volumes for the fermentation of soluble material (A) were not significantly different between these two treatments. In experiment 2, the effect of ammonia concentration on the digestibility and gas production degradability parameters (B, C and Lag) of NDF prepared from cellulose, guinea, star grass, Brachiaria decumbens and wet season kikuyu grass was studied using harvested ruminal bacteria as the inoculant. The response of NDF degradability to nitrogen availability was broadly consistent with earlier *in vitro* and *in situ* studies, indicating that, the ammonia concentration required to maximize the rate of NDF gas production varies according to the availability of fermentable NDF and that a minimum of approximately 100 mg N/l was required to achieve the maximum rate of degradation of NDF. The effect of nitrogen availability on NDF gas production degradability appeared to be mainly, if not entirely on the rate of gas production rather than its extent.

5.2 INTRODUCTION

Ruminal micro-organisms have a requirement for fermentable nitrogen in order that they may synthesis the nitrogen containing constituents of their cell mass by amino acids, nucleic acids. The fermentable nitrogen can be in many forms, the simplest of which is ammonia which is the preferred nitrogen source for some species of bacteria (Hungate, 1960). Rumen micro-organisms utilize ammonia for synthesizing protein for growth and fermentation of feeds. Several earlier studies have examined the influence of rumen ammonia concentration on microbial protein synthesis. Based on study in vivo, Hume *et al.*, (1970) reported that the ammonia concentration at which microbial synthesis of protein was maximal was 88 mg N/l but, that the flow of microbial protein from the rumen was highest with an ammonia concentration of 133mg N/l. Allen and Miller (1976) noted that the greatest flows of non-ammonia nitrogen through the abomasum were achieved when the ammonia concentration in the rumen were between 160 and 220 mg N/l. Satter and Slyter (1974), using continuous culture fermenters, found that the precise limiting ammonia concentration was about 20mg/l but, suggested that the ammonia concentration of 50 mg N/l was a practical requirement for maximal microbial growth. Schaefter *et al.*, (1980), using pure cultures, reported a requirement for a concentration of ammonia of 14 mg N/l to achieve 95% of maximal microbial growth rate.

When nitrogen is not limiting microbial growth there is good evidence to suggest that the amount of microbial protein synthesized is directly related to the amount of substrate fermented (Hungate, 1966). Taking this into account, several

studies have aimed to define the level of ammonia needed to support maximum rate of fermentation. Miller *et al*, (1973) recommended 289 mg ammonia N/l to optimize the rate of fermentation. Using *in situ* method Mehrez *et al.*, (1977) noted that an ammonia concentration of 200 mg N/l was required to obtain the maximum rate of disappearance of barley in sheep. Similarly, Wallace (1979) observed that increased *in situ* DM and CP degradation rates of barley grain were accompanied by increased bacterial growth when rumen ammonia concentration was increased from 97 to 214 mg N/l. Kerbs and Leng (1983) found that the rate of *in situ* degradability of cotton wool was enhanced with increasing levels of rumen ammonia up to 210 mg N/l but that levels up to 50 mg N/l were sufficient to maximize the rate of disappearance of oaten chaff. Boniface *et al* (1986) found that maximum rate of disappearance (*in situ*) of hay grass was observed at ammonia levels of about 50 mg N/l. Erdman *et al.*, (1986) reported that *in situ* degradation rates plateaued at ammonia concentration in excess of 200 mg N/l and concluded that the nitrogen requirement to achieve maximum degradation was dependent on the potential degradability of feed. Oosting *et al.*, (1989), using an *in vitro* technique based on Tilley and Terry (1963), noted that 100 mg N/l was required for maximum *in vitro* degradation of feed. Recently, Dryhurst and Wood (1998), using an *in vitro* gas production technique based on Theodorou *et al.*, (1994) studied the effect of nitrogen concentration on the gas production dynamics of barley straw. They concluded that a minimum ammonia concentration of about 80 mg N/l was needed to achieve the maximum degradation of carbohydrate by rumen microbes but the nitrogen concentration indicated may underestimate the true concentration of nitrogen available to the microbes since they mention that the contribution of inoculum ammonia was about 28 mg N/l.

General guidelines on the level of ammonia concentration needed by rumen microbes to maximize the microbial growth and fermentation process therefore range from 50 mg N/l (Satter and Slyter 1974; Boniface *et al.*, 1986) to over 200 mg N/l (Miller *et al*, 1973; Mehrez *et al.*, 1977 and Erdman *et al.*, 1986).

In the above reports the nitrogen supplied by the animal (endogenous), inoculum or substrate were not controlled. Thus the nitrogen concentrations indicated may underestimate the true nitrogen availability to the rumen microbes. The objectives of this work were 1) to study the effect of harvesting ruminal micro-organisms to remove the fermentable nitrogen present in ruminal fluid on the gas production profiles, 2) to study the effect of fermentable nitrogen availability on NDF gas production degradability using a harvested ruminal micro-organisms.

5.3 MATERIAL AND METHOD

Animals and diet

Two ruminally fistulated Suffolk sheep fed 750g sheep pellets (NDF and CP 338 and 175 g/kg DM) and 250g grass nuts (NDF and CP 345 and 150 g/kg DM) twice a day at 8.00 and 17.00 h were used. Water was available to the sheep *ad libitum.*

Inoculum preparation

A sample of rumen contents containing both solid and liquid material was taken before morning feeding and collected in a prewarmed vacuum flask. In the laboratory ruminal fluid was filtered through two layers of muslin cloth, the solid material was squeezed lightly. The microbial activity of the strained ruminal fluid were tested by measuring the bacterial DM indirectly (see Table 5.1) as described in chapter 2. The strained ruminal fluid was mixed with anaerobic medium (Menke and Steingass, 1988) before or after centrifugation according to the design of each experiment. Once prepared, the suspension of micro-organisms was kept at 39 °C with CO_2 bubbling through for approximately 20 min before addition to syringes.

Experimental treatments

Experiment 1.

The aim of this experiment was to compare the gas production profile of harvested rumen micro-organisms with that of strained ruminal fluid. In six runs (n = *6*), the strained ruminal liquor (2 l) was divided into two portions. The first portion was mixed with the buffer solution (1:2) and kept at 39 °C with CO_2 bubbling through until the incubation time whilst the second portion was immediately centrifuged at 1000 X G

for 5 min at 30 °C to harvest the protozoa and fungi (Henning *et al.*1991). The pellet was washed with 50 ml of the buffer media and kept at 39 °C while the supernatant was recentrifuged at 26000 X G for 15 min at 30 °C to harvest the bacteria (Henning *et al.*1991; Pell and Schofield, 1993). The supernatant was discarded whilst the bacterial pellet was mixed with 50 ml of the buffer media. Both pellets were mixed together and returned to the original volume with the buffer. The mixture of the pellets was diluted with the buffer solution (1:2) for the gas production incubation. For each portion gas production was measured in the absence of substrate and from the incubation of 200mg DM ∝-cellulose (Sigma, Chemical Co Ltd. Poole, Dorset, UK), oatfeed, ryegrass (*Lolium perenne*), cocksfoot (*Dactyylis glomerata*), wet and dry season kikuyu grasses (*Pennisetum clanestinum*) (Poas Region, Costa Rica) in 100 cm^3 glass syringes (Fortuna, Germany). Forage samples were analysed for neutral detergent fibre (NDF) using a modified micro technique (Pell and Schofield, 1993), crude protein (CP) using standard Kjeldahl nitrogen determinations and for ash (Table 5.2).

Experiment 2.

The aim of this experiment was to study the influence of nitrogen availability on the gas production degradability parameters. In four runs ($n = 4$), the strained ruminal fluid (1 l) was divided into five portions and immediately centrifuged (to remove the fermentable nitrogen from the inoculum) as described in experiment 1. The mixed pellets of each portion were returned to the original volume and diluted with the buffer solution (1:2) for the gas production incubation. Nitrogen was added as ammonium bicarbonate to give the following concentration in the final buffer mixture: 0, 25, 50, 100 and 200 mg N l^{-1}. To maintain a constant level of bicarbonate in the buffer, the

difference between the standard amount of ammonium bicarbonate and the amount needed to yield the above concentrations was added to the buffer in the form of sodium bicarbonate. Ammonia concentration (mg N/l) of strained ruminal fluid, harvested rumen micro-organisms, buffer solution and final culture (harvested rumen micro-organisms and buffer solution) were determined (see Table 5.3) by using an ammonia electrode. Gas production was performed simultaneously using a single source of inoculum. About 2 g NDF of guinea (*Panicum maximum*), star (*Cydon nlenfluensis*), Brachiaria grass (*B.decumbens*) and wet season kikuyu grass were prepared by refluxing each forage with neutral detergent solution (NDS) (without sodium sulfite) according to the method of Van Soest and Robertson (1985) described by Blummel and Becker (1997). After refluxing, the NDF was recovered on a sintered glass crucible and rinsed with distilled water fifteen times. For each ammonium concentration, gas production was measured in the absence of substrate and from the incubation of 200mg dry NDF of the above forage samples and ∝-cellulose in 100 cm^3 glass syringes (Fortuna, Germany). Each combination of ammonium concentration and substrate was replicated four times. NDF residues were collected at the end of the incubations for the estimation of NDF digestibility (NDFD) using a modified micro technique (Pell and Schofield, 1993) by emptying the contents of each syringes into 100 cm^3 medical flat bottles. Each syringe was washed three times with 5 cm^3 of NDF solution, the washings being added to the appropriate bottle.

Table 5.1. Ruminal fluid microbial mass for experiment 1 and 2.

Microbial activity	Experiment 1		Experiment 2				
	Ruminal micro-organisms		Ammonia concentration (mg N/l)				
	Harvested	Unharvested	0	25	50	100	200
Observed bacterial absorbance (A)	0.150		0.163				
Estimated bacterial DM (mg/10 cm^3)	130.2		141.5				
Observed blanks gas volume (cm^3)	21.5	23.3	17.5	20.3	20.0	23.8	22.5

Gas production measurements and analytical procedures

The *in vitro* gas production was performed as described by Jessop and Herrero (1996). The cumulative gas production for each syringe was recorded at 1,2,3,4,5,6,8 h; thereafter every 4 h until 60 h, and then 72, 84, 96 and 120 h. Cumulative gas volumes were corrected for fermentation of soluble material (experiment 1) by subtracting the gas produced up to 4 h (A) (Herrero and Jessop, 1996), and fitted to the model Gas = $B(1-exp^{-C(t-Lag)})$ (Krishnamoorthy *et. al.*, 1991) in experiment 2 using Marquart algorithm as implemented by GraFit (Leatherbarrow, 1992). B is the asymptotic gas production from the fermentation of NDF (cm^3), C is the fractional rate of gas production (/h), t is time (h) and lag is the lag phase before the fermentation of NDF begins (h).

Table 5.2 Chemical composition of forage samples

Forage sample	CP (g/kg DM)	NDF (g/kg DM)	Ash (g/kg DM)
ryegrass	193 ± 4.4	476 ± 16.1	82 ± 7.2
kikuyu (wet season)	259 ± 12.2	534 ± 22.1	127 ± 11.3
kikuyu (dry season)	60 ± 7.1	751 ± 27.4	103 ± 6.7
cocksfoot	91 ± 5.5	508 ± 12.3	73 ± 6.3
oatfeed	87 ± 3.7	271 ± 7.7	39 ± 2.5
star	43 ± 3.4	755 ± 23.8	96 ± 4.8
B.decumbens	129 ± 6.8	592 ± 18.5	122 ± 9.6
guinea	96 ± 7.0	626 ± 14.2	118 ± 12.8

**se* is standard error

Statistical analysis

Paired T Tests were used to compare the degradability parameters of feed between harvested and unharvested ruminal micro-organisms in experiment 1. Analysis of variance was used to compare both gas production parameters and NDFD between different ammonia concentrations in experiment 2. The statistical package MINITAB (1993) was used for these purposes. Least significant differences were calculated from the standard error of the differences between means.

Table 5.3. Ammonia concentration (mg N/l) of strained ruminal fluid, harvested rumen micro-organisms, buffer solution and final culture (harvested rumen micro-organisms and buffer solution) in experiment 2.

Treatment	Ammonia concentration (mg N/l)			
	Strained ruminal fluid	Harvested rumen micro-organisms	Buffer solution	Final culture
1		5.1	0	5.1
2		4.9	25	29.9
3	177	4.5	50	54.5
4		5.0	100	105
5		4.8	200	204.8

5.4 RESULTS

The effect of harvesting ruminal micro-organisms on gas production dynamics

The estimated parameter values for *in vitro* gas production dynamics of cellulose, wet and dry season kikuyu grass, ryegrass, cocksfoot, and oatfeed incubated either in harvested or unharvested ruminal liquor microbes are presented in Table 5.4. In most cases, feed samples incubated in strained ruminal fluid (unharvested) had a slightly higher gas from soluble fraction (A), asymptotic gas production from the fermentation of NDF (B) and rate of gas production (C) and lag phase before the fermentation of NDF begins (h) than on those incubated in harvested ruminal fluid microbes. However, there were no significant ($P>0.05$) differences in all estimated parameters between feed incubated either in harvested or unharvested ruminal fluid.

Table 5.4 Gas production degradability parameters of cellulose, ryegrass, cocksfoot, oatfeed and wet and dry seasons kikuyu grass incubated either in harvested or unharvested ruminal fluid micro-organisms. Each value is a mean of six replicates (*n=6*)

Gas production degradability parameter		Ruminal fluid micro-organisms		
		Harvested	Unharvested	SEM
cellulose	B (cm^3)	90.6	91.3	0.67
	C (/h)	0.097	0.102	0.0035
	Lag (h)	4.7	4.5	0.22
kikuyu (wet season)	A (cm^3)	8.6	8.7	0.11
	B (cm^3)	38.6	39.1	0.67
	C (/h)	0.056	0.058	0.0035
	Lag (h)	5.4	5.2	0.22
kikuyu (dry season)	A (cm^3)	5.2	5.6	0.11
	B (cm^3)	29.5	32.6	0.67
	C (/h)	0.0438	0.0453	0.0035
	Lag (h)	5.7	5.7	0.22
ryegrass	A (cm^3)	19.6	19.2	0.11
	B (cm^3)	43.9	41.5	0.67
	C (/h)	0.069	0.066	0.0035
	Lag (h)	3.7	3.6	0.22
cocksfoot	A (cm^3)	12.1	12.3	0.11
	B (cm^3)	39.4	39.4	0.67
	C (/h)	0.057	0.061	0.0035
	Lag (h)	4.1	3.9	0.22
oatfeed	A (cm^3)	9.1	9.3	0.11
	B (cm^3)	27.5	27.7	0.67
	C (/h)	0.046	0.048	0.0035
	Lag (h)	3.7	3.8	0.22

The influence of nitrogen concentration on gas production dynamics and NDFD

The NDF digestibility (NDFD) and the estimated gas production degradability parameters of NDF fractions of cellulose, wet season kikuyu grass, *B.decumbens*, guinea and star incubated in a buffered harvested ruminal fluid micro-organisms with different nitrogen (ammonium bicarbonate) concentration (0, 25, 50, 100 and 200 mg N/l) are shown in Table 5.5. In most cases, the overall influence of increasing the level of nitrogen concentration from zero to 200 mg N/l on the estimated gas production parameters were similar between cellulose (except the lag phase), wet season kikuyu grass, *B.decumbens* and guinea.

The asymptotic gas production from the fermentation of NDF (B) and NDF digestibility of cellulose, wet season kikuyu grass, *B.decumbens* and guinea incubated in nitrogen free medium were significantly ($P<0.05$) lower than those incubated in 25, 50, 100 and 200 mg N/l whereas the asymptotic gas production (B) and NDFD of star were not significantly influenced by the availability of nitrogen. However, there were no significant differences in the asymptotic gas production from the fermentation of NDF (B) and NDFD of cellulose, wet season kikuyu grass, *B.decumbens* and guinea when the nitrogen concentration increased from 25 to 200 mg N/l.

Nevertheless, the rate of NDF gas production (C) of cellulose, wet season kikuyu grass, *B.decumbens* and guinea was significantly ($P<0.05$) increased as nitrogen concentration was increased from 0 to 100 mg /l, after which there were no significant responses to nitrogen concentration. The rate of gas production of star NDF was significantly ($P<0.05$) increased until the nitrogen reached the concentration of 50 mg

N/l but no significant responses to the increase of nitrogen concentration were observed above that.

The lag phase before the fermentation of NDF begins (h) of guinea and decreased ($P<0.05$) as the nitrogen concentration increased from 0 to 50 mg N/l but thereafter no effect of nitrogen concentration was noted. The lag phase before the fermentation of NDF begins (h) of cellulose, wet season kikuyu grass and *B.decumbens* appeared to be more sensitive to the nitrogen concentration where the nitrogen concentration of 100 mg N/l was enough to minimize the lag phase and was significantly ($P<0.05$) increased as the concentration of nitrogen decreased from 100 to 0 mg N/l.

As expected the results indicate that there were significant ($P<0.05$) differences in gas production degradability parameters (B, C and Lag) and NDFD between feed samples. In addition to that there were also a significant ($P<0.05$) interaction in the rate of gas production (C) and the lag time (Lag) between ammonia concentration and feed samples but no significant interaction between ammonia concentration and feed samples in the asymptotic gas production from the fermentation of NDF (B) and NDFD was found.

Table 5.5 Gas production degradability parameter of NDF and NDFD of cellulose, guinea, star, *B.decumbens* and wet season kikuyu incubated in a mixture of harvested ruminal fluid micro-organisms and buffer medium with varying levels of ammonium concentration (mg N/l). Each value is a mean of four replicates ($n=4$). Means with different superscripts within the same column are significantly different ($P<0.05$).

Feed sample	Ammonium concentration (mg N/l)	Gas production degradability parameter			
		B (cm^3)	C (/h)	Lag (h)	NDFD (g/kg DM)
cellulose	0	80.1^a	0.051^a	8.2^a	978^a
	25	89.9^b	0.077^b	6.7^b	999^b
	50	90.2^b	0.089^c	5.3^c	1000^b
	100	91.5^b	0.098^d	4.6^d	1000^b
	200	90.9^b	0.101^d	4.4^d	1000^b
kikuyu (wet season)	0	60.2^a	0.043^a	8.7^a	628^a
	25	66.7^b	0.048^b	7.3^b	703^b
	50	67.3^b	0.052^c	6.1^c	711^b
	100	67.9^b	0.057^d	5.0^d	715^b
	200	68.6^b	0.059^d	4.9^d	728^b
B.decumbens	0	58.8^a	0.040^a	9.1^a	617^a
	25	64.4^b	0.046^b	7.8^b	680^b
	50	65.2^b	0.051^c	6.5^c	686^b
	100	65.9^b	0.056^d	5.0^d	698^b
	200	66.1^b	0.058^d	4.8^d	707^b
guinea	0	50.1^a	0.032^a	9.3^a	524^a
	25	56.4^b	0.037^b	7.2^b	604^b
	50	56.5^b	0.042^c	5.6^c	609^b
	100	56.7^b	0.048^d	5.3^c	618^b
	200	58.5^b	0.049^d	5.1^c	634^b
star	0	38.5^a	0.021^a	11.4^a	408^a
	25	39.7^a	0.026^b	10.3^b	419^a
	50	40.5^a	0.030^c	8.4^c	430^a
	100	40.8^a	0.032^c	8.0^c	443^a
	200	40.6^a	0.030^c	7.9^c	441^a
SED		1.28	0.0017	0.42	22.7
Effect of ammonia concentration		*	*	*	*
Effect of feed		*	*	*	*
Ammonia concentration x Feed		ns	*	*	ns

$^*P<0.05$, $^{ns}P>0.05$

5.5 DISCUSSION

The effect of harvesting ruminal microbes on gas production dynamics

In order to investigate the effect of limiting the availability of nutrients on the degradation characteristics of feed, the potential endogenous contribution of the specific nutrient under study should be considered. The results in Table 5.4 indicate that microbes can be harvested from ruminal fluid without altering the gas production degradability parameters using the centrifugation speeds recommended by Henning *et al.*, (1991). This increases the potential use of the *in vitro* gas production technique.

The influence of nitrogen concentration on gas production dynamics and NDFD

The primary objective of this work was to study the effect of nitrogen availability on the degradability and digestibility of NDF. The results showed that each was sensitive to the availability of nitrogen (Table 5.5). The asymptotic gas production from the fermentation of NDF (cm^3) and NDFD of cellulose, B.decumbens, guinea and wet season kikuyu grass increased with increasing ammonia concentration but maximal responses were observed at ammonia concentration of 25 mg N/l. A change in NDFD might be the cause of differences in the asymptotic gas production from the fermentation of NDF (cm^3) between feed samples incubated either in zero ammonia concentration (the clarified inoculum contribution was about 4.9 mg N/l) or other ammonia concentration (25, 50, 100 and 200 mg N/l). This could be due to a deficiency of nutrient supply to the microbes in the form of ammonia which would reduce the efficiency of rumen microbial growth and subsequently the maximal fermentation to be achieved. Dryhurst and Wood (1998), noted that ammonia concentrations (0, 20, 40, 80, 120 and 165 mg N/l) did not alter the total gas production and DMD of barley straw

when the incubation time was extended until 166 h. However, the nitrogen concentration stated in their study was an underestimate of the true concentration of nitrogen available to the microbes since fermentable nitrogen would have been available from the inoculant (they state this as 28 mg N/l) and from the feed source.

Stoichiometric relationships have shown that around 0.47 cm^3 of gas should be produced per mg of NDF fermented (Wolin, 1960; Herrero and Jessop, 1996). Taking NDFD value (Table 5.5) of pure cellulose incubated in zero ammonia concentration as an example to estimate the total gas predicted to be produced from the fermentation of NDF, it can be observed that about 92 cm^3 gas should be produced from 200 mg DM cellulose with a digestibility of 97.8 % whereas the actual total gas observed was 80.1 cm^3. This could be attributed to the differences in particles size between the initial and residual cellulose where the particles size of cellulose will be reduced after the attachment and fermentation by rumen microbes and resulted in more undegradable materials passing through the pores of the sintered glass crucible and assumed to be fermented. Thus, care should be taken in the feed particle size when using a modified micro technique (Pell and Schofield, 1993) to measure the NDFD of feed.

The results also indicate that an ammonia concentration of 100 mg N/l was required to maximize the rate of gas production from cellulose, B.decumbens, guinea and wet season kikuyu grass which is in agreement with *in vivo* work by Hume *et al.*, (1970) and *in vitro* Tilley and Terry (1963) work by Oosting *et al.*, (1989). This also agrees with the work by Dryhurst and Wood (1998), using an *in vitro* gas production technique based on Theodorou *et al.*, (1994) if the contribution of nitrogen supplied by

the inoculum they used was taken into account. Nevertheless, the results also indicated that the gas production degradability parameters and NDFD of star feed were less influenced by the availability of nitrogen and ammonia concentration of 50 mg N/l were sufficient to maximize the rate of gas production degradability which is consistent with the *in vitro* finding of Satter and Slyter (1974) and *in situ* findings of Kerbs and Leng (1983) (for oaten chaff) and Boniface *et al* (1986).

The different responses between substrates to the availability of nitrogen could be related to the relationship between nitrogen requirement and carbohydrate availability to microbes as suggested by Erdman *et al.*, (1986). The effect of the availability of both nitrogen and digestible cell wall (dcw (NDF*NDFD)) on the rate of gas production is shown in Figure 5.1. It can be seen that the rate of gas production was highly dependent on the availability of both nitrogen and fermentable carbohydrate where the microbial demand for nitrogen to maximize the rate of gas production was increased as the dcw of substrate increased. This finding would support the above suggestion by Erdman *et al.*, (1986) and explain the interaction in the rate of gas production which was observed (Table 5.5) between ammonia concentrations and feed samples. However, the ammonia concentration of 100 mg N/l was enough to maximize cellulose (totally fermentable and available to rumen microbes) rate of fermentation and to reach the value of 0.1 /h which is the maximum cellulose rate of fermentation proposed by Russell *et al.*, (1992).

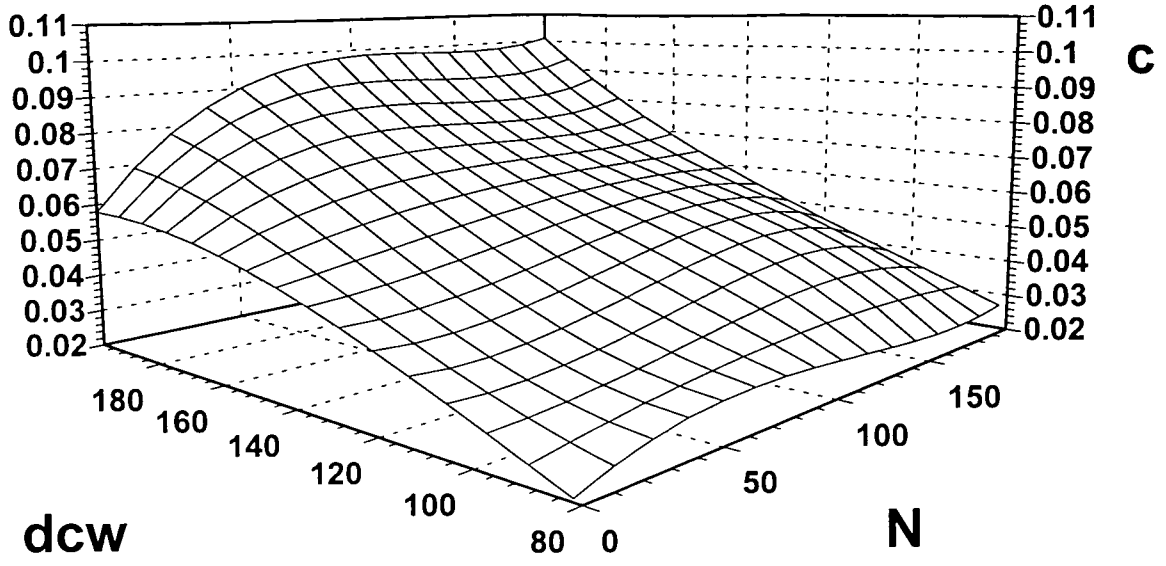

Figure 5.1 The effect of nitrogen (N (mg/l)) and digestible cell wall (dcw (mg / 200 mg NDF)) availability on the rate of gas production (C (/h)).

The higher levels of ammonia concentration reported by Miller *et al*, 1973; Mehrez *et al.*, 1977; Kerbs and Leng, 1983 (for cotton wool) and Erdman *et al.*, (1986) are difficult to explain since the methodology they used was different.

Correcting the balance of nutrient supply to rumen microbes, ensures there are no deficiencies of microbial nutrients and rumen microbes grow efficiently, ensuring that maximal rate of fermentation is achieved, can improve the degradability of forages (Leng, 1990). The gas production technique promises to be suitable for such investigations.

5.6 CONCLUSION

1) The results of this study indicated that harvesting ruminal fluid micro-organisms did not alter the gas production degradability parameters.

2) The ammonia concentration required to maximize NDF fermentability is varied according to the availability of both fermentable NDF and nitrogen (Figure 5.1).

3) The minimum ammonia concentration need to maximize the rate of NDF fermentation is 100 mg N/l.

4) Protein–energy interactions can be studied using the *in vitro* gas production technique.

Chapter 6

The potential use of ovine, bovine and equine faeces as sources of inocula for the *in vitro* gas production method to replace ruminal fluid

6.1 Abstract

The suitability of using ovine faecal fluid (OFF), bovine faecal fluid (BFF) and equine faecal fluid (EFF) as source of inocula for the *in vitro* gas production technique to replace ruminal fluid was studied. Bacterial DM, bacterial absorbance and blanks gas volume were significantly different between OFF, BFF, EFF and ovine ruminal fluid (ORF). Gas production degradability parameters and molar proportion of VFA from cellulose, glucose, ryegrass, wet season kikuyu grass and star grass incubated in buffered BFF or EFF were significantly different from those incubated in buffered ORF. Glucose, ryegrass and wet season kikuyu grass incubated in buffered OFF had similar gas production degradability parameters and molar proportion of VFA to those incubated in buffered ORF. In contrast, cellulose and star grass incubated in OFF had a significant longer lag time, lower rate and total gas production, and different molar proportion of VFA compared to those incubated in buffered ORF. No significant differences in total VFA concentrations and NDFD of feed samples incubated either in OFF, BFF, EFF or ORF were observed. Results from this study indicate that faecal fluid particularly from sheep has the potential to be used as source of inocula for the *in vitro* gas production technique instead of ruminal fluid. However, more research is required to confirm this finding and improve the activity of faeces micro-organisms.

6.2 Introduction

In vitro techniques have been used extensively to measure digestibility (Tilley and Terry, 1963) and the kinetics of feed degradation (Goering and Van Soest 1970; Menke *et al.*, 1979). However, these techniques rely on using ruminal fluid as a source of inocula to mimic ruminal fermentation of feedstuffs. Use of ruminal fluid as source of inocula for the *in vitro* techniques requires fistulated animals; these are expensive to maintain, time consuming, in some circumstances unavailable and not favoured by animal welfare organisations.

For the two-stage *in vitro* technique of Tilley and Terry (1963) several studies have shown a high correlation between digestibility of forages as determined using either ovine ruminal fluid, ovine faeces or bovine faeces as the microbial inoculum (El Shaer *et al.*, 1987; Omed *et al.*, 1989; Akhter *et al.*, 1994; Akhter *et al.*, 1995; Nsahlai and Umunna 1996; Macheboeuf *et al.*, 1998). In contrast, using ovine or bovine faeces as sources of inocula for the *in vitro* gas production technique have been shown to effect gas production profiles (Aiple *et al.*, 1992; Harris *et al.*, 1995; Nsahlai and Umunna 1996; Altaf *et al.*, 1998; El Meadaway *et al.*, 1998; Mauricio *et al.*, 1998b) and total VFA concentrations (El Meadaway *et al.*, 1998) of different feedstuffs when compared to ruminal fluid. El Meadaway *et al.* (1998) reported evidence that bovine faeces suffer from low microbial activity due to a lower cellulotic bacterial count when compared to ruminal fluid. Therefore, extensive dilution of faecal fluid (Altaf *et al.*, 1998; Mauricio *et al.*, 1998a & b) might also minimise the density of faecal micro-organisms and subsequently their activity. Increasing the microbial activity of faecal fluid by decreasing its dilution with buffer

up to a minimum (1:2) could be one solution to that problem. Knowledge about the relationship between bacterial DM, bacterial absorbance between faeces and ruminal fluid is lacking and information on differences in VFA production and NDFD between feed samples incubated either in buffered ovine, bovine and equine faeces or ovine ruminal fluid as inoculum is sparse.

The objectives of this study were to investigate the suitability of using ovine, bovine and equine faeces as source of inocula for the *in vitro* gas production technique to replace ruminal fluid. This was assessed by:

1- Comparing the microbial mass between ovine, bovine, equine faeces and ovine ruminal fluid.

2- Comparing gas production degradability parameters, VFA production and NDFD of feed samples incubated either in buffered ovine, bovine and equine faeces or ovine ruminal fluid as inoculum.

6.3 Material and Method

Animals and diet

In a split plot design, faeces were collected from four sheep fed 750g sheep pellets (NDF and CP 338 and 175 g/kg DM) and 250g grass nut (NDF and CP 345 and 150 g/kg DM) twice a day at 8.00 and 17.00 h, steers fed hay (NDF and CP 576 and 110 g/kg DM) *ad libitum* and ponies fed on alfalfa (NDF and CP 538 and 72 g/kg DM) *ad libitum*. Ruminal fluid was obtained from the same sheep via permanent rumen fistula.

Inoculum preparation

A sample of faeces was taken immediately after defecation from each animal following the morning feeding, collected in a plastic bag and transported to the laboratory in a polystyrene box to maintain temperature. In the laboratory, faeces were mixed with buffer medium (Menke and Steingass 1988), which was previously flushed with CO_2 in a proportion of 1:1 (wt/vol) in a kitchen blender (El Shaer *et al.* 1987). The mixture was filtered through two layers of muslin cloth and diluted again with buffer medium by a factor of three (2:1).

A sample of rumen contents containing both solid and liquid material was taken before morning feeding and collected in a prewarmed vacuum flask. In the laboratory ruminal fluid was filtered through two layers of muslin cloth, the solid material was squeezed lightly. The microbial activity of the strained ruminal fluid were tested by measuring the bacterial DM indirectly as described in chapter 2. The

strained ruminal fluid was diluted with anaerobic medium (Menke and Steingass, 1988) until the bacterial absorbance of a 50 fold dilution reached any value between 0.111 and 0.134 in order to ensure a minimum bacterial DM of 0.09 g (Chapter 2 and 3).

Once prepared, the suspension of micro-organisms from either faeces or ruminal fluid was kept at 39 °C with CO_2 bubbling through for approximately 20 min before addition to gas syringes.

Measurement of Bacterial DM

Bacterial DM was measured directly by centrifuging 30 ml of the buffered faecal or ruminal fluid at 1000 X G (to remove protozoa and fungi and particulate matter) for 5 min at 30 °C. The pellet was discarded and the supernatant was recentrifuged at 26000 X G for 15 min at 4 °C. The supernatant was discarded whilst the bacterial pellet was resuspended in NaCl (9g/l) solution and centrifuged again. The supernatant was discarded and the bacterial pellet was dried at 80 °C to constant weight for determination of bacterial DM (Henning *et al.*, 1991). Bacterial DM was also measured indirectly by reading the absorbance at 600 nm (Brown *et al.*, 1989; Wells and Russell, 1996) of faecal or ruminal fluid that had been diluted fifty fold with buffer.

Gas production measurements and analytical procedures

The *in vitro* gas production was performed as described by Jessop and Herrero (1996). In triplicate samples, gas production was measured without added substrate (blank) and from the incubation of 50mg DM D-glucose (Merck Ltd, Merck House, Poole, Dorset, UK), 200mg DM α- cellulose (Sigma, Chemical Co Ltd. Poole, Dorset, UK), ryegrass (*Lolium perenne*), wet season kikuyu grass (*Pennisetum clanestinum*) (Poas Region, Costa Rica) and star grass (*Cynodon nlenfluensis*). The chemical composition of forage samples are given in Table 6.1. The cumulative gas production for each syringe was recorded at 1,2,3,4,5,6,8 h; thereafter every 4 h until 60 h, and then 72, 84, 96 and 120 h. Cellulose and glucose gas production data were fitted to the model Gas = $B(1-exp^{-C(t-Lag)})$ (Krishnamoorthy *et. al.*, 1991) whereas rye, wet season kikuyu grass and star gas production data were fitted to the model Gas = $A\ (1-exp^{-CA(t)})+B(1-exp^{-C(t-Lag)})$ using Marquart algorithm as implemented by GraFit (Leatherbarrow, 1992). A is a measure of the gas produced from the soluble fraction (cm^3), CA is the fractional rate of soluble gas production (/h), B is the asymptotic gas production from the fermentation of NDF (cm^3), CB is the fractional rate of gas production from the fermentation of NDF (/h), C is the fractional rate of gas production from the fermentation of cellulose or glucose, t is time (h) and lag is the time before the fermentation of NDF begins (h).

Volatile fatty acid (VFA) analysis and NDFD measurement

After the final reading of gas production (120 h) for all samples including blanks was taken, a small aliquot of the culture fluid of each sample and the blank (100 µl) was taken and mixed with 25 µl internal standard of 20% orthophosphoric acid solution containing 100 mM of 2-ethylbutirc acid (Tolera *et al.* 1997). The mixture was shaken and centrifuged at 26000 x g for 15 min using a micro centrifuged machine. The supernatant of each sample was transferred to a previously labelled 5 ml capped tube and stored at –18 °C until subsequent volatile fatty acid (VFA) analysis. Analysis for VFA content of the clear supernatant samples was carried out by injecting 1.0 µl into a gas chromatogram (Hewlett Packard 6890, California, USA) fitted with high performance capilliary column (Hewlett Packard, HP-INNOWax (Crosslinked Polyethylane Glycol), Film thickness: 0.25 µm, Length: 30 m). A flow rate of 2 cm^3 nitrogen per minute was used with an initial temperature of 120 °C which was held for a minute after which the temperature was increased at the rate of 10 °C per minute to 265 °C for a further 2 min. Integration of the results were achieved using a personal computer fitted with HP ChemStation Software (Hewlett Packard, California, USA). A standard solution of a mixture of VFAs was used to calculate the concentration of VFAs in the samples. VFA concentration in the samples was obtained after subtracting concentration of VFAs from the blank. VFA parameters were quantified as a total VFA (mmoles / l), and acetate, propionate and butyrate molar proportions (molar %). The remaining final culture and residual forage particles were used to measure NDF residues for the estimation of NDF digestibility (NDFD) using a modified micro technique (Pell and Schofield, 1993).

Table 6.1 Chemical composition of forage samples

Forage sample	CP (g/kg DM)	NDF (g/kg DM)	Ash (g/kg DM)
ryegrass	193 ± 4.4	476 ±16.1	82 ± 7.2
kikuyu (wet season)	259 ± 12.2	534 ± 22.1	127 ± 11.3
star	43 ± 3.4	755 ± 23.8	96 ± 4.8

Statistical analysis

Analysis of variance using Genstat statistical package (1997) was used to compare bacterial DM, bacterial absorbance, blanks' gas volume and estimated gas production degradability parameters between different source of inoculums. Least significant differences were calculated from the standard error of the differences between means.

6.4 Results

Bacterial DM, bacterial absorbance and the blank's gas volume for ovine, bovine, equine faecal fluid and ovine ruminal fluid are shown on Table 6.2. It can be seen that equine faecal fluid had a significantly ($P<0.05$) higher bacterial DM and bacterial absorbance than the other sources of inoculum, followed by ovine faecal fluid, bovine faecal fluid and sheep ruminal fluid respectively. Ovine faecal fluid had a significantly ($P<0.05$) higher blank's gas volume than the other inocula and was followed by ruminal fluid, bovine and equine faecal fluid respectively.

Table 6.2 Microbial activity of different sources of inoculum. . Each value is a mean of four replicates ($n=4$). Means with different superscripts within the same column are significantly different ($P<0.05$).

Source of inoculum	Bacterial DM (mg/ 10 cm^3)	Bacterial Absorbance (A)	Blanks' Gas Volume (cm^3)
Ruminal fluid	94.2^a	0.114^a	17.3^a
Ovine faecal fluid	233.6^b	0.312^b	20.8^b
Bovine faecal fluid	199.7^c	0.247^c	14.5^c
Equine faecal fluid	281.9^d	0.369^d	12.3^d
SED	9.4	0.0039	0.51

The estimated gas production degradability parameters and VFA concentrations of glucose, cellulose, rye grass, wet season kikuyu grass and star incubated either in ovine ruminal fluid (ORF), ovine faecal fluid (OFF), bovine faecal fluid (BFF) or equine faecal fluid (EFF) are presented in Table 6.3 and 6.4. Degradability parameters differed ($P<0.05$) between feed samples and between sources of inocula (Table 6.4). There were also significant ($P<0.05$) interactions

between inoculum and feed samples for all gas production degradability parameters except CA.

Cellulose and star incubated either in ovine ruminal fluid (ORF), ovine faecal fluid (OFF), bovine faecal fluid (BFF) or equine faecal fluid (EFF) had a similar responses in which the asymptotic gas production of cellulose (B) and star (A+B) was significantly ($P<0.05$) lower when incubated in OFF, BFF and EFF than ORF. Also, fermentation of cellulose and star with OFF, BFF and EFF were characterised by a significant ($P<0.05$) longer lag phase (lag) and slower fractional rate of gas production (C, CA and CB) than with ORF.

For glucose, rye grass and wet kikuyu grass no significant differences ($P>0.05$) in the gas production degradability parameters, were observed when OFF was used as inocula, compared to ORF. Whereas glucose, rye grass and wet kikuyu grass incubated in BFF or EFF had significantly ($P<0.05$) lower gas production degradability parameters than ORF and OFF.

There were no significant ($P>0.05$) effects of inoculum on the total VFA concentrations for all substrates and the NDFD of rye grass, wet season kikuyu grass and star but the total VFA concentrations and NDFD varied significantly ($P<0.05$) between feeds.

However, molar proportion of acetic, propionic and butyric acids were significantly ($P<0.05$) affected by inoculum. When OFF, BFF and EFF were used as

sources of inocula for the *in vitro* gas production technique, significant ($P<0.05$) lower acetic and higher propionic acid concentrations, compared to ORF were observed for cellulose and star, while butyric acid concentration was not significantly ($P>0.05$) different between ORF and OFF. But butyric acid concentration was significantly ($P<0.05$) higher for cellulose and star incubated in BFF and EFF than ORF.

There were no significant ($P>0.05$) differences in molar proportion of VFAs of glucose, rye grass and wet season kikuyu grass between ORF and OFF except that wet season kikuyu grass had a significantly ($P<0.05$) lower butyric acid when incubated in OFF than ORF. However, with BFF and EFF an increase ($P<0.05$) in propionic and a decrease ($P<0.05$) in acetic acid concentrations were observed for glucose, rye grass and wet season kikuyu grass. BFF and EFF had higher ($P<0.05$) molar proportion of butyric acid for glucose, and wet season kikuyu grass (EFF only) and significant ($P<0.05$) lower molar proportion of butyric acid for rye grass compared to ORF.

There were significant ($P>0.05$) interactions in molar proportion of acetic and propionic acid between inoculum and feed samples.

Table 6.3 Estimated gas production degradability parameters and VFA concentrations of 200 mg DM cellulose and 50 mg DM glucose incubated either in ovine ruminal fluid (ORF), ovine faecal fluid (OFF), bovine faecal fluid (BFF) or equine faecal fluid (EFF). Each value is a mean of four replicates ($n=4$). For each feed sample, means with different superscripts within the same row are significantly different ($P<0.05$).

Parameter	Cellulose				Glucose				SED
	ORF	OFF	BFF	EFF	ORF	OFF	BFF	EFF	
B (cm^3)	90.0^a	83.6^b	78.6^c	70.3^d	17.5^a	16.9^a	14.7^b	12.3^c	0.92
C (/h)	0.097^a	0.087^b	0.075^c	0.069^d	0.205^a	0.208^a	0.178^b	0.163^c	0.0027
Lag (h)	4.6^a	5.2^b	6.8^c	8.2^d	-1.4^a	-1.5^a	0.4^b	1.5^c	0.34
Total VFA (mmoles/l)	76.2	73.7	74.8	68.7	14.1	15.3	14.9	12.8	5.21
Molar proportions of VFA:									
Acetate	60.3^a	54.4^b	46.7^c	40.3^d	59.6^a	57.7^a	48.4^b	42.6^c	1.76
Propionate	29.6^a	34.8^b	40.8^c	47.9^d	29.9^a	30.7^a	38.7^c	45.9^d	0.87
Butyrate	10.1^a	10.8^a	12.5^b	11.8^b	10.5^a	11.6^b	12.9^c	11.5^b	0.41

$^*P<0.05$, $^{ns}P>0.05$

Table 6.4 Estimated gas production degradability parameters, NDFD and VFA concentrations of 200 mg DM Rye grass, Kikuyu (wet season) and star incubated either in either in ovine ruminal fluid (ORF), ovine faecal fluid (OFF), bovine faecal fluid (BFF) or equine faecal fluid (EFF). Each value is a mean of four replicates (*n=4*). For each feed sample, means with different superscripts within the same row are significantly different (*P<0.05*).

	Rye grass				Kikuyu (wet season)				Star					Effect of		
Parameter	**ORF**	**OFF**	**BFF**	**EFF**	**ORF**	**OFF**	**BFF**	**EFF**	**ORF**	**OFF**	**BFF**	**EFF**	**SED**	**I**	**F**	**I x F**
B (cm^3)	42.6^a	41.9^a	38.4^b	34.8^c	38.6^a	37.2^a	33.2^b	31.7^b	31.6^a	27.4^b	25.0^c	21.8^d	0.92	*	*	*
CB (/h)	0.068^a	0.072^a	0.058^b	0.047^c	0.057^a	0.054^a	0.046^b	0.038^c	0.034^a	0.027^b	0.024^b	0.015^c	0.0027	*	*	*
Lag (h)	3.6^a	3.8^a	4.7^b	6.2^c	5.3^a	5.2^a	6.3^b	8.8^c	8.1^a	8.9^b	10.7^c	14.3^d	0.34	*	*	*
A (cm^3)	18.5^a	19.0^a	15.3^b	13.1^c	8.1^a	8.3^a	6.2^b	4.8^c	5.2^a	$4.7^{a,b}$	4.0^b	4.1^b	0.42	*	*	*
A+B (cm^3)	61.1^a	60.9^a	53.7^b	47.9^c	46.7^a	45.5^a	39.4^b	36.5^c	36.8^a	32.1^b	29.0^c	25.9^d	0.96	*	*	*
CA (/h)	0.314^a	0.303^b	0.259^c	0.267^c	0.451^a	0.462^a	0.411^b	0.384^b	0.490^a	0.455^b	0.446^b	0.422^b	0.018	*	*	
NDFD (g/kg DM)	847	863	849	851	731	727	719	721	456	452	446	446	6.8		*	
Total VFA (mmoles/l)	48.3	50.4	46.1	43.1	36.7	34.3	38.3	33.2	24.9	22.8	25.6	21.7	5.21		*	
Molar proportions of VFA:																
Acetate	64.4^a	65.6^a	56.2^b	51.9^c	65.6^a	66.3^a	58.1^b	55.4^b	71.2^a	66.9^b	62.3^c	60.1^c	1.76	*		*
Propionate	23.1^a	23.5^a	33.0^b	36.1^c	23.6^a	24.1^a	30.4^b	32.2^c	18.4^a	21.5^b	26.4^c	26.2^c	0.87	*		*
Butyrate	12.5^a	11.9^a	10.8^b	12.0^c	10.8^a	9.6^b	11.5^a	12.4^c	10.4^a	$10.6^{a,b}$	11.3^b	13.7^c	0.41	*		

$^*P<0.05$, $^{ns}P>0.05$, ^{I} Inocula, ^{F} Feed, $^{I\,X\,F}$ Interaction between Inocula and Feed.

6.5 Discussion

The primary objective of this study was to determine the feasibility of using ovine, bovine and equine faecal fluid as sources of inocula for the *in vitro* gas production technique to replace ruminal fluid. The results of this study showed (Table 6.2) that the initial microbial mass for ovine, bovine and equine faecal fluid as indicated by the bacterial DM and bacterial absorbance were significantly higher than ovine ruminal fluid but that their blanks gas volume (indirect monitoring of microbial activity) was lower than predicted (Chapter 1 and 2). However, estimating bacterial DM by either taking the absorbance at 600 nm of the inoculum or from the blanks gas volume (Chapter 2 and 3) is a method for predicting microbial density of inocula and not its activity. It may be that the proportion of microbes that are not active is considerably greater in faecal fluid when compared to ruminal fluid.

In addition the results in Table 6.3 and 6.4 indicated that gas production degradability with BFF and EFF is characterised by longer lag time, lower gas production (A, B and A+B) and slower rate of gas production (C, CB and CA) than ORF. The higher levels of propionic acid when BFF and EFF were used as inoculum compared with ORF (Table 6.3 and 6.4) may be responsible for these differences in gas production degradability parameters. Since higher levels of propionate are associated with lower gas production due to the lower CO_2 production associated with this VFA (Wolin 1960). El- Meadaway *et al.* (1998) compared the quantity of cellulolytic bacteria between cattle ruminal and faecal fluid and reported that cattle faecal fluid is associated with a lower cellulolytic bacterial numbers compared to cattle ruminal fluid. They also concluded that the lower initial cellulolytic bacterial

numbers in cattle faecal fluid are likely responsible for the increase in lag time and a lower rate of gas production when faeces from cattle was used as inoculum. Several authors have reported similar differences in the gas production profiles when faeces were used as source of inocula for the *in vitro* gas production technique. Aiple *et al.* (1992) investigated the suitability of a buffered faecal suspension as the inoculum for the *in vitro* gas production technique. They reported that hay, barley and straw incubated in a buffered faecal suspension had a significantly longer lag time and lower rate gas production than ruminal fluid but the total gas production was similar. El- Meadaway *et al.* (1998) also reported a similar trend of gas production profiles using buffered cattle faeces suspension as the inoculum for different feedstuffs. Mauricio *et al.* (1998a) and Altaf *et al.* (1998) compared bovine ruminal liquor and faeces as source of inocula for the *in vitro* gas production technique and reported a significant correlation between the two inoculum for the total and potential gas production but lag phase and rate of gas production from bovine faeces were poorly related to ruminal fluid. Mauricio *et al.* (1998b) examined the suitability of using bovine faeces as source of inocula for the *in vitro* gas production technique to replace ruminal fluid. They found that feedstuffs incubated in buffered bovine faecal fluid had a significantly longer lag time and lower rate and total gas production than those incubated in buffered bovine ruminal fluid. In contrast, Harris *et al.* (1995) studied the use of dairy cow faeces rather than ruminal fluid in gas production technique for assessing digestion kinetics *in vitro*. They found that the rate of gas production of winter beans and spring barley grain, and the total gas production of soyabean and rapeseed were significantly greater when incubated in a buffered cow faeces than ruminal fluid but in this study correction of blanks gas volume was not mentioned.

The discrepancy between studies could be due to differences in the initial microbial mass of ruminal fluid.

However, the results of this study indicate that OFF has the potential to replace ruminal fluid as source of inocula for the *in vitro* gas production technique. This conclusion was reached when glucose, ryegrass and wet season kikuyu grass incubated in OFF had similar gas production kinetics and VFA concentrations to that incubated in ruminal fluid. This is in agreement with Aiple *et al* (1992) who reported that faeces from sheep was superior to that of faeces from cattle and pigs to be used as source of inoculum to replace ruminal fluid for the *in vitro* gas production technique. Nsahlai and Umunna (1996) also reported that gas production using sheep faeces inoculum was strongly correlated to gas production using sheep ruminal fluid inoculum particularly at 48 h of incubation. In contrast, Goncalves and Borba (1996) compared gas production profiles of oatfeed, perennial ryegrass and Italian ryegrass between sheep ruminal and faecal fluid and reported that the utilisation of suspension of sheep faeces gives a lower gas production profiles than sheep ruminal fluid.

For a dense and active microbial population in the hindgut and subsequently in the faeces, the diet of donor animals should provide enough fermentable substrate for the lower digestive tract (Aiple *et al.* 1992). Therefore, differences in the gas production profiles between ovine, bovine and equine faecal fluid could be attributed to the differences in their diets. It could be that pellets from sheep faeces provide a more stable environment for the microflora to survive than viscous faeces from cattle and equine. Aiple *et al.* (1992) studied the effect of the diet of host animal (sheep) on

the gas production after 48 h of incubation using faeces from animals kept on four different diets and reported that differences in the quality of donor animal diet can led to different results and recommended the use of diet with 40% concentrate and 60% hay.

The results also indicate that gas production profiles when OFF was used as source of inocula were variable among feed samples where glucose, ryegrass and wet season kikuyu grass incubated in OFF had a similar gas production kinetics and molar proportion of VFA concentration to that incubated in ORF. Whereas cellulose and star grass incubated in OFF had a significant differences in gas production degradability parameters and compared to those incubated in ORF. This might imply that faeces micro-organisms need a quick source of energy such as sugar to survive and optimise their activity and that was not either available in cellulose or enough in star grass. This might suggest a pre-incubation with a known amount of a quickly fermentable carbohydrate is needed for faecal fluid in order to maximise its microbial activity particularly if the purpose of using faecal fluid as source of inocula for the *in vitro* gas production technique was to investigate gas production dynamics of low quality feedstuffs.

The results in Table 6.3 and 6.4 also show that there were no significant differences in NDFD and total VFA concentration between inocula. However, several authors have reported a significant positive correlation between *in vitro* and *in vivo* DM or OM digestibility when either ruminal or faecal fluid were used as source of inocula (El Shaer *et al.*, 1987; Omed *et al.*, 1989; Aiple *et al.*, 1992; Akhter

et al., 1994; Akhter *et al.*, 1995; Nsahlai and Umunna 1996; Altaf *et al.*, 1998; El Meadaway *et al.*, 1998; Mauricio *et al.*, 1998a; Macheboeuf *et al.*, 1998; Lowman *et al.*, 1999).

6.6 Conclusion

The results of this study indicate that ovine, bovine and equine faeces have potential as a source of inocula instead of ruminal fluid for the *in vitro* gas production technique. Ovine faeces maybe a more suitable alternative to ruminal fluid than bovine and equine faeces. However, further research is needed to improve the activity of faecal micro-organisms.

Chapter 7

General discussion

7.1 Importance of accurate estimation of gas production degradability parameters

The consumption of feed by ruminants is the first step in the process that converts feed into valuable products such as meat, milk and wool for human consumption or use. One of the biggest challenges to ruminant nutritionist has been to quantitatively predict food intake and the consequential supply of nutrients to the animal which are the most important factors determining animal performance. Chemical characterization of ruminant feeds into various fractions alone is not sufficient to provide quantitative information about nutrients supplied to the animal and available for production. Information concerning the rate and extent of digestion is required in order to predict the amount and type of nutrients that can be consumed and used by the animal (Mertens, 1993). Therefore, knowledge on rumen degradation characteristics of feed organic components is necessary and essential. The Cornell Net Carbohydrate and Protein System is already using this type of information to evaluate ruminant feeds.

Simulation models have been used to integrate knowledge of processes of ruminal degradation, digestion and passage of feed to enable prediction of both the pattern of nutrients supply from rumen fermentation (Jessop, 1999) and feed intake (Mertens and Ely 1979; Forbes, 1980; Fisher et al., 1987; Illius and Gordon 1991; Poppi et al., 1994; Jessop, 1999). Prediction of both nutrient supply and feed intake by simulation models has been based on the knowledge of feed chemical

composition, degradation parameters and disappearance rate from the rumen by fermentation and absorption or passage to abomasum.

The *in vitro* gas production technique has the potential to characterize ruminant feedstuffs. In this thesis, several factors have been found to effect the pattern of gas production and hence the gas production degradability parameters. It is important to know to what extent underestimation or overestimation of gas production degradability parameters might influence the prediction of nutrients supply and feed intake. Consequently, each of the gas production degradability parameters (A, B, C and Lag) for ryegrass, wet season kikuyu grass and 3rd stage of FC2 *B.brizantha* leaf were varied by ± 15% sequentially with other factors and parameters being held constant, using the simulation model introduced by Jessop (1999). The sensitivity of predicted energy and protein supply and of DM intake for 50 kg sheep to this variation is shown in Table 7.1. It can be seen that decreasing or increasing **A** and **B** values by 15% mainly affected the sensitivity of predicting energy and protein supply whereas a 15% change in **C** and **Lag** influenced the sensitivity of predicting energy supply, protein supply and DMI. However, attempts have been made to integrate various factors that might influence forage intake in ruminants and some such models (Illius and Gordon 1991; Poppi et al., 1994) have undertaken a sensitivity analysis of intake to components of their models. Poppi et al., (1994) show that the fractional degradation rate of the NDF or cell wall fraction exerts a large influence in intake (in cattle) with a change of 0.01 (/h) altering predicted intake by 630 g DM day^{-1} when other factors were held constant. Illius and Gordon (1991) show that the composition of forage in terms of cell content (i.e.

soluble material) to cell wall (i.e. insoluble material) exerts a large effect on prediction of intake.

The above investigations show the importance of obtaining accurate estimates of forage degradability parameters. Therefore, caution should be taken if the *in vitro* gas production technique was use to determine the degradability parameters of ruminant feedstuffs. Since the results of this thesis indicated that lowering microbial concentration of ruminal fluid by extensive dilution with buffer media (chapter 2), frequently sampling of ruminal fluid (chapter 2) and the diets of donor animals (chapter 3) resulted in underestimation of gas production degradability parameters. Also, the results showed that physical form of feed sample incubated in the *in vitro* gas production technique might under or over estimate the gas production degradability (chapter 4). Taking the rate of gas production as an example, the results showed that extensive dilution with buffer media reduced hay gas production by 16% (chapter 2), frequent sampling of ruminal fluid resulted in underestimation of cellulose gas production by 34% (chapter 2) and changing the diets of host animals by increasing the ratio of hay to concentrate reduced bacterial concentration and hence decreased the rate of gas production of hay by 18% (chapter 3). Moreover, the results of this thesis indicated that using a dry 1mm ground sample of red fescue underestimated the rate gas production for the fresh red fescue by 22% (chapter 4).

However, maximising feed intake and nutrient supply to the animal is desirable in ruminant nutrition. This can be achieved by correcting the balance of

nutrient supply to rumen microbes such as ammonia concentration (chapter 5) and subsequently improving the degradability of foods.

Table 7.1 Effect of 15% decrease or increase in the estimation of each gas production degradability parameters (A, B, C and Lag) of ryegrass, wet season kikuyu grass and 3rd stage of FC2 *B.brizantha* leaf on the sensitivity of predicting energy, protein supply and feed DM intake (DMI) for 50 kg sheep using the simulation model of Jessop (1999).

Forage sample	Degradability parameters	Sensitivity of predicting*		
		Protein supply	Energy supply	DMI
Ryegrass	A	0.67	0.64	0.34
	B	0.27	0.70	0.39
	C	0.52	0.59	0.70
	Lag	0.54	0.56	0.61
Kikuyu (wet season)	A	0.58	0.61	0.38
	B	0.57	0.63	0.47
	C	0.59	0.52	0.67
	Lag	0.67	0.63	0.59
B.brizantha	A	0.63	0.58	0.35
	B	0.53	0.67	0.41
	C	0.51	0.60	0.65
	Lag	0.44	0.51	0.47

*Sensitivity was calculated as the % change in response (protein or energy supply or DMI), divided by % change in each of gas production degradability parameters (A, B, C and Lag).

7.2 Standardising microbial activity of ruminal fluid

One of the objectives of this thesis was to standardise the level of microbial activity of ruminal fluid. Achievement of this was made possible by the strong relationship observed between the bacterial DM content of ruminal fluid and its absorbance (chapter 2 and 3). Use of all data collected during this project (reported in chapters 2, 3 and 6) gives the relationship shown in Figure 7.1. Bacterial DM was linearly related ($R^2 = 0.99$, $p<0.001$) to bacterial absorbance such that:

Bacterial DM (mg / 10 cm^3 of strained rumen fluid) =

865 (s.e ± 3.1)* Bacterial Absorbance (A)

The slope of this regression equation is very similar to those obtained in chapter 2 (868) and 3 (863). This relationship suggested that bacterial concentration of ruminal fluid can be estimated from its absorbance at 600 nm which could be a good way of monitoring the microbial activity of ruminal fluid to improve the reproducibility and reliability of the *in vitro* gas production technique within and between laboratories. However, it was observed that the minimum initial bacterial DM needed for the *in vitro* gas production technique is 0.09 g/10 cm^3 (chapter 2 and 3), hence caution should be taken in diluting ruminal fluid particularly for those research groups (Waghorn and Stafford, 1993; Pell and Schofield, 1993; Theodorou *et at.*, 1994; Davies *et at.*, 1995; Mauricio *et at.*, 1999) who dilute ruminal fluid extensively with buffer media. Also, diluting ruminal fluid by a factor of three (Menke *et at.*, 1979; Beuvink *et at.*, 1992;Cone *et at.*, 1996) is not always the case of getting a high microbial concentration and mimicking the *in vivo* fermentation (chapter 2).

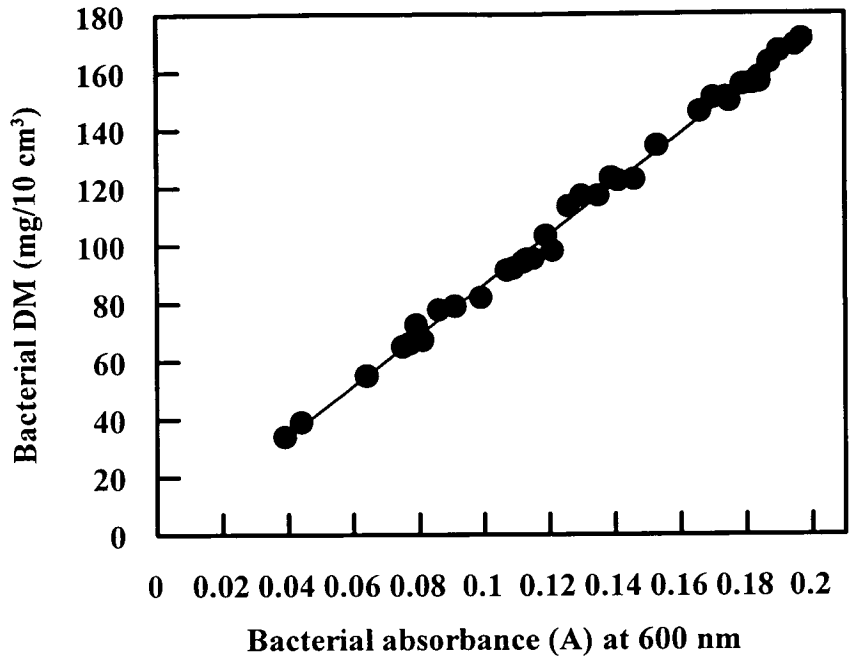

Figure 7.1 The relationship between bacterial DM mg / 10 cm^3 of strained rumen fluid and bacterial absorbance A (n=36).

Furthermore, it was observed that bacterial DM was strongly related to the volume of gas produced in incubation with no added substrate (blanks gas volume) (chapter 2 and 3) suggesting that the quantity of bacterial DM in ruminal fluid can be estimated from blanks gas volume. In a similar manner to above, ruminal fluid bacterial DM and blanks gas volume measured in this thesis (chapter 2, 3 and 6) were used to produce a regression equation with large number of replicates (Figure 2). The regression equation obtain is:

Bacterial DM (mg / 10 cm^3 of strained rumen fluid) =

6.06 (s.e ±0.049)*Blanks' Volume (cm^3).

The slope obtained from the above equation is not much different from those (5.89, chapter 2 and 6.34, chapter 3) obtained earlier in the thesis.

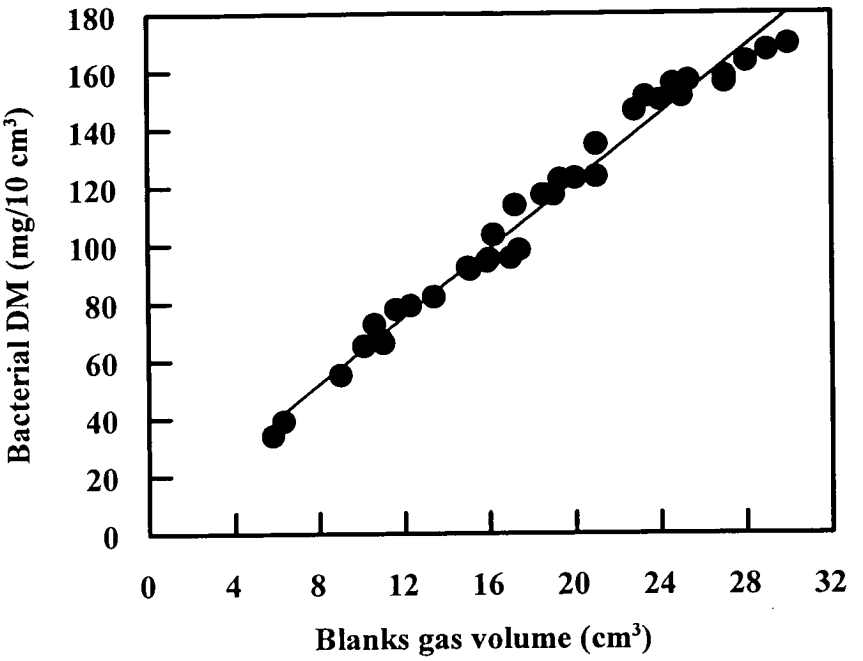

Figure 7.2 The relationship between bacterial DM mg / 10 cm^3 of strained rumen fluid and blanks gas volume (n=36).

7.3 The use of ovine, bovine and equine faeces as sources of inoculum for the *in vitro* gas production technique

The use of ruminal fluid as inoculum for the *in vitro* gas production technique requires fistulated animals which are expensive to maintain and not favoured by animal rights organizations. Therefore, an alternative source of inoculum for the *in vitro* gas production technique would be desirable. The results of this thesis (chapter 6) showed that faeces from ovine, bovine and equine have the potential to be used as sources of inoculum for the *in vitro* gas production technique in place of ruminal fluid. The results also indicated that faeces from ovine were superior compared to that of faeces from bovine and equine and could be used as source of inoculum for the *in vitro* gas production technique to estimate the degradability of good quality

forage. Use of ovine, bovine or equine faeces as inoculum this has some potential but needs further development.

7.4 Conclusions

Results from this thesis indicated the following points:

1- Dilution of ruminal fluid extensively with buffer media resulted in underestimation of the pattern and degradability parameters of gas production without altering NDFD.

2- Daily sampling of ruminal fluid decreased the initial bacterial concentration and subsequently affected the kinetics of gas production.

3- Variation in the ratio of hay to concentrate in the diet of the donor animal led to a change in the initial bacterial and gas production degradability parameters.

4- Standardisation of the level of microbial activity between days could be achieved by estimating the bacterial DM before the incubation indirectly from the absorbance of ruminal fluid and a minimal bacterial DM of 0.09 g/10 cm^3 was recommended.

5- Physical form of feed sample incubated *in vitro* using gas production technique influenced the pattern of gas production.

6- The *in vitro* gas production technique can be used to study protein-energy interaction and a minimum nitrogen concentration of 100 mg/l requires achieving the maximum degradation of NDF.

7- Ovine, bovine or equine faeces have the potential to be used as source of inoculum for the *in vitro* gas production technique to replace ruminal fluid.

7.6 Recommendations

1- Dilution of ruminal fluid with the buffer media should be set according to initial bacterial DM and a minimal bacterial DM of 0.09 g/10cm^3 is recommended. This can be done by estimating the bacterial DM indirectly from the bacterial absorbence of the rumen fluid.

2- Ruminal fluid should not be taken frequently from the same animal. Allowing an interval of two days between samplings is recommended to enable rumen microbial level to recover.

3- The ratio of about 40% concentrates in donor animal diet is recommended to be a minimum ratio that should be offered to the donor animal in order to obtain sufficient microbial matter for gas production technique (although it should be remembered that studies *in vivo* have shown that increasing the ratio of concentrate to above 70% had reduced cellulolytic activity within the rumen).

4- The indirect estimation of bacterial DM from bacterial absorbance of ruminal fluid should be used for monitoring the effect of donor diet and its ability in providing enough microbial matter for the *in vitro* gas production technique.

5- Macerated fresh forage sample is recommended for the purpose of studying the fermentation dynamics or to evaluate the nutritional value particularly for grazing animals when using the *in vitro* gas production technique.

6- To investigate the effect of limiting the availability of nutrients on the fermentation dynamics of feed, the potential endogenous contribution of the specific nutrient under study should be considered. Harvesting ruminal microbes by centrifugation is recommended for such investigations since it has not altered the pattern of gas production.

7.6 Possibilities for future research

In view of the results obtained from this thesis, the following section describes the avenues of research, which are potentially important and may provide valuable and interesting results. These avenues include:

1) The *in vitro* gas production technique is recognised as being of potential value in characterisation of ruminant feedstuffs. The variation in gas production profiles between days of this method has been shown in this work to be minimised by standardising the level of microbial activity. However, each laboratory has developed its own method and procedure for obtaining a gas production profiles. Quite substantial variation between laboratories has been observed (Rymer et al., 1998). It would be important to investigate the extent to which such variation could be reduced by adoption of this technique to standardise microbial activity.

2) Evaluating the nutritional value of grazing animal forages using the *in vitro* gas production technique require the use of fresh sample. Crushing samples prior to the *in vitro* gas production incubation does not effectively mimic mastication by the animal. Therefore, research in sample preparation and developing a method that mimics ruminant mastication is urgently needed.

3) The major disadvantage of using ruminal fluid is the requirement for fistulated animals. It has been shown that faeces have the potential to be used as source of micro-organisms for the *in vitro* gas production technique to replace ruminal fluid (chapter 6). However, faeces usually give a longer lag phase and a

lower rate of gas production which could be due to their lower microbial activity. A pre-incubation of quickly fermentable substrate for a specific length of incubation still need to be tested which might improve faeces microbial activity.

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Appendix

A.1 General Material and Methods

In vitro gas production determination

This section describes methods and materials used. In brief, the *in vitro* gas production method was used in all cases studied in this thesis. Full details about this technique are given below.

The preparation of forage samples

All forage samples that used in this thesis (except chapter 4) were first dried for 24 hours at 60^{o}C and then milled through a screen width of about 1.0 mm for gas production tests.

Gas production test

Hohenheim method was used in this thesis to determine gas production according to the methdology described by Menke & Steingass (1988).

1- Equipment

a. Water bath with ventilator, accuracy approx. ±1^{o}C.

b. A perspex lid fitted with holes to hold the syringes in the water bath as a modification by Blümmel & Ørskov (1993).

c. Glass syringe (100 ml, Fortuna, Germany), fitted with a plunger, silicone tube and plastic clip.

d. Precision balance.

e. Steel container with carbon dioxide.

f. Filling equipment for rumen fluid and buffer solutions which consist of:

g. - Automatic pipette

h. - Winchester (3 litres)

i. - Magnetic stirrer.

2- Reagents

- Disodium hydrogen phosphate (Na_2HPO_4).

- Potassium dihydrogen phosphate (KH_2PO_4).

- Magnesium sulphate ($MgSO_4 . 7H_2O$)

- Sodium hydrogen carbonate ($NaHCO_3$).

- Ammonia hydrogen carbonate ($(NH_4)HCO_3$).

- Calcium chloride ($CaCl_2 . 2H_2O$)

- Manganese chloride ($MnCl_2 . 4H_2O$)

- Cobalt chloride ($CoCl_2 . 6H_2O$)

- Iron chloride (Fe Cl . $6H_2O$)

- Resazurin

- Sodium hydroxide (1M NaOH)

- Sodium sulphide ($Na_2 S . 7H_2O$)

- Vaseline (Paraffin, soft white).

3- Preparation of the solutions.

The solution was made up by the following procedure:

(1). Main element solution: 5.7 g Na_2HPO_4 + 6.2 g $KHP0_4$ + 0.6 g $MgSO_4$.$7H_2O$ in 1 litre distilled water.

(2). Trace element solution: 13.2 g $CaCl_2$. 2 H_2O + 10.0 g $MnCl_2$. $4H_2O$ + 1.0 g $CoCl_2$. $6H_2O$ + 0.89 $FeCl_2$. $6H_2O$ in 100 ml distilled water.

(3). Buffer solution: 35 g $NaHCO_3$ + 4 g $(NH_4)HCO_3$ in 1 litre distilled water.

(4). Resazurin solution: 100.0 mg resazurin in 100 ml distilled water.

(5). Reduction solution: 2 ml 1-M-NaOH + 285 mg Na_2S . $7H_2O$ in 47.5 ml distilled water.

These solutions were made up and stored except the reduction solution (5) which was prepared each time shortly before the incubation.

Depending on the number of syringes (see Table A.1), the solutions were poured into a Winchester and incubated in a water bath at 39°C.

Carbon dioxide gas was flushed through this mixture, then the reduction solution (5) was added. When the mixed solution became to colourless, the rumen fluid (strained through muslin) was added in the ratio of 2 parts buffer medium to 1 part rumen fluid. The mixture was kept under CO_2 gas in a water bath at 39^oC and stirred by magnetic stirrer.

Table A.1 Ruminal fluid buffer medium preparation for the in vitro gas production technique based on a dilution factor of three (1:2 v/v).

	Number of Syringes	Buffer Media					Redaction Solution		
Ruminal fluid : Buffer Media (1:2 v/v)		Distil water ml	Trace elements ml	Buffer solution ml	Main elements ml	Resazurin ml	NaOH 1M ml	Sodium Sulphide mg	Distilled water ml
10 : 20	1	10	0.003	5	5	0.03	0.04	5.7	0.95
100 : 200	10	100	0.03	50	50	0.26	0.4	57	9.5
150 : 300	15	150	0.04	75	75	0.39	0.6	86	14
200 : 400	20	200	0.05	100	100	0.51	0.8	114	19
300 : 600	30	300	0.08	150	150	0.77	1.2	171	28.5
400 : 800	40	400	0.1	200	200	1.03	1.6	228	38
500 : 1000	50	500	0.13	250	250	1.29	2	285	47.5
600 : 1200	60	600	0.15	300	300	1.54	2.4	342	57
700 : 1400	70	700	0.18	350	350	1.8	2.8	399	66.5
800 : 1600	80	800	0.2	400	400	2.06	3.2	456	76

4- Procedure

Approximately 200 ± 5 mg of the sample was accurately weighed into a 100 ml glass syringe, the plungers were lubricated with a little vaseline to ease the sliding of plungers and prevent leakage of gas. The syringes were prewarmed to 39^{o}C before the injection of 30 ± 2.0 ml of buffer mixture: rumen fluid (which was prepared acording to the guidelines procedure for standardising the initial microbial concentration desribed in figure 2.4) into each syringe by fixing the silicone rubber tube that connected with the 'needle' of the syringes to the automatic pipette. The plastic clip which was fixed on the silicone tube was closed.

The position of the plunger was read and the initial volume and time was recorded. Then the syringes were gently shaken, followed by incubation in a water bath (39 ± 1^{o}C).

Gas production was recorded and the syringes were shaken at 1,2,3,4,5,6,8 h; thereafter every 4 h until 60 h, and then 72, 84, 96 and 120 hours of incubation period.

If the reading of gas volume was to exceed 60 ml, the reading was recorded, the tube clip opened and the plunger was returned vertically to the 30 cm^{3} mark. Then the incubation was continued.

To correct the gas volume which was produced from the fermentation of the ingredients in the rumen fluid, three blanks (syringes containing rumen fluid, buffer

mixture without feed sample) were used for each treatment. The mean gas production value of these blanks were deducted from the recorded gas production of all samples.

Ammonia concentration and pH measurements

Ammonia (chapter 5) and pH concentration (chapter 3) of the mixture of rumen fluid and buffer media were determined by using ammonia and pH electrode machine.

Analytical methods of the residues in the syringes after incubation

NDF determination

The incubated material in the syringe was transferred into a 75 ml conical flask. Then the syringes were washed twice with 5 ml NDF solution (to remove the remaining material which attached the internal wall of the syringe). The flasks were covered by plastic film, then stored in a fridge until analysed. Then the flask content was transferred into a 100 ml bottle fitted with cup and washed by 20 ml of NDF solution and then autoclaved at 105^{o}C for 1 hr (Pell and Schofield, 1993). The content the bottle was filtered through a pre-weighed dry sintered glass crucible using a filtration apparatus and washed 3 times with hot distilled water. The crucible contents were washed with ethanol and acetone. The crucibles and their contents were oven-dried at 100^{o}C for 24 hours. The crucibles were cooled in a dessicator, then re-weighed. The NDF was determined by weight difference.

Analytical methods of the forage samples

A) *Ash determination*

Approximately 3g of the dried samples were accurately weighed into pre-weighed dry crucible and ashed in a muffle furnace at 500^{o}C overnight. The crucibles were cooled in a dessicator, then re-weighed and the ash content was calculated.

B) *Determination of Neutral Detergent Fibre (NDF)*

NDF was determined according to the method of Goering and Van Soest (1970). This method is defined below :

One g of sample was accurately weighed into a 250 cm^3 beaker. 50 cm^3 of neutral detergent solution was added and heated to boiling and boiled gently for 30 minutes. The beaker was removed from the heat, and then 50 cm^3 of cold neutral detergent solution and 4 cm^3 of a-amylase solution was added, heated and boiled gently for a further 30 minutes. Then the above mixture was filtered immediately through a sintered glass crucible and washed twice with hot distilled water, using a filtration apparatus. After that the sintered crucible was placed in the crystallising basin and a mixture of 30 cm^3 hot distilled water and 4 cm^3 a-amalase was added. Then the crucible was returned to the holder and filter, and the sample was washed twice with hot distilled water and twice with acetone. After, the crucible and its contents were dried in an oven at 100 ^{o}C for 24 hours. Then the crucible and its contents were cooled in dessicator and weighed (W1). Finally, the crucible and contents were ashed in a furnace at 500 ^{o}C for 3 hours and then returned to the 100 ^{o}C oven. The crucible and contents were cooled in a dessicator, and then re-weighed (W2). The loss in

weight on ignition (W1-W2) x 1000 was the neutral detergent fibre of 1g sample (NDF g/kg^{-1}).

C) *Crude Protein (CP) determination of forage samples*

The crude protein (NX6.25) content in forage samples was determined by the Kjeldahl method.

A.2 Publication arising from work in this thesis

Nagadi, S., Herrero, M. and Jessop, N. S. 1998. A comparison of the gas production profiles of fresh and dry forage. *Proceeding of the British Society of Animal Science*: pp. 162 (abstract).

Nagadi, S., Herrero, M. and Jessop, N. S. 1999. Effect of frequency of ovine ruminal sampling on microbial activity and substrate fermentation. *Proceeding of the British Society of Animal Science*: pp. 154 (abstract).

Nagadi, S., Herrero, M. and Jessop, N. S. 2000. The influence of microbial activity on the pattern of gas production. *Animal Science*, (submitted).

Nagadi, S., Herrero, M. and Jessop, N. S. 2000. The influence of diet of the donor animal on the initial bacterial concentration of ruminal fluid and gas production degradability. *Animal Feed Science and Technology* (submitted).

Nagadi, S., Herrero, M. and Jessop, N. S. 2000. The effect of fermentable nitrogen availability on the gas production degradability of NDF. *Animal Feed Science and Technology* (submitted).