IN VITRO ASSESSMENT OF THE NUTRITIVE VALUE OF MIXTURES OF LEAVES FROM TROPICAL FODDER TREES

Mauricio Rosales M

Wolfson College
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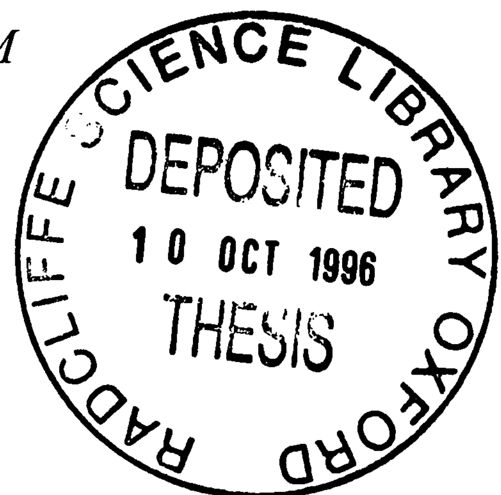

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"It is a humbling fact for grass pasture experts to realize that probably more animals feed on shrubs and trees, or on associations in which shrubs and trees play an important part, than on true grass or grass-legume pasture, short- and tall-grass ranges and steppes." (Imperial Agricultural Bureaux, Joint Publication No. 10, June 1947).

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I dedicate this thesis to the memory of my late father Dr Luis A. Rosales and grandfather Dr Luis O. Rosales.

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Abstract

Previous work in animal nutrition has focused on single feeds and assumed additivity in ration systems. In the tropics, farmers are likely to feed mixtures of feeds, including tree fodders, which may not be simply additive in nutritional terms. This study has increased our understanding of the mechanisms that determine the associative effects on the *in vitro* fermentation of mixtures of fodder tree leaves. Associative effects are governed by a synchronisation in the fermentation rates of the components of the mixture. This is in turn dependent on the fermentability of their chemical constituents. Effects were demonstrated by changes in the fermentation kinetics of gas production curves.

The chemical components of fodder tree leaves that affect the fermentation, and the time at which the effect occurs, were identified, using two media of different nitrogen contents.

The fermentation of mixtures of pure chemical entities in various combinations was then examined. The greatest associative effects were found when the mixture had components of similar fermentability. It is proposed that associative effects are a function of the synchronisation of fermentation of the different components and was shown to occur at the point when the rate was maximal.

With two types of protein (casein and bovine serum albumin (BSA)), utilisation of a protein by rumen microbes was shown to be a function of its fermentability and not of its solubility. This is also influenced by the type of associated carbohydrate.

Fodder tree leaves were then combined with different pure chemical entities. Associative effects between fodder tree leaves and carbohydrates were shown to occur and the responses were similar to those obtained with mixtures of pure carbohydrates and proteins.

The effect of tannins and phenolic compounds was studied using quebracho tannin as a model, and in five of the tree species. They were shown to affect the fermentability of both carbohydrates and proteins. The effect was greater with carbohydrates of medium to low fermentability. They also reacted with both soluble and insoluble protein. Forages with phenolic compounds showed both positive and negative effects. The effects were possible due to a synchrony or asynchrony in the release of protein.

In mixtures of leaves from different species, associative effects were related to their fermentability. Again, this appeared to be the result of the synchronisation of the release of nutrients. Associative effects with fodder tree leaves were of a composite nature and can be both positive and negative.

The implications of these findings in relation to *in vivo* digestion and animal production are discussed. Due to the diversity of fodder trees, there is the potential to develop feeding systems based on mixtures which make better use of available resources. This will also contribute to improved efficiency in the management and use of natural resources, and take advantage of natural plant diversity in the tropics.

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Chapter 1

GENERAL INTRODUCTION

In recent years, the scientific world has conceived the terms "biodiversity" and "megadiversity" to refer to the huge genetic reservoir in natural ecosystems. The tropical zone contains the greatest genetic diversity in the world, diversity which is expressed in the large number of vascular plants per unit area (McNeely *et al.*, 1990; Reid and Miller, 1989). However, this richness is being threatened by the pressure imposed by the increasing population. Of particular concern is the process of deforestation and its irreversible ecological effects (Myers, 1989). The major causes of deforestation are conversion of forest to agricultural land and cattle grazing, logging and demand for fuelwood. Recently, a range of environmentally beneficial farming practices seems to be emerging as a synthesis based on both old, proven ideas and a new understanding of natural nutrient cycles and ecological relationships (WRI, 1992). There are several examples of this new generation of farming systems in the tropics where multipurpose trees play a critical role in the sustainability of the system, by supplying protein for livestock, firewood, and sinks for carbon dioxide and controlling erosion (Murgueitio, 1990; Preston and Murgueitio, 1992; Moog, 1992b; Devendra, 1993; Wilson, 1995).

A recent review of feeding systems used in warm climates (Roggero *et al.*, 1996) suggested that sustainability depends on making use of diverse local biological resources. This concept calls for wider use of the diversity of fodder tree species as providers of animal forage. Despite the fact that the list of trees and shrubs with potential use as fodder comprises more than 300 species, the emphasis has been placed on very few. The danger of this over-dependence on so few species is illustrated by the psyllid epidemic (*Heteropsylla cubana*) in *Leucaena leucocephala* (Moog and Sison, 1986; NFTA, 1987; Moog, 1992a), and the disappearance of some valuable fodder species, e.g., *Terminalia avicennioides* in Niger, due to its replacement with *Gliricidia sepium* (Baumer, 1992). Given the diversity of fodder trees, there is an urgent need to study and recommend promising species for specific agro-ecological environments and animal production systems, both in terms of plant productivity and nutritional value.

Fodder trees have always played a significant role in feeding domestic animals. Until relatively recently, these feed resources have been generally ignored by scientists because of inadequate knowledge of their potential use and the lack of initiatives to develop more innovative systems of feeding (Devendra, 1992). The conventional approach to fodder trees is to study and exploit "single"

species. The reality is that, in many parts of the tropical world, animals eat or are fed with "mixtures" of tree leaves. Cafeteria trials have been widely used to determine relative palatability differences within different plant species. Apart from showing the animal's preferences for particular fodder species, they also show that, given the opportunity, ruminants will feed on mixtures of forages. Mixed foliage can be given as a supplement to rice straw and others crop residues or may constitute the whole ration. In Nepal and Indonesia, farmers traditionally feed a mixture of fodder tree leaves to their animals (Paudel and Tiwari, 1992; Rangkuti *et al.*, 1990). This practice has been observed throughout developing countries, especially with small ruminants (Devendra and Pun, 1990; Gill and Powell, 1993).

There is negligible published information about the reasons why farmers feed mixtures and the choice of appropriate mixtures currently relies on the farmers' traditional knowledge. Paudel and Tiwari (1992) reported that the great diversity of species used in Nepal may be explained by the improved nutritive value of a combination of species compared to single species on their own. For Devendra and Pun (1990), the underlying reasons are associated with reducing the toxic effects associated with a particular feed and increasing the variety and palatability of the diet. These interpretations highlight two important aspects of the nutritive value of mixtures of fodder trees: the deleterious factors and the associative effects.

Plants contain more than 1200 different classes of chemical compounds that are produced by secondary metabolism. Most of these compounds have storage, defence or reproductive functions in the plants. Many of them appear to be the natural result of the co-evolution of plants with herbivorous mammals, although some have probably evolved as mechanisms of protection against insect pests and plant diseases, in which case their effects on higher animals may be coincidental (Paterson, 1993). About 8,000 polyphenols, 270 non-protein amino acids, 32 cyanogens, 10,000 alkaloids and several saponins have been reported to occur (Liener, 1980; Kumar, 1992a). Tannins are the most common secondary plant compounds, but the implications for animal feeding are not entirely clear, with both harmful and beneficial effects possible (Waterman, 1988; Rosales *et al.*, 1989; Mueller-Harvey and McAllan, 1992; Gottlieb, 1992). A major characteristic is their propensity to form chemical complexes. Recent studies have revealed that tannins not only bind strongly with proteins but also with many other compounds like polysaccharides, nucleic acids, steroids, alkaloids and saponins (Mueller-Harvey and McAllan, 1992). It has been suggested that using mixtures of fodder plants with variable tannin levels improves nitrogen utilization by ruminants (by reducing soluble protein degradation in the rumen) (Barry, 1988; Waghorn *et al.*,1987; Lascano and Palacios, 1993) and dilutes the effect of the deleterious compounds (Lowry, 1990).

In addition, the amount of nutrients which a ruminant can extract from one feed can be modified by the type and quantity of other feeds consumed the same day (Gill and Powell, 1993). These interactive processes can have substantial consequences for intake and digestibility of feeds and on animal performance, in general. Associative effects between components of a mixed diet occur when, as a consequence of the interactive processes, the nutritional value of the mixture is not equal to the sum of its individual components. These effects can be positive or negative. Evidence for associative effects was described at the beginning of the century (Ewing and Wells, 1915) and although they have been studied, there is little information concerning their mode of action. Most of the studies leading to an understanding of the mode of action of the associative effects relate to the effect of a source of rapidly fermentable carbohydrates (like barley or maize silage) on roughage digestion (Mould *et al.*, 1983a; Moss *et al.*, 1992; Margan *et al.*, 1994). There are some studies of the effect of mixtures of temperate (Cassida *et al.*, 1994; Glenn, 1989) and tropical (Brown and Pitman, 1991) grasses and legumes but, so far, there are no studies on mixtures of fodder tree leaves.

Given the diversity of fodder trees and their complex chemistry, there is a potential to develop feeding strategies based on strategic mixtures that result in "enhanced" nutritive value. This can be achieved by capitalising on the interactive processes, such as:

- protecting dietary protein with natural tannins in order to increase the amount of nitrogen which by-passes the rumen;
- diluting the effects of deleterious compounds;
- inducing associative effects that result in an increased voluntary intake; and
- inducing associative effects on digestibility between the components of the diet.

The aim of this work is to study the nutritive value of mixtures of fodder tree leaves in order to begin to understand the factors that determine their associative effects and the interactions between tannins and other feed components. This will provide the basis for predicting appropriate mixtures of tree foliage that result in overall improvement in nutritive value and ultimately in a more diverse supply of high quality tree fodder for ruminants in the tropics.

Chapter 2

LITERATURE REVIEW

2.1 NUTRITIVE VALUE AND FEEDING VALUE

There are no clear definitions in the literature of the terms 'nutritive value' and 'feeding value'. Sometimes there is disagreement between authors and very often the terms are regarded as synonymous. For the purposes of this thesis, the nutritive value of a forage is its potential to contribute to the nutrient supply of the animal. This is a function of its chemical composition and its digestibility. The chemical composition comprises both nutritive and anti-nutritive factors. Digestibility, in a broad sense, includes degradability and fermentability. These, in turn, are functions of the chemistry of the forages.

The feeding value is the capacity of a forage to promote animal production. This is a complex function of intake, nutrient supply and animal metabolism. This is a highly interactive process. In ruminants, the utilisation of nutrients derived from one feed can be modified by the presence in the rumen of other feeds consumed the same day.

2.2 FODDER TREES

Tree crops are highly appropriate for the tropics. They capture a large amount of solar energy and, in high and low rainfall areas, they provide sustainable yields of biomass. They reduce erosion and improve soil structure and fertility. In marginal areas where dry seasons are prolonged, the deep roots of trees allow greater exploitation of water and mineral reserves in the soil profile. Some trees also provide green biomass of high protein content at a time when feed resources are scarce and mostly low in nitrogen (Preston and Leng, 1987). Most tree species are not primarily grown for fodder, despite the fact that fodder trees have always played a significant role in feeding domestic animals in the tropics. Most of the multipurpose species have economic value other than as fodder but being grown for such purposes makes them readily available for livestock feeding (Moog, 1992b). Fodder trees are an integral part of the smallholder farming systems in the tropics such as; (i) three strata forage system, (ii) integrated tree cropping systems; (iii) agroforestry systems; (iv) food-feed intercropping; (v) relay cropping; (vi) alley cropping, and (vii) grazing and stall feeding systems (Devendra, 1992).

Traditionally trees have been grown for specific purposes (Nitis, 1989), among these are:

- as live fences and windbreaks;
- to reduce wind and water erosion;
- shade for crops and livestock;
- climbers for vine crops;
- food production for human (fruits) and livestock consumption (pods and leaves);
- poles for livestock housing;
- to store crop residues and to dry grasses;
- as materials for farm implements, timber for housing and wood for handicrafts;
- religious ceremonies and traditional medicine;
- a source of pollen for bee keeping;
- fuelwood.

The advantage of using trees as sources of forage is the high protein content which does not decrease as the tree matures. They also provide leaves when grass is unavailable, e.g. in the dry season. In arid regions of Africa, the leaves of trees and shrubs can contribute over 60% of the biomass produced by rangeland. However, it is not only in rangeland systems that trees have a role to play in providing feed for livestock. Livestock may browse in forests, while indigenous forest trees can also be harvested for feeding to ruminants in cut and carry systems. Pure stands of fodder trees are also grown, either to allow ruminants restricted access for browsing or to harvest and offer to ruminants or pigs kept in confinement, or to be processed and used as poultry feed (Gill *et al.*, 1992). Apart from the good nutritive value and reasonable palatability to animals, fodder trees should also fulfil desirable agronomic requirements like: ease of establishment, management and harvesting, high productivity and persistence under repeated cutting or grazing, resistance to pests and diseases, minimal fertilizer requirement, good competitive ability, ability to produce seed or reliable vegetative propagation, and availability of different species (Ivory, 1990).

DIVERSITY OF FODDER TREES AND SHRUBS

A huge diversity of tree and shrub species has been documented. Blair (1990) presented a list (gathered from several sources) of trees and shrubs of potential value as animal fodders. The compendium included 270 different species from about 74 genera. To produce a complete inventory is a complex task because of the considerable diversity within genera and within species. For example, Lowry *et al.* (1994) reported some 100 species in the genus *Albizia*, and some 112 species of the genus

Erythrina were reported by Kass (1994). The diversity within species is illustrated by the 26 accessions of *Aeschynomene americana* and 96 accessions of *Cajanus cajan* that ILCA (1985) listed in its forage germplasm catalogue. There are increasing reports in the literature on the evaluation of new fodder resources (shrubs and trees): 16 species from the Philippines (Moog, 1992b), 45 from Costa Rica, 40 from Guatemala, (Benavides, 1994) and 20 from Colombia (Rosales *et al.*, 1992).

Although the final list consists of several hundred trees and shrubs, for most of them there are no quantitative data on their contribution to animal production, and the real feed value is known for a very limited set of species (see Table 2.1). This reflects the lack of knowledge on the nutritive value of most fodder shrubs and trees and highlights the need to evaluate these materials.

Table 2.1: *Examples of Important tree fodders and shrubs for feeding animals in the tropics.*

Common name	Scientific name
Acacia	*Acacia catechu*
	Acacia nilotica
	Acacia siberiana
Cassava	*Maniot esculenta*
Calliandra	*Calliandra calothyrsus*
Erythrina	*Erythrina variegata*
Ficus	*Ficus exasperata*
	Ficus bengalensis
	Ficus religiosa
Gliricidia	*Gliricidia sepium*
	Gliricidia maculata
Jackfruit	*Artocarpus heterophyllus*
Leucaena	*Leucaena leucocephala*
Pigeon pea	*Cajanus cajan*
Prosopis	*Prosopis juliflora*
Sesbania	*Sesbania grandiflora*
	Sesbania sesban
Tamarind	*Tamarindus indica*

Source: *Devendra, 1992*

FOLIAGE PRODUCTION

The first criterion in selecting a species is the amount of biomass which will be available for use at a time when either animal demand is high or the availability of other feeds is low (Gill *et al.*, 1992). Browse production is influenced by many environmental factors such as climatic, edaphic and topographic conditions and management background involving exploitation by animals, lopping and burning forested areas (Walker, 1980). Regardless of the production systems of fodder trees, foliage yield is affected by such factors as plant density and harvesting management, i.e., age at first harvest, height and frequency of cutting and season of harvest (Ivory, 1990). Thus, only broad generalisations

on the relative yields of different species can be made from values given in the literature. Trees may be grown in pure stands, for restricted browsing, or cut-and-carry systems. Yields of *Leucaena* foliage up to 11.8 t dry matter (DM)/ha have been reported for 3 different spacings of K341 and K8 *Leucaena* grown in Hawaii (Pound and Martinez, 1983). A range from 5.6 t fresh weight/ha to 30 t fresh weight/ha has been reported when comparing the effects of plant spacing, within and between rows in an alley cropping trial with *Leucaena* grown in Kenya (Macklin *et al.*, 1988). Leaves may also be harvested from individual trees. In these cases, the units for expressing yield are kg/tree. These yields compare with values of 1490 g DM/tree and 1280 g DM/tree for *Leucaena leucocephala* and *Gliricidia sepium* respectively, grown in an acid ultisol in southern Sumatra and cut at a height of approximately 1m every 3-4 months over a 4 year period (Blair, 1990).

NUTRITIVE VALUE OF FODDER TREE LEAVES

The success of the rationing systems in temperate countries has encouraged researchers to replicate them in developing countries as these standards are relatively predictable, but the problem is that they require feed resources that are inappropriate on socio-economic grounds in most developing countries. This success has also led some researchers from both industrialized and developing countries to the belief that the chemical composition of the feed is its nutritive value. It is not difficult to find articles in journals or seminar proceedings in which the nutritive value of trees is expressed as a simple chemical composition (proximate analysis). Ration systems based on additive chemical composition may not be appropiate however with mixtures of tropical feeds. The relevance of conventional feeding standards to developing countries, particularly in the tropics, has been questioned from the economic (Jackson, 1980) and nutritional points of view (Preston and Leng, 1987). Tropical feeding systems involve the use of "mixed" feeds widely different from one another (Vadiveloo and Fadel, 1992). This is particularly relevant when fodder trees and shrubs are used in the feeding system, as they are considered as a source of high protein feed for supplementing the basal diet and so stimulating feed intake (Palmer *et al.*, 1990).

METHODS OF ANALYSIS OF THE NUTRITIVE VALUE

The prediction of the quality of the feed, expressed as the nutritive value, is obviously a key factor in the development of feeding systems and many methods of chemical analysis have been proposed. Since the middle of the 19th century, scientists in temperate industrialized countries have attempted to develop analytical systems to characterise the value of feed to livestock. They include measurements of the chemical composition of the feed and its digestibility (INRA, 1989). One of the most popular

systems is that of *proximate analysis of feed*, which was devised about 100 years ago by two German scientists, Henneberg and Stohmann (McDonald *et al.*, 1981). The limitations of the methods are discussed below.

Proximate analysis

The system which is most frequently quoted in the literature is that of proximate analysis. This system of analysis divides the feed into six fractions, as shown in Table 2.2.

Table 2.2: *Components of different fractions in the proximate analysis of foods.*

Fraction	Component
Moisture	Water (and volatile acids and bases if present).
Ash	Essential elements:
	Major: Ca, K, Mg, Na, S, P, Cl.
	Trace: Fe, Mn, Cu, Co, I, Zn, Si, Mo, Se, Cr, F, V, Sn, As, Ni.
	Non-essential elements: Ti, Al, B, Pb.
Crude protein	Proteins, amino acids, amines, nitrates, nitrogenous glycosides, glycolipids, B-vitamins, nucleic acids, urea, ammonia.
Ether extract	Fats, oils, waxes, organic acids, pigments, sterols, vitamins A, D, E, K.
Crude fibre	Cellulose, hemicellulose, lignin
Nitrogen free extractives	Sugars, fructans, starch, pectins, organic acids, resins, pigments, water soluble proteins.

Source: *McDonald et al., 1981*

The chemical composition of a feed is routinely used as a rapid and economical method for predicting digestibility and other measures of the nutritive value. This is based more on a statistical association and less on a causal relationship between the content of analysed constituents and feed quality (Van Soest, 1982). As such, no single compositional parameter can adequately predict nutritive value across a range of feeds, although combining the results from several analyses may improve its prediction (Vadiveloo and Fadel, 1992).

The dry matter (DM) content is determined as the loss in weight which results from drying a known weight of food at 100°C to constant weight. This method is satisfactory for most foods but with a few, such as silage, significant losses of volatile material may take place.

The ash content is determined by ignition of a known weight of the feed at 500°C until all carbon has been removed. This residue is the ash and is taken to represent the inorganic constituents of the food. However, some loss of volatile material in the form of sodium, chloride, potassium, phosphorus and

sulphur will take place during ignition. The ash content is thus not truly representative of the inorganic material in the feed either qualitatively or quantitatively.

The crude protein (CP) content is calculated from the nitrogen content of the food, determined by a modification of the Kjeldahl sulphuric acid digestion technique. In this method, all nitrogen present in the feed, except that in the form of nitrate and nitrite, is converted to ammonia. This ammonia is liberated by adding sodium hydroxide to the digest, distilling off and collecting in standard acid solution. The nitrogen content is multiplied by 6.25 to obtain the crude protein value, based on the assumption that plant proteins contain approximately 16 percent nitrogen. This is not "true protein" since the method includes nitrogen from sources other than protein, such as nucleic acids, alkaloids, etc.

The ether extract fraction (EE) is determined by subjecting the feed to a continuous extraction with petroleum ether for a defined period. The residue is obtained by evaporation of the solvent. As well as true fat, it contains waxes, organic acids, alcohols, pigments; designation of the fraction as "oil" or "fat" is therefore incorrect. The ether extract determination is usually omitted in forage analysis as the amount of lipid present in feeds is very low.

The carbohydrate component of feed is contained in two fractions; the crude fibre and the nitrogen-free extractives. The former is determined by subjecting the residue from ether extraction to successive treatments with boiling acid and alkali of defined concentration; the organic residue is the crude fibre (CF). When the sum of the amounts of moisture, ash, crude protein, ether extract and crude fibre (expressed in g/kg) is subtracted from 1000, the difference is designated the nitrogen free extractives (NFE). The crude fibre fraction contains cellulose, lignin and hemicellulose, but not necessarily all of these materials are present in the feed: a variable proportion of them is contained in the nitrogen-free extractives, depending upon the species and the stage of growth of the plant material. The complexity of the NFE is well illustrated by the constituents shown in Table 2.2. The crude fibre was intended originally to provide a measure of the indigestible part of the food, but quite a large part of it may in fact be digested by ruminants animals (McDonald *et al.*, 1981). The division of carbohydrate between NFE and CF has proved to be of limited use in predicting the extraction of nutrients by the animal (Van Soest, 1982).

This scheme has been critically reviewed and alternative schemes have been developed which include further partition of the main components. The main modification aims to characterise better the carbohydrate component. The partitioning of the carbohydrate fraction into structural and non-

structural carbohydrates correspond to its function in the plant. Non-structural carbohydrates are composed of total sugars and starch. This last is also referred to as storage carbohydrate. In this thesis, the sugar fraction is partitioned into reducing and non-reducing sugars. The reducing sugars, which are more common, are able to function as reducing agents because free, or potentially free, aldehyde groups are present in the molecule. This aldehyde group is readily oxidised to carboxylic acid at neutral pH by mild oxidizing agents and enzymes. All monosaccharides or simple sugars are reducing sugars (eg. ribose, arabinose, xylose, glucose, mannose, fructose, etc). Oligosaccharides can be reducing or non-reducing sugars. For example, maltose, cellobiose and rutinose are reducing sugars whereas sucrose, the major product of plant photosynthesis, is not a reducing sugar (Allen, 1989).

The original crude fibre method has been replaced by determinations which are chemically more meaningful. These are the acid detergent fibre (ADF) and the neutral detergent fibre (NDF). This method was proposed by Van Soest (1976). ADF represents essentially the crude lignin and cellulose fractions of plant material but also includes silica. NDF consists essentially of lignin, cellulose and hemicellulose and is regarded as a measure of the plant cell wall material. Also, most of the silica is removed during extraction. The determination of ADF is particularly useful for forages as there is a good statistical correlation between this and digestibility (McDonald *et al.*, 1981).

None of these alternative schemes guarantee that the summation of the individual results will account for exactly 100% of the original material (Allen, 1989).

Data exist in the literature on the chemical composition of fodder trees, especially on *Gliricidia* and *Leucaena* species (see Table 2.3). Compared to grasses, fodder trees have relatively higher concentrations of crude protein, minerals and neutral detergent fibre plus acid detergent lignin (Wilson, 1969., Palmer *et al.*, 1990., Dicko and Sikena, 1992., Gill *et al.*, 1992). The differences in the nutrient content between fodder trees and grasses are even more striking when dry season values are compared. The crude protein content of dry, mature tropical grasses often falls below the minimum 6% required for maintenance, while most fodder trees remain green with high protein contents (Smith, 1992). In a review of the nutritive value of browse in West Africa, le Houérou (1980) concluded that browse contains double the amount of metabolisable energy of dry grass because of its lower content of fibre.

Table 2.3: *Nutrient content of selected fodder trees and shrubs compared to Panicum maximum.*

	g/kg (on a dry matter basis)					
Fodder Species	**Organic Matter**	**Crude Protein**	**Crude Fibre**	**Acid Deter Fibre**	**Neutral Deter Fibre**	**Total Ash**
Albizia lebbeck	850	217	362	246	354	73
Gliricidia sepium	900	230	207	287	428	97
Leucaena leucocephala	890	224	130	289	420	94
Sesbania grandiflora	882	235	-----	217	271	101
Panicum maximum	884	120	300	559	760	130

Source: *Smith 1992.*

Considerable variation in crude protein content occurs between species of trees and even edible parts of the same plant (Dicko and Sikena, 1992). In general, leaves are higher in CP than twigs, almost twice as much in the case of southern African browse (Walker, 1980). They also contain more CP than pods but the latter were found to have higher organic matter and digestibility (Göhl, 1981). Variations between species in crude protein content from 60 to 230g/kg in dry matter have been reported (Guérin, 1987). Variation in the concentration of nitrogen within species is also considerable. For *Acacia seyal* leaves, a range of 111 - 293g/kg CP in DM can be found in the literature, 156 - 300g/kg for *Gliricidia sepium*, 152 - 269g/kg for *Leucaena leucocephala* and 99 - 219g/kg for *Prosopis cineraria* (Gill *et al.*, 1992). Leguminous species were found to contain 25 to 50% more crude protein than non-leguminous plants (Wilson, 1969, Nitis, 1989). There are less data available in the literature on mineral concentrations in fodder trees and shrubs, particularly the unconventional species. Available data for the favoured species like *Gliricidia* and other species suggest that, except for a disproportionately high Ca:P ratio in leguminous species, most browse species contain adequate levels of macrominerals to cover animal requirements and that this could be better exploited. More studies are however required to fully characterise trace mineral contents of these fodder resources (Smith, 1992).

The analyses just described do not take full account of another class of compounds, frequently referred to as secondary plant compounds, which are known to have anti-nutritive effects. The leaves of woody plants tend to contain more of these compounds, particularly phenolics, than do herbaceous plants (Bate-Smith, 1962). In addition, tropical plants also tend to produce secondary compounds which have adverse effects on herbivores, as a strategy to protect them from defoliation (Levin and York, 1978). The key to assessing the nutritive value of tree leaves lies in the ability to estimate the presence and effects of these compounds.

FEEDING VALUE OF TREES AND SHRUBS

Although there is considerable evidence of the beneficial responses in production due to the use of some fodder trees and shrubs as supplements, more information is needed for the majority of the species. This section highlights the feeding value of some the fodder trees selected for this study.

It has been well established that *Leucaena leucocephala* and *Gliricidia sepium* can be successfully used to improve livestock production (Palmer *et al.*, 1990). *Leucaena leucocephala* has been shown to increase milk and meat production in ruminants (Pound and Martinez, 1983; Jones, 1994) and *Gliricidia sepium* also has proved to improve productivity (Carew, 1983) and reproductive parameters (Mejía *et al.*, 1991). Esnaola and Ríos (1994) showed that supplementation of a basal diet of *Pennisetum purpureum* with different levels of *Erythrina poeppigiana* in lactating goats increased milk production linearly from 326 to 820g/animal/day as the levels of the tree leaves increased. The highest milk production corresponded to a level of supplementation of 1.5kgDM/100kg live weight (LW). *Erythrina poeppigiana* also increased daily milk production linearly from 8.7 to 9.4kg/cow as intake of its foliage increased from 0 to 0.6%LW (Romero *et al.*, 1993). It has also been shown that *Trichanthera gigantea* fed as a supplement to a basal diet of *Pennisetum purpureum* increased milk and meat production in goats in a cut-and-carry system (Rosales and Galindo, 1987). It has been reported that *Inga spectablilis* is high in condensed tannins (Rosales *et al.*, 1992) and that, when given at a high level, reduces milk and intake in lactating goats. It is used as supplement for cattle without negative effects on the animals (Rosales and Galindo, 1987).

2.3 FORAGE AS FEED

Animal cells are devoid of the enzymes required to break down structural carbohydrates but some microorganisms possess cellulases and hemicellulases and hence bacteria in the intestine of monogastric species are able to digest limited amounts of cellulose and hemicellulose (Van Soest, 1982). On the other hand, ruminants have evolved a capacious set of stomachs, a fermentation vat, which harbours microorganisms capable of digesting fibrous materials such as cellulose. The microbial fermentation in the rumen precedes host enzyme digestion in the abomasum and small intestine and thus allows the ruminants to utilise plants which have a high fibre content and low nutritional value for simple-stomached animals (Forbes and France, 1993). Rumen microorganisms, bacteria, protozoa and fungi, ferment feed constituents (polysaccharides, sugars, proteins, etc.) generating the adenosine triphosphate (ATP) they need for maintenance and growth - the latter involving the use of energy for the synthesis of monomers and their polymerization (e.g., for synthesis of amino acids from ammonia

and carbon compounds, and for the elongation of polypeptide chains) in addition to the end products of the fermentation (Nolan, 1993).

FERMENTATION IN THE RUMEN

Fermentation of carbohydrates

Dietary structural and non structural carbohydrates are the main fermentation substrates in the rumen. They are degraded to their constituent hexoses and pentoses before being fermented to volatile fatty acids (VFAs). The VFAs are principally acetic, propionic and butyric. The other main end products of the fermentation are carbon dioxide and methane (Czerkawski, 1986) (see Figure 2.1). To the microbes, the VFAs are waste products but to the host animal they represent the major source of absorbed energy and with most diets account for approximately 80% of the energy disappearing in the rumen (the remainder being lost as heat and methane). The VFAs are absorbed across the epithelium of the reticulo-rumen and CO_2 and CH_4 are lost by eructation. (France and Siddons, 1993).

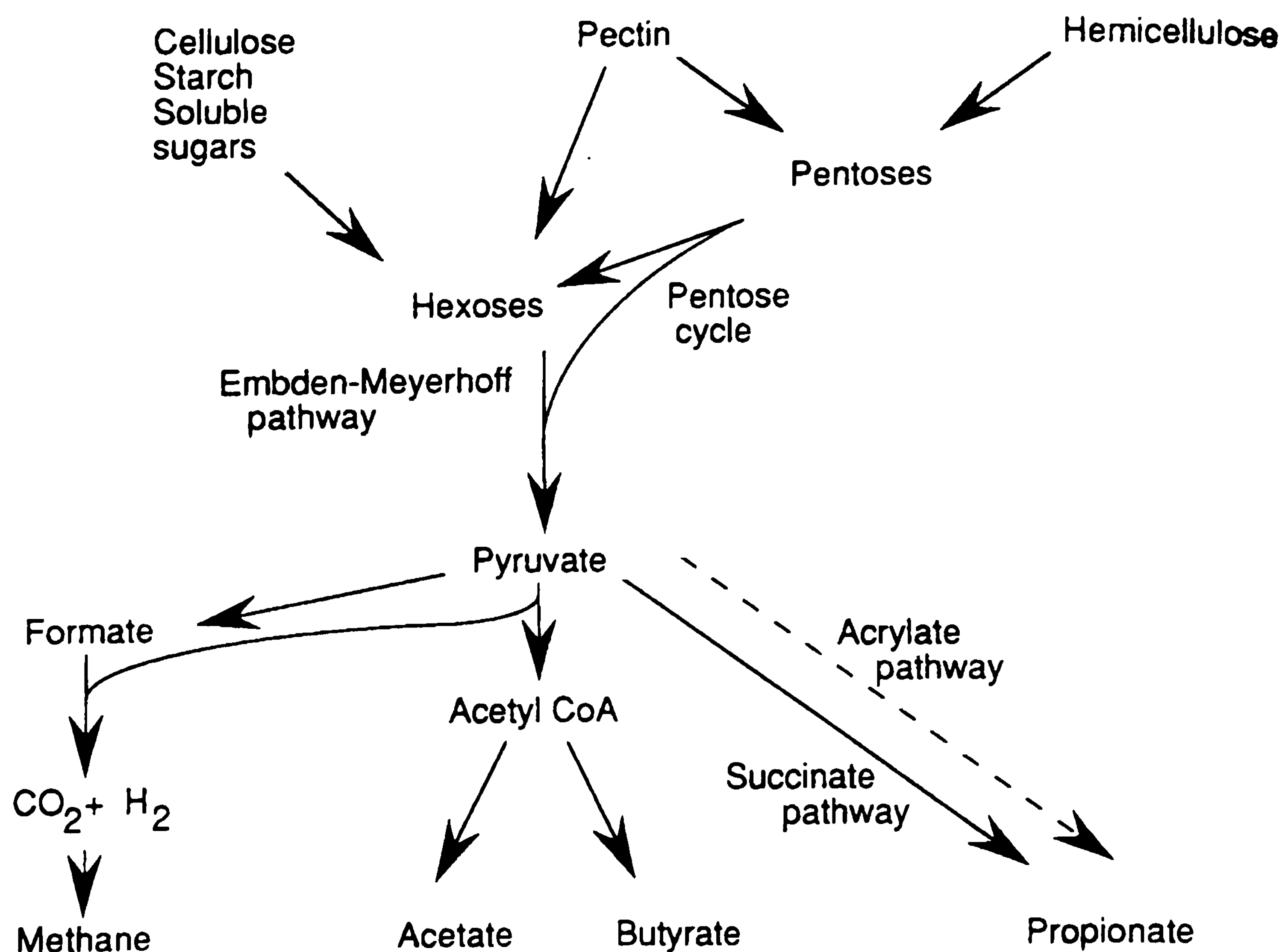


Figure 2.1: *A schematic representation of the major pathways of carbohydrate metabolism in the rumen.*
Source: *France and Siddons, 1993.*

Fermentation of protein

Protein is also degraded by microbes in the rumen. The amino acids and peptides produced are used for microbial protein growth. The feed and endogenous protein is subjected to varying degrees of proteolysis and deamination in the reticulo-rumen producing ammonia (NH_3) (see Figure 2.2). Ammonia is the most important source of nitrogen for protein synthesis in the rumen (Wallace and Cotta, 1988).

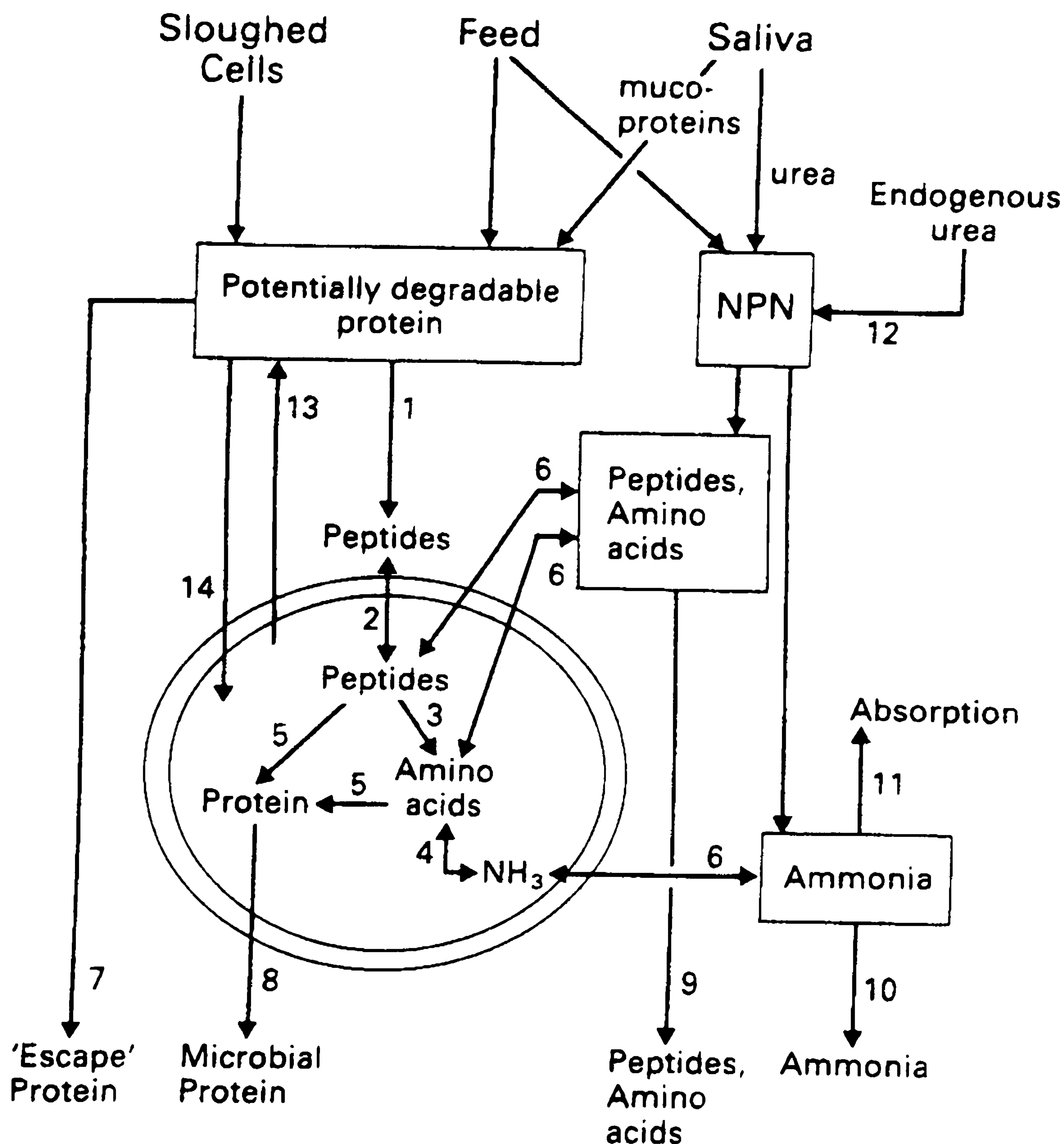


Figure 2.2: *A model of nitrogen transactions in the rumen. The ovals delineate the microbial cell wall and numbers adjacent to arrows refer to individual pathways as follows: 1, Proteolysis by bacterial, protozoal and fungal proteases; 2, carrier-mediated peptide uptake across microbial cell walls; 3, peptidolysis; 4, amination/deamination; 5, protein synthesis; 6, microbial assimilation/excretion or equilibration of amino acids and ammonia; 7, protein not hydrolysed before efflux from rumen (UDP); 8, microbial protein efflux; 9, efflux of extracellular peptides and amino acids; 10; efflux of extracellular ammonia; 11, absorption of ammonia through rumen wall; 12, movement of endogenous urea through the rumen wall; 13, N compounds excreted by living cells and debris of lysed cells; 14, engulfment of proteinaceous particles by protozoa.*
Source: *Nolan, 1993.*

Microbial protein synthesis

Fermentation and microbial growth in the rumen are often viewed as disparate processes, the first generating ATP and the second utilizing it. In the cells these processes are coupled and virtually inseparable. Therefore an understanding of microbial metabolism must simultaneously encompass the dynamics of the energy and protein transactions (Nolan, 1993). Microbial protein synthesis is known to depend largely on the balance between the supply of energy (ATP) which is derived from the rumen fermentation of dietary digestible organic matter and the supply of ammonia which is released from the breakdown of dietary rumen degradable protein, recycled urea and salivary protein (Thomas and Martin, 1988). At best, if the amino acid breakdown products of proteolysis are assimilated directly and entirely into microbial protein, there will be a small energy cost for the uptake of amino acids into the cell and the polymerization of amino acids into microbial protein. If degradation proceeds to deamination, more energy is needed to resynthesise amino acids from the ammonia produced. Because the resultant microbial protein is of fairly high quality, nutritional value is not necessarily lost. At worst, as occurs in many nutritional regimes, a large proportion of the ammonia released by deamination is not assimilated into microbial protein because energy production for microbial growth is not synchronized with the ammonia release. The ammonia cannot then be assimilated and it diffuses across the rumen wall to be ultimately lost as urea in the urine (Wallace, 1988).

Microbes present in the rumen have the ability to convert non-protein nitrogen (NPN) into microbial protein. The NPN will only be converted into microbial protein if other nutrients required by the microbes are available in sufficient quantities. These include sulphur, specific amino acids, peptides and energy. The maximum efficiency of microbial protein production will only be achieved if ammonia is released from the source of NPN at the same slow rate as the energy from the forage (Minson, 1990). Breakdown and resynthesis of the components of feed makes inefficient use of energy and does not supply sufficient protein to support high levels of production (Wallace and Cotta, 1988; Broderick *et al*, 1991; Preston and Leng, 1987). Therefore, the objectives of research in this field have been two-fold. One is to capitalise on the microbial capacity to form protein from ammonia by feeding NPN. The other is to minimise the breakdown in the rumen, and thereby to increase the bypass dietary protein, reaching the lower tract (Wallace and Cotta, 1988).

Controlling the rate and extent of degradation of dietary protein to balance the protein supply from microbial synthesis is of great interest to ruminant nutritionists. The extent and rate of dietary protein degradation in the rumen is dependent on a number of factors, although it is generally considered that the solubility of protein in the rumen liquid is the major determinant and this varies between proteins

from different sources (Broderick *et al.*, 1991). It also depends on the level of proteolytic activity, which is highly variable, and on the time the protein spends in the rumen as well as other factors such as pH and microbial species present. Various chemical and physical treatments have been applied to protein supplements to reduce their degradability and increase the fraction that escapes to the intestines. Heat, aldehydes (formaldehyde), tannins, alkalis and coating with lipids have all been used with varying success (Nolan, 1993).

IN VITRO AND *IN VIVO* DIGESTIBILITY METHODS

Analytical methods can give an indication of the potential value of a feed for supplying a particular nutrient, but the actual value of the feed to the animal can be arrived at only after making allowances for availability of these nutrients during digestion. The following methods take account of the action of the rumen microbes. These widely used methods for predicting the nutritive value for ruminants are known as the *in vitro* and *in vivo* or *in sacco* dry matter digestibility (DMD).

The *in vitro* DM digestibility (referred to as *in vitro* DMD) involves the incubation in the laboratory of aliquots of feed with an inoculum of micro-organisms from the rumen, and measurement of the amount of substrate which disappears over a set (48h) time period. In theory, the values produced give a good indication of nutritive value but, in practice, a high degree of error is associated with the technique (McDonald *et al.*, 1981). Data on digestibility of fodder trees measured *in vivo* indicate a range for leaves of 44 - 60%. This compares with values of 40 - 70% for tropical grasses and 55 - 75% for temperate grasses (Van Soest, 1982). A range from 38 to 78% was found for woody species in Botswana (Skarpe and Bergstrom, 1986). Similar findings were reported by McKay and Frandsen (1969) and Walker (1980). A wide range of *in vitro* dry matter digestibility from 17 to 67% has been reported (Vercoe, 1987). Table 2.4 shows the digestibility of selected fodder trees.

Table 2.4: *Digestibility of selected fodder tree leaves compared to Panicum maximum.*

Fodder Species	*In vivo* Organic Matter Digestibility (%)	*In vitro* Organic Matter Digestibility (%)
Albizia lebbeck	53.2	59.5
Gliricidia sepium	53.9	------
Leucaena leucocephala	64.9	57.5
Sesbania grandiflora	61.8	66.7
Panicum maximum	53.2	37.0

Source: *Smith 1992.*

Several studies have developed *in vitro* techniques based on incubation of forages with rumen liquid (Tilley and Terry, 1963), with cellulose-type enzymes (Van Soest, 1982) or on gas produced by incubation with rumen liquor (Menke *et al.*, 1979). Another method which is commonly used is to estimate the rate of digestion of components of the feed, such as fibre or nitrogen, by incubating aliquots of the feed in dacron bags in the rumen (*in situ*) for a range of time periods (Ørskov *et al.*, 1980).

The gas production technique (Theodorou *et al.*, 1994) is a new, simple and sensitive *in vitro* method which can be used to provide information on the fermentability of temperate and tropical crops, by-products and leaves from fodder trees. It uses the accumulation of gas pressure and gas volume in the head-space of culture bottles containing dried and ground forages mixed with an anaerobic medium inoculated with rumen liquid. In this method, a pressure transducer connected to a digital readout voltmeter and a gas-tight syringe assembly is used to measure and release the accumulated gas pressures from the incubating culture bottles. The gas production method provides precise data relating to the fermentation kinetics of ruminant feeds and ranks feeds with respect to their *in vitro* fermentability. This technique has been also proved useful to study the role of tannins on the fermentability of tropical forage legumes (Sanderson, 1993; Longland *et al.*, 1994).

2.4 SECONDARY COMPOUNDS AND PLANT TOXICITY

The term secondary compounds is used to describe a group of chemical constituents in plants thought not to be involved in the biochemical processes of plant growth and reproduction (Palmer *et al.*, 1990). These secondary metabolites are thought to have a defensive role that ensures survival of the plant (Coley *et al.*, 1985), by protecting against insect predation or by restricting grazing by herbivores (Swain, 1979). The compounds have been implicated in limiting the utilization of feeds. They can act by inhibiting digestion, having toxic effects, or inhibiting some enzymes and/or metabolic processes or as precursors of other anti-nutritional compounds (Palo, 1987).

In summary, Barry and Blaney (1987) stated that secondary compounds can be toxic to animals or cause reduction in their productivity by reducing feed intake. These plant constituents do not affect all herbivores equally; there are examples of plants being toxic to monogastric species but not to ruminants, because the toxin is rendered harmless by the rumen bacteria.

There are more than 1200 classes of secondary compounds. These include among others: polyphenols, cyanogenetic glycosides, alkaloids, saponins, steroids, toxic proteins and amino acids, non-protein

amino acids, phytohemagglutinins, triterpenes and oxalic acid (Kumar, 1992a; Liener, 1980). Table 2.5 shows some of the secondary compounds reported to occur in fodder trees. Discussion here will be restricted to tannins which are the most common secondary compounds in tropical fodder trees.

Table 2.5: *Secondary compounds in the leaves of trees and shrubs documented as being used in livestock feeding.*

Substances	Species
Non-protein amino acids	
Mimosine	*Leucaena leucocephala*
Indospecine	*Indigofera spicta*
Glycosides	
Cyanogens	*Acacia giraffae*
	A. cunninghamii
	A. siberiana
	Bambusa bambos
	Barteria fistulosa
	Manihot esculenta
Saponins	*Albizia stipulata*
	Cassia latifolia
	Sesbania sesban
Phytohemaglutinins	*Bauhinia purpurea*
Ricin	*Ricinus communis*
Robin	*Robinia pseudoacacia*
Polyphenolic compounds	
Tannins	All vascular plants
Lignin	All vascular plants
Alkaloids	
N-methyl-β-phen ethylamine	*Acacia berlandieri*
Sesbanine	*Sesbania vesicaria*
	S. drummondii
	S. punicea
Triterpenes	
Azadirachtin	*Azadirachta indica*
Limonin	*Azadirachta indica*
Oxalate	*Acacia aneura*

Source: *Kumar, 1992a.*

TANNINS

Vegetable tannins are water soluble polyphenolic compounds, having a relative molecular mass between 500 and 3000 (see Figure 2.3) (Haslam, 1981) and, besides giving the usual phenolic reactions, they have some special properties such as the ability to bind strongly with proteins, polysaccharides, nucleic acids, steroids, alkaloids and saponins (Mueller-Harvey and McAllan, 1992, Haslam, 1986). The mechanism of vegetable tannage is generally accepted to be the formation of a hydrogen-bonded network between hydroxyl groups of vegetable tannins and relevant groups in collagen and hydrophobic interactions between vegetable tannins and certain regions or groups of the collagen polymer (see Figure 2.4) (Spencer *et al.*, 1988).

Traditionally, tannins have been divided into two groups: the condensed and hydrolysable tannins. However, a new group, the complex tannins has been proposed (Tang *et al.*, 1992). It is generally thought that condensed tannins are less harmful than hydrolysable tannins, although both have the ability to bind protein.

Nutritional effects of tannins

Studies of the effects of tannins on animal nutrition have involved a wide range of plants and have covered a wide variety of animal species. In the vast majority of cases there has been little or no characterisation of tannins present in the feedstuffs used (Mueller-Harvey and McAllan, 1992). In general, tannins cause growth depression and an adverse effect on protein and dry matter digestibility (Liener, 1980). They can also produce liver necrosis, act as a pectinase inhibitors and as carcinogenic agents (NAS, 1973).

Tannins are known to impart an astringent or bitter taste and the level of tannins in the diet may therefore reduce the palatability. However the effects of tannin levels in the diet may also be quite negligible or indeed they may even enhance intake (Mueller-Harvey and McAllan, 1992).

Levels between 0.2 and 2% of tannins have been shown to depress dry matter, protein and amino acid digestion, reduce energy utilisation and growth and lead to lower feed efficiency ratios in poultry. Leg abnormalities have been found in chicks receiving high tannin sorghum grain diets. Histopathological effects in chicks include decreases in blood haemoglobin, red and white cell counts and necrosis of the kidney and liver. Decreases in egg production and yolk discolouration have also been reported (Mueller-Harvey and McAllan, 1992).

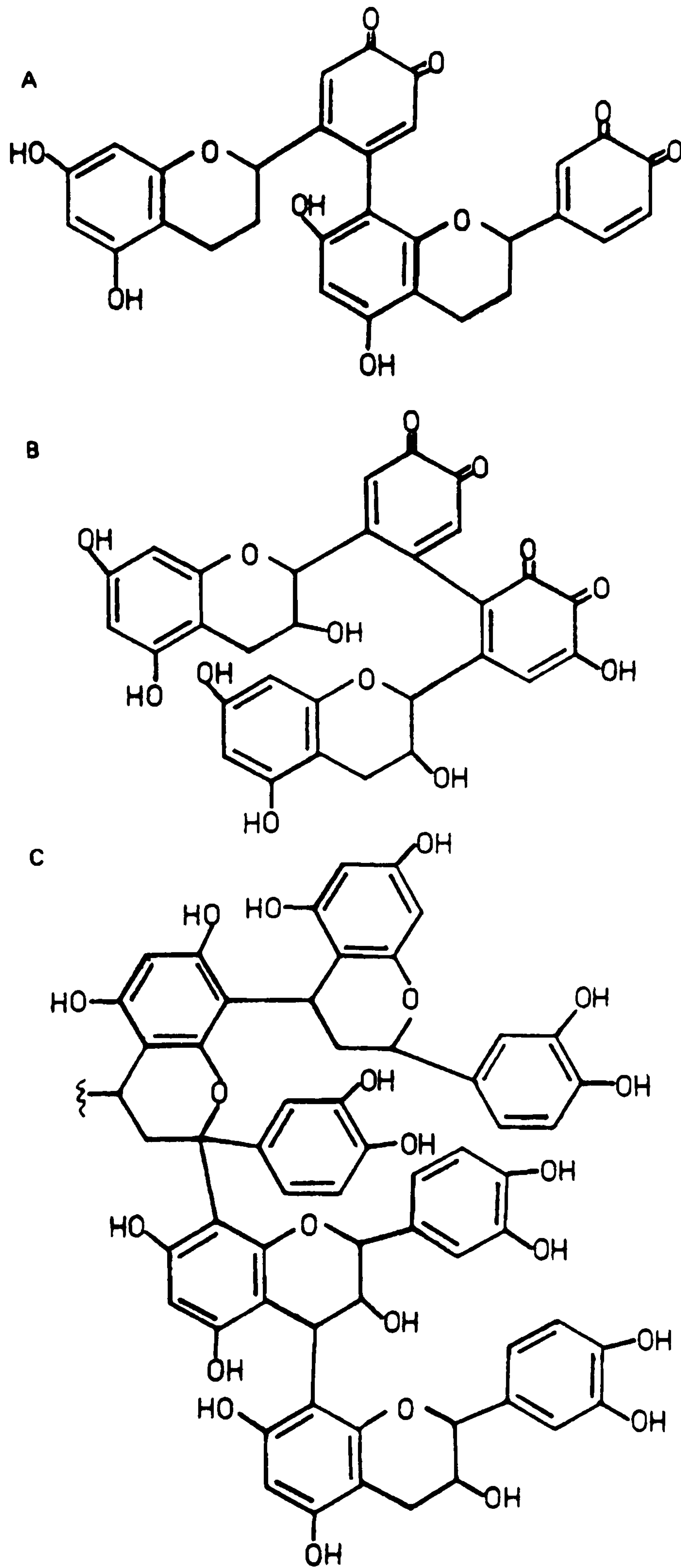


Figure 2.3: *Formulae of condensed flavonoid substances and tannins. A. Head to tail polymer. B. Tail to tail polymer. C. Possible structure of condensed tannin.*
Source: *Van Soest, 1982.*

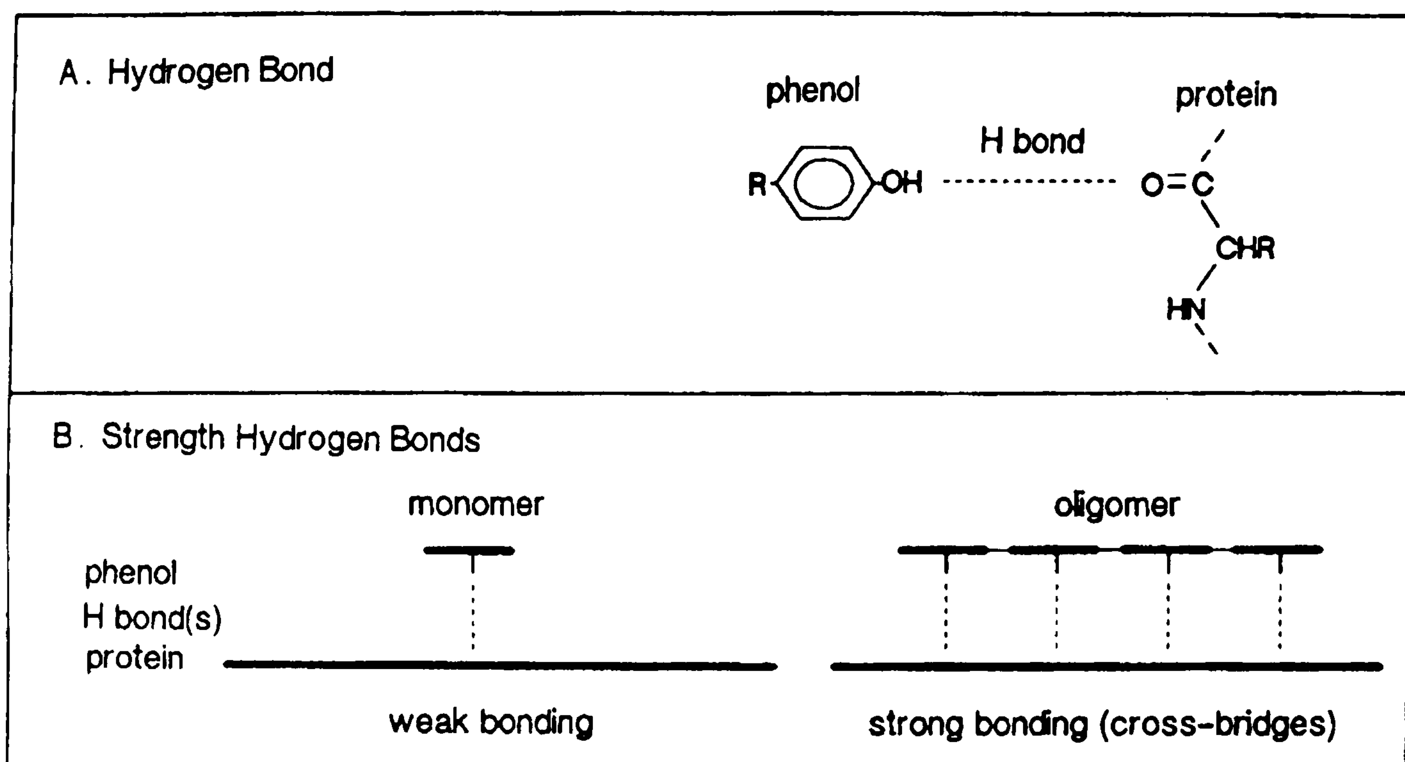


Figure 2.4: *Hydrogen bonding of tannic compounds with proteins. A. Location of the hydrogen bond between the hydroxyl group of the phenol and the carboxyl group of the protein's peptide. B. The role of cross bridged hydrogen bonds on the strength of the tannin-protein interaction.*
Source: *Field and Lettinga, 1992.*

In pigs, reductions in dry matter digestibility and gross energy have been reported. Physiological abnormalities resulting from continuous ingestion of free tannin by young pigs include anorexia, dyspnoea, hydrothorax and oedema of the lungs, hepatic degeneration and dilation of the heart. In general, feeding with high tannin diets results in poor performance, particularly in terms of feed conversion efficiency (Liener, 1980). The antinutritional effects of tannins in fodder trees are summarized in Table 2.6.

The effect of tannins in ruminant feeding is not consistent, with possible harmful and beneficial effects (Mueller-Harvey and McAllan, 1992., Barry and Duncan, 1984). The two types of tannins differ in their nutritional and toxic effects. The condensed tannins (CT) have a more profound digestibility-reducing effect than hydrolysable tannins (HT), whereas the latter may cause varied toxic manifestations due to hydrolysis in the rumen.

Rumen microbes have been shown to degrade flavonoids. Strains of *Butyrovibrio* and *Peptostreptococcus* are prominent in the cleavage of heterocyclic rings. However, there are few data available on the degradation of tannins by the rumen microflora (Deschamps *et al.*, 1983; Field and Lettinga, 1992). Since rumen microorganisms may modify or metabolise ingested tannins, the extensive adaptation of the rumen microflora to different plant constituents could be of particular importance in reducing the potential toxicity of ingested tannins. These ingested tannins may act in

the rumen in a number of ways such as:

- affect the species and composition of the microflora;
- complex with and inhibit extracellular enzymes produced by the microflora;
- complex and render unavailable dietary nutrients;
- they (or metabolic products) may be absorbed from the rumen and prove toxic at the tissue level (Mueller-Harvey and McAllan, 1992).

Table 2.6: *Examples of anti-nutritional effects of tannins in shrubs and tree forages.*

Species	Predominant Tannin	Animal	Nutritional effect
Acacia aneura	CT	Sheep	Reduction in N digestibility, decreased wool yield, growth and S absorption.
A. cyanophylla	CT	Sheep	Reduced feed intake, negative N digestibility, loss in weight.
A. nilotica (pods)	CT	Sheep	Low growth rate, reduced N and NDF digestibility.
A. siberiana (pods)	HT	Sheep	Low growth rate, reduced N and NDF digestibility.
Albizia chinensis	CT	Goat	Reduced *in sacco* N digestibility.
Leucaena leucocephala	CT	Poultry	Poor N retention, low apparent metabolisable energy value.
Manihot esculenta	CT	*In vitro*	Inhibits digestibility.
Prosopis cineraria	CT	Sheep	Reduction of feed intake, protein, digestibility, decreased wool yield & growth, decreased iron absorption.
Robinia pseudoacacia	CT	Rat	Reduced N digestibility.
		Rabbit	Reduced feed intake & growth.
Terminalia oblongata	HT	Sheep	Reduction in feed intake, toxicity, but not effect upon digestibility.

CT: Condensed tannin
HT: Hydrolysable tannin
Source: *Kumar, 1992a.*

Evidence is increasing that tannins can have some benefits. Tannins have been found to have bloat-safe properties. It has been suggested that tannins inhibit the production of stable foam in the rumen, helping to control bloat (Lees, 1992). Low concentrations (2% dry matter) of tannins from *Lotus* have been shown to reduce the carcass fatness in growing lambs, whereas high tannin diets increased levels of growth hormone in sheep blood (Barry and Manley, 1986). Dietary condensed tannins from *Lotus pendunculatus* (2-3%) have been shown to impart beneficial effects because they reduce the protein degradation in the rumen by the formation of a protein-tannin complex (Barry and Blaney, 1987).

Tannins and bypass protein

Protein that is slowly degradable in the rumen may provide amino acids and peptides for microbial growth in addition to providing bypass protein. Tannins are known to protect dietary protein against microbial attack in the rumen. Thus if a freshly harvested tropical legume given as a supplement is to provide bypass protein then it should be selected for a relatively high content of tannins, even though this will depress fibre digestibility. The benefits of including bypass protein in the diet have been widely documented (Preston and Leng, 1987).

The tannin-protein complex can be either reversible (i.e., with the formation of hydrophobic and/or hydrogen bonds) or irreversible (i.e., oxidation of the phenolic compounds to reactive quinone) (Fahey and Jung, 1989). The reversible tannin-protein complexes have maximum stability in the pH range 4 - 7. Above and below this pH range the complex is readily dissociated. In this way, the tannin-protein complex after escaping rumen fermentation (about pH 5 - 7) would be digested readily by the enzymes in the gastric (about pH 2.5) and pancreatic (about pH 8 - 9) secretions (Palo, 1987).

2.5 ASSOCIATIVE EFFECTS

DEFINITION

Associative effects of feedstuffs were first described by Ewing and Wells (1915) as the influence of one ingredient of the diet on the others. In 1931, Kriss wrote "the net energy values of individual feedingstuffs are fundamentally variable in the sense that their values depend to a considerable extent on the combination in which they are fed with other feedingstuffs. The associative effects of combinations are unpredictable in the present state of knowledge". These observations were then confirmed by Forbes *et al.* (1933) when measuring the net availability of metabolisable energy (ME)

of maize meal in fattening steers they found that it differed markedly according to the basal ration on which the animal was fed. If maize was added to a ration of oat straw, corn meal and molasses, a higher efficiency was obtained than if it was added to a ration of alfalfa hay, linseed meal and bran. The latter was in turn higher than that found when maize was added to a basal ration of timothy hay. Forbes and Kriss were unable to explain this effect. Other workers subsequently found similar effects. Hamilton (1942) showed one of the most impressive examples of associative effects in the depression of the digestibility of cellulose by the addition of starch or glucose to the diet. It occurred when starchy grains were added to roughage diets, particularly those low in nitrogen content. Watson (1945) gathered enough evidence to show that, when mixtures of different feedingstuffs are given to animals, the apparent digestibility of the mixture was not necessarily the same as the weighted sum of the apparent digestibilities of its components. This phenomenon was called 'associative digestibility'.

In 1962, Blaxter, when outlining the basis of the new ME system, acknowledged the associative effects of feedstuffs both in ME and in digestibility and proposed an explanation. He hypothesised that the associative effects on ME found by Forbes *et al.* (1933) arose from the different type of fermentation produced when maize was used to supplement the different rations. The depression in cellulose digestibility found by Hamilton (1942) could be accounted for by the depletion in soluble nitrogen (and possible other essential nutrients) in the fluid phase of the rumen resulting from the rapid growth of starch-fermenting organisms. This had a consequent negative effect on the cellulolytic flora. In a similar way, the addition of protein-rich material could cause an increase in the apparent digestibility of a low nitrogen roughage. One of the fundamental assumptions in the new ME scheme was that the values for individual feeds could be added together to obtain the metabolizable energy value of whole rations (principle of additivity). This implied a rejection of all associative effects on the digestibility of the feed. Blaxter argued that, although these effects existed, their magnitude was usually not large, particularly if time was allowed for an equilibrium to be established between the microflora in the gut and the change in diet. They were not likely to limit the usefulness of the scheme in practice. Since then, the prevalent feeding systems based on ME, both in Europe and the USA, have assumed additivity of ME (and digestibility of all other nutrients) in ration formulation.

Additivity is then the key factor which needs to be tested in order to quantify the associative effects. Its importance was highlighted by Sauvant and Giger (1989), who considered associative digestibility to be the lack of additivity between digestible nutrients. Oldham and Emmans (1990) defined associative effects as the interactions between feed values which influence animal performance in ways which are not predictable on the basis of strict additivity. This last definition includes associative effects in the feeding value and not just in the nutritive value. Apart from the effects of specific

nutrients on fibre digestibility and associated feed intake, Oldham and Emmans also discussed the influence of nutrient balance on nutrient partitioning as a consequence of the associative processes.

It is clear that the digestion process is an interaction between all the components of the diet. Associative effects between the components of a mixed diet occur when, as a consequence of the interactive process, the nutritional value of the mixture cannot be predicted from the sum of its individual components. Associative effects occur with both digestibility and intake, and intake can influence the associative effects on digestibility. It is necessary to separate true associative effects from the greater intake of more digestible feeds. If the mixture of feeds results in an increase in voluntary intake because one component, for example, is highly palatable, the resultant increase in digestibility cannot be attributed to an associative effect itself (i.e., the lack of additivity). The increased digestibility may be the result of a greater intake of a more digestible component. On the other hand, intake can be reduced if one of the components of the mixture has an astringent effect (like tannins). The associative effect on intake is referred to as a substitution effect. It occurs when an increased intake of a supplementary feed results in a decreased intake of the basal feed, to an extent that varies directly with the digestibility of the basal feed (Forbes, 1986). While there are some cases where the substitution effect might be explained on simple additivity grounds (replacement of 'fibre' on a 1:1 basis), this is certainly not a general explanation. The substitution effect is an important associative effect for which the criterion of evaluation is solely through the measurement of animal performance (Oldham and Emmans, 1990).

QUANTIFICATION OF THE ASSOCIATIVE EFFECTS ON DIGESTIBILITY

Sauvant and Giger (1989) described a method to quantify associative effects on the digestibility of straw based on a quadratic model (see Figure 2.5). Several authors (Mould *et al.*, 1983b; Moss *et al.*, 1992; Margan *et al.*, 1994; and Cassida, *et al.*, 1994) have used a similar method which is described below. In their method, the mixed diet is given in increasing proportions of one component and decreasing proportions of the other one at the same time (from ratio 100:0 to 0:100). The usual ratios are 100:0, 75:25, 50:50, 25:75, 0:100. Fewer levels make the analysis less precise. The data are examined by linear and quadratic regressions and if the quadratic trend is significant then associative effects are shown to exist. In the absence of associative effects, the digestibility should change linearly with the increasing ratios of the mixture (there is additivity of the two components).

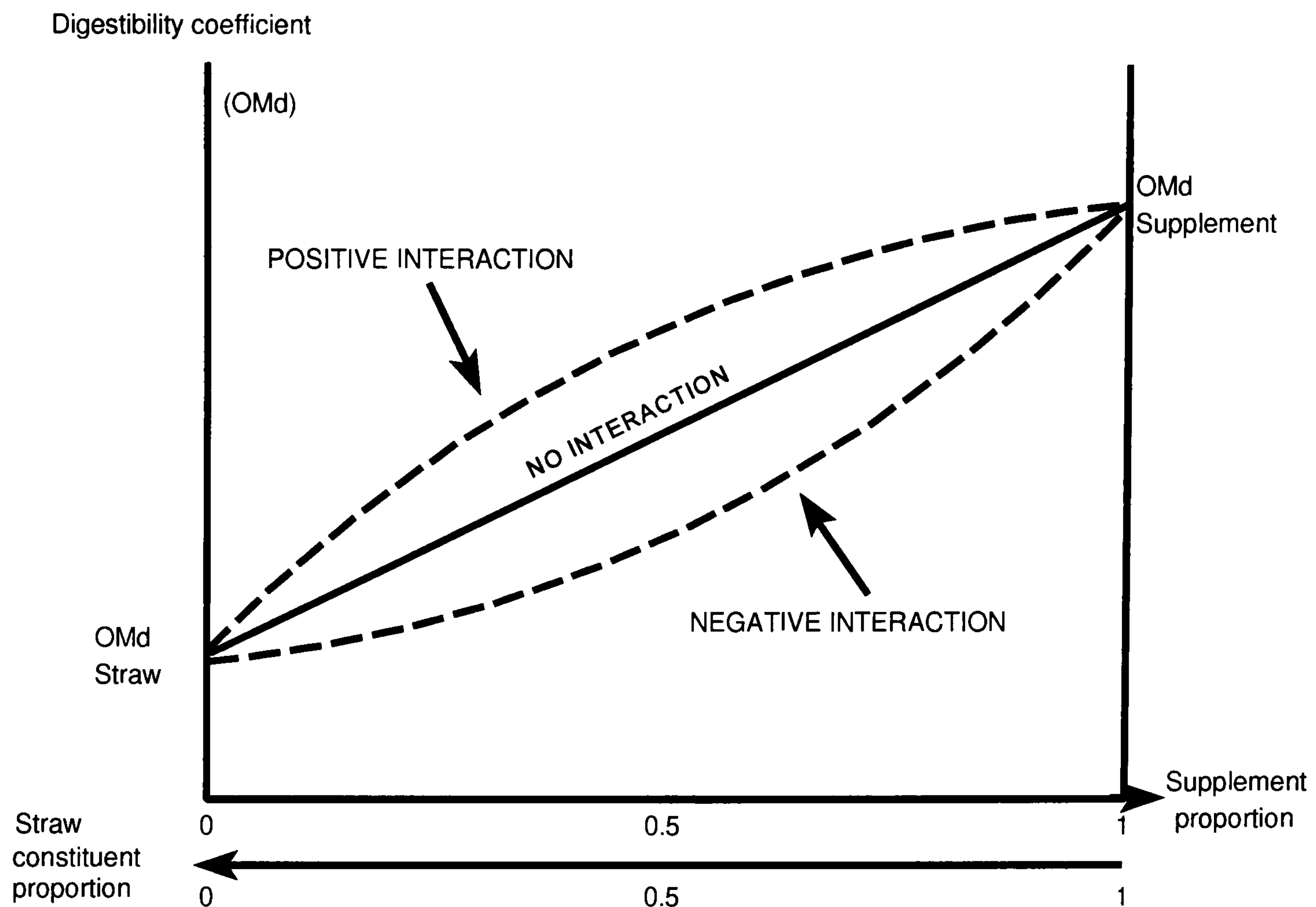


Figure 2.5: *Associative effects represented as the quadratic variation of the diet digestibility, as proposed by Sauvant and Giger (1989).*

Other authors (Brown and Pitman, 1991), working *in vitro*, have preferred to regress the *in vitro* digestibility values against the ratio, using the values for the 100:0 and 0:100 mixtures. Associative effects are calculated as the deviation of the actual *in vitro* digestibility values from the regression line established using the 100:0 and 0:100 values. The drawback of this last method is that a greater number of replicates is needed for the end values in order to increase the precision of the expected values calculated by the regression.

In animal experiments, intake can influence the associative effects as previously explained. To overcome this problem, the animals are given a restricted intake to a maintenance level or just below, enough to maintain liveweight and to ensure that the feed offered is completely consumed. This also ensures that there is the same level of consumption for all ratios. Water intake can also affect the associative digestibility if the consumption is great enough to affect gut fill or rate of passage (Cassida *et al.*, 1994).

MODE OF ACTION OF THE ASSOCIATIVE EFFECTS

Several authors have hypothesised about the likely mode of action of the associative effects. Minson (1990) suggested that 'synergism' or positive associative effects would only occur where one species is deficient in an essential nutrient, which is provided by the second forage. For the most common associative effect reported in the literature, that is the depression of fibre digestion by the addition of a feedstuff rich in starch or sugars, the general consensus seems to be that when this carbohydrate-rich feed is mixed with a feedstuff with a particularly low nitrogen content, the soluble nitrogen is depleted due to the rapid growth of amylolytic organisms. This shortage of nitrogen causes a reduction in the growth rate of cellulolytic organisms and therefore the cellulose digestibility is decreased (Blaxter, 1962; Garnsworthy and Cole, 1990). Cheeson and Forsberg (1989) highlighted the importance of the form of the fermentable carbohydrate and Oldham (1984) stressed the amount and form of dietary crude protein. As for the effect found when high concentrations of fat in roughage diets decrease the digestibility of fibre in the rumen, Devendra and Lewis (1974) suggested that this may be due to physical coating of the fibre by the fat, toxic effects of fat on rumen microbes, surfactant effects of fatty acids on cell membranes or the formation of insoluble cation soaps. Sauvant and Giger (1989) suggested that the associative effects depended on reticulo-rumen microbial activity and particle transit.

Associative effects of different components of a diet on digestibility are widely recorded especially for feedstuffs used in temperate countries. Despite this, few experiments have been set up to explain the mode of action of these effects, that is experiments that are specifically designed to test the deviation from linearity (i.e., test the additivity of the components). Also, the majority of the studies have used combinations of forages or low quality roughage and a readily available energy source. Fewer experiments have examined the possibility of associative effects between different forages. The suppression of fibre degradation when a fibrous roughage is fed with a readily degradable substrate was demonstrated to be of a composite nature, due in part to a decrease in rumen pH and in part to the amount of rapidly degradable substrate provided (Mould *et al.*, 1983a). This was demonstrated by feeding four chopped roughages (straw, dried grass and two hays) with three sources of rapidly fermentable carbohydrates: barley, maize and molasses. Bicarbonate salts were infused intraruminally to keep the pH at the same level as the high roughage diets. The results showed that the extent of the depression in fibre degradation could not be alleviated completely by the addition of the bicarbonate salts. This may be due to the adaptation of the rumen microflora (the number of cellulolytic microorganisms was lower in diets that caused a low pH). Figure 2.6, shows both pH and carbohydrate components of the associative effects.

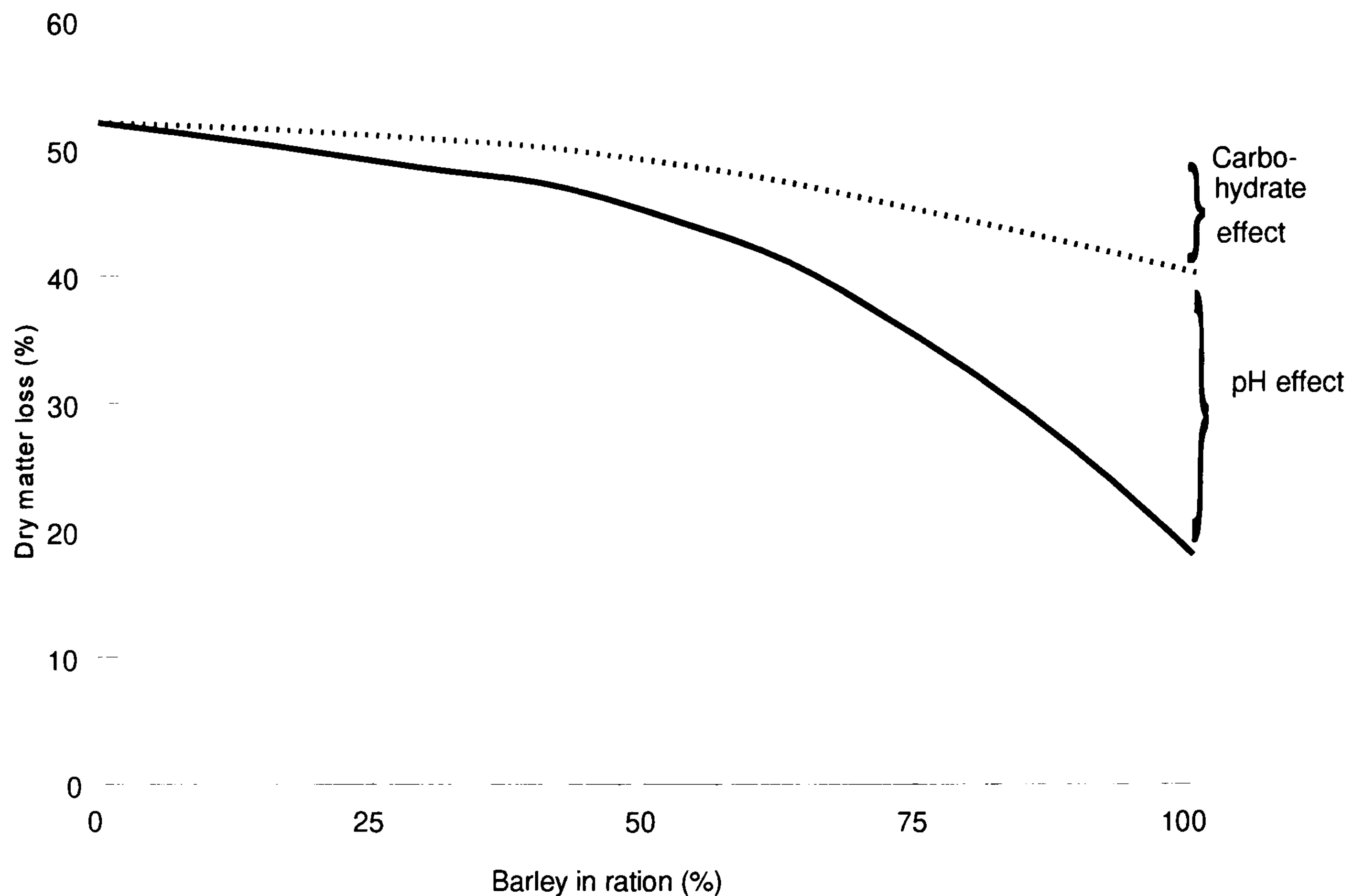


Figure 2.6: *Depression of the dry matter weight loss of ground hay due to the supplementation of a hay-based diet with pelleted whole barley without bicarbonate salt addition (solid line) and with bicarbonate addition (dotted line).*
Source: *Mould et al., (1983a).*

Moss *et al.* (1992) measured the associative effects, *in vivo* and *in vitro*, of a range of mixtures of grass silage:straw, grass silage:maize silage, and maize silage:lucerne silage. For the mixtures of maize:lucerne and maize:grass, negative associative effects were found for the digestibility of the organic matter (OMD) that were suggested to be due to the starch content of the maize inhibiting fibre digestion. However, the inhibition was less marked with mixtures of maize:lucerne. The authors suggested that this was possibly due to the higher buffering capacity of lucerne compared to the grass. The negative associative effects found for the grass:straw mixture were considered to be due to the lack of readily fermentable carbohydrates limiting the utilization of the soluble nitrogen in the mixtures.

Associative effects of energy and protein for a range of mixtures of maize silage and red clover were measured in sheep kept in metabolism crates (Margan *et al.*, 1994). To account for differences in intake, half of each group was fed *ad libitum* and the other half at a level designated to maintain live weight. Results showed positive associative effects for voluntary ME intake, digestibilities of energy, nitrogen (N) and cell wall organic matter, and energy and nitrogen balances. It was hypothesised that the more efficient use of dietary energy and nitrogen could be attributed to improved microbial

synthesis of rumen-degraded clover N and/or synchronous absorption from the duodenum of non-ammonia N from the clover and undegraded maize starch from the silage.

Negative associative effects for the digestibility of fibre were found between mixtures of tyfon (*Brassica campestris*) and hay fed to sheep (Cassida *et al.*, 1994). The inhibition of fibre digestibility (NDF, ADF and cellulose) was attributed to the likely reduction in rumen pH caused by the readily fermentable carbohydrates. Contrary to what is reported in other experiments, associative effects on the digestibility of dry matter were not found. In this experiment, because dry matter and water intake increased simultaneously with the proportion of tyfon in the diet, the effects on dry matter intake, digestibility of organic matter, rate of passage, degradation rates and carbohydrate intake were confounded. Moreover, the addition of hay to tyfon resulted in increased daily weight gain. The authors suggested that these results could be related to a modification of rumen fermentation and indicated the need for further research to elucidate these effects.

Reid *et al.* (1987) studied the effect of feeding mixtures of two grass hays: orchard grass (*Dactylis glomerata*) and perennial ryegrass (*Lolium perenne*) and two legumes: alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*) individually and in different combinations to growing lambs. The effect of an increasing proportion of legume in the mixture on dry matter digestibility (DMD), neutral detergent fibre digestibility (NDFD) and digestible dry matter intake differed with species combination. For all combinations, a quadratic regression for DMD and DNDF indicated a small, negative associative effect for mixtures of grasses and legumes compared with pure species. DMI showed a positive quadratic effect with increasing level of legume inclusion, indicating a positive associative effect on intake. Mineral utilization (apparent absorption and retention) did not differ with species combination, and generally improved with increasing legume content in the mixture. The authors found that with the exception of calcium retention (negative quadratic effect), there was little evidence for significant associative effects between grasses and legumes in mineral utilization.

Glenn (1989) evaluated mixtures of lucerne (*Medicago sativa*) and orchardgrass (*Dactylis glomerata*) in the rumen. To account for differences in intake, the animals (steers) were divided into two groups: one fed on restricted level of the mixtures and the other *ad libitum*. Associative effects were found for rumen microbial protein synthesis and tissue N retention. The author suggested these results indicated synergism in rumen fermentation of NDF and N from mixtures of the two plant species.

With regard to tropical feeds, associative effects were tested *in vitro* using grass:legume mixtures including the grasses bahiagrass (*Paspalum notatum*) and limpograss (*Hemarthria altissima*), and the legumes aeschynomene (*Aeschynomene americana*) and hairy indigo (*Indigofera hirsuta*) (Brown and Pitman, 1991). Positive associative effects in *in vitro* NDF digestion were observed when *Aeschynomene americana* was combined with mature grasses of low nitrogen content. The magnitude of the associative effects was similar to the increase in *in vitro* NDF digestion of pure grass when rumen fluid from a donor animal fed low quality hay plus supplementary nitrogen was used, suggesting that *Aeschynomene americana* provided soluble and/or degradable N to the *in vitro* fermentation. The fermentation kinetics were studied and it appeared that the likely mode of action of the positive associative effects was a reduced lag time in the initiation of fibre digestion. This work highlighted the importance of N from the legumes in the associative effects of the mixtures. It also showed that associative effects may change over time during the process of fermentation, as the associative effects were found at 48 but not at 96 hours.

Associative effects reported in the literature are often concealed by other types of interactions between feedstuffs that can occur in the animal, such as the effect of a supplement or the substitution effect. Although in some cases these effects can be explained on grounds of simple additivity this is not always the case. The difficulty of separating these effects is mainly that the experiments were not set up initially to test, or to explain, the associative effects. Table 2.7, shows examples of the effect of increasing levels of fodder tree supplementation on productivity of different species of ruminants. These feeding trials with fodder tree leaves were design to establish the optimum ratio of supplementation of a basal diet or to study substitution effects on intake. The data show that in all cases there was an associative effect on DM intake and that, bearing in mind the increased DM intake, there may be associative effects on DM digestibility between the basal diet and the leaves from a single fodder tree species. Associative effects of the same type using mixtures of fodder tree leaves may be possible.

To the knowledge of the author of this thesis, there have been no studies so far of associative effects on digestibility of mixtures of fodder tree leaves. It is self-evident that the mechanisms that may produce the associative effects in mixtures of tree leaves are still uncertain. According to the works of Glenn (1989) and Brown and Pitman (1991) (see above) in mixtures of tropical grasses and legumes, the likely mode of action of the associative effects was a synergism in rumen fermentation of NDF and N. This suggests that the mode of action of associative effects of mixtures of fodder tree leaves may be also a synergism in the fermentation of nutrients from the components of the mixture. Several authors (Johnson, 1976; Sniffen *et al.*, 1983; Henning *et al.*, 1991; Sinclair *et al.*, 1993) have

proposed that microbial growth efficiency and hence animal performance may be improved by a synchronisation of energy and nitrogen supply to the rumen. Most authors have defined synchrony in terms of the rates of fermentation of carbohydrates and nitrogen in the rumen (Garnsworthy *et al.*, 1995).

Table 2.7: Effect of increasing levels of forage tree legume supplementation on productivity of cattle and goats.

Browse species	Animal Species	Basal Diet	Level of browse (%DM)	Voluntary Intake g/kg/day	Dietary DMD %
Leucaena leucocephala	Cattle	Grass	0	20.2	42
			20	26.1	44
			40	28.8	46
			60	28.8	44
			100	22.1	51
Leucaena leucocephala	Goats	Barley straw	0	17.9	48
			33	29.5	60
			65	30.9	57
			100	27.1	62
Albizia chinensis	Goats	Hay	0	18.9	46
			27	27.8	56
			61	27.4	49
			100	24.6	48
Sesbania sesban	Goats	Barley straw	0	17.7	48
			33	28.7	61
			66	31.7	64
			100	27.8	64

DM = Dry matter

DMD = Dry matter digestibility

Source: *Norton 1994.*

Johnson (1976) hypothesised that, given three rumen fermentation rates (for soluble sugars, starch and cell wall carbohydrates), the ideal situation in the rumen with regard to the ammonia pool would be that it demonstrates a curve somewhat similar to the carbohydrate fermentation curve, resulting in a synchrony in the fermentation rates (synchrony in the release of nutrients) (see Figure 2.7). Sniffen *et al.* (1983) proposed that rumen fermentation should be balanced in order to maximise a balanced microbial growth (i.e., maximising the digestion of forage cell wall while optimising the growth rate and efficiency of rumen bacteria). According to the author, this could be achieved by a balance of degradable protein and fermentable carbohydrates. This balance can be obtained by synchrony. For example, a rapidly released unit of nitrogen could be used with a slowly released unit of carbohydrate that had been eaten by the animal some hours previously (Garnsworthy *et al.*, 1995). The effect of two diets, synchronous or asynchronous with respect to the hourly supply of energy and nitrogen on rumen

fermentation and microbial protein synthesis, was studied in sheep (Sinclair *et al.*, 1993). The results showed that synchronising the rate of supply of N and energy-yielding substrates to the rumen microorganism can improve microbial protein flow at the duodenum and the efficiency of microbial protein synthesis.

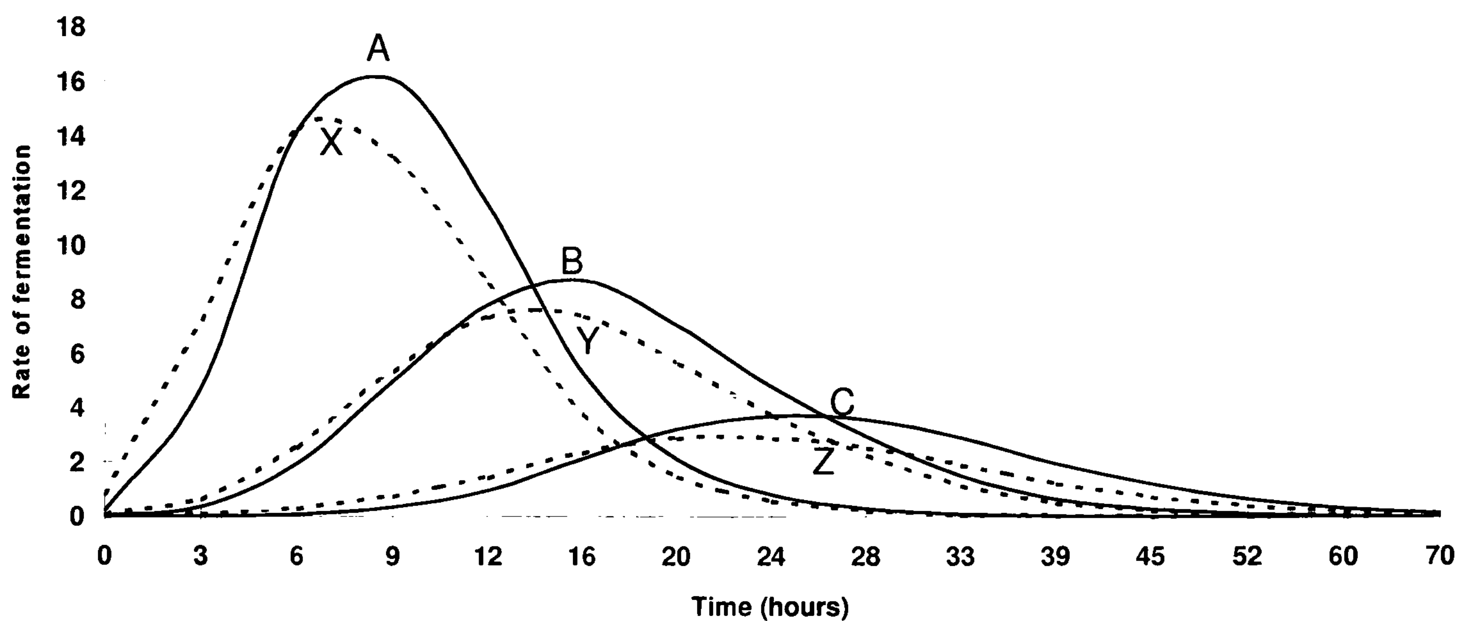


Figure 2.7: *Illustration of the theoretical rumen fermentation rates over time after ingestion of three forms of carbohydrates (A=soluble sugars, B=starches and dextrins, C=cell wall carbohydrates) and rumen ammonia curves (X,Y,Z= N-sources) required to support protein synthesis from the fermentation of these carbohydrates.*
Source: *Johnson, 1976.*

2.6 MIXTURES OF FODDER TREE LEAVES

Farmers in many parts of the world feed mixtures of fodder tree leaves to their animals as a supplement or as the whole ration (Paudel and Tiwari, 1992; Rangkuti *et al.*, 1990; Devendra and Pun, 1990; Gill and Powell, 1993). The use of mixtures ensures a more diverse supply of forages and therefore reduces the risk of dependence on a single plant species. Nitis *et al.*, (1990) described a three-strata forage system in Bali which involves grasses and ground legumes (first stratum), shrubs and legumes (second stratum) and fodder trees (third stratum).

Farmers also overcome and reduce toxicity problems by feeding mixtures of fodder tree leaves with and without sun drying. This process not only extends the choice of feeds available but also dilutes and reduces problems of palatability and side effects. Little is known about the optimum dietary levels of feeds from individual shrubs and trees (especially for those with deleterious principles), about how to reduce the incidence of deleterious effects, nor about suitable mixtures in economic feeding systems for individual ruminants (Devendra, 1993).

Ruminants (especially goats) when left to browse *ad libitum* will prefer a varied diet. Cafeteria trials can be use to determine animal preferences for particular mixtures. Le Houérou (1991) evaluated the intake of 9 species of native and exotic shrubs offered either alone or in a mixture to ewes in Libya. The results showed that consumption of mixed shrubs was higher than that of a single species.

If information on the nutritive and feeding value of many trees and shrubs is scarce, there is even less information on the nutritive and feeding value of mixtures of leaves. Most of the information available involving the use of two or more fodder tree species relates to their replacement value (Van Eys *et al.*, 1986; Richards *et al.*, 1994b; Adejumo, 1995). There are, however, some studies on mixtures that indicate their potential.

Foliage from *Leucaena leucocephala*, *Calliandra calothyrsus* and their mixture (1:1) was used to supplement a diet of maize husks for goats. The daily weight gain of animals supplemented with the mixture *Leucaena:Calliandra* was greater than those animals supplemented with *Calliandra* only (22.6 and 19g). Daily weight gain of animals supplemented with *Leucaena* only was the highest of all (28.5), but dry matter intake (DMI) was also much greater (331.6g/day). There were no differences in the intake of *Calliandra* (315.2g/day) or the mixture (317.4g/day) (Phiri *et al.*, 1992).

Bosman *et al.*, (1995) fed West African Dwarf goats with *Gliricidia sepium* and a mixture of *Gliricidia sepium* combined with *Leucaena leucocephala*. (*Leucaena* only was not included in the experiment). Diets were offered at seven different levels in two experiments varying from 60 to 120g DM/kg$^{0.75}$/day, in increments of 10g, in experiment 1, and from 40 to 130g DM/kg$^{0.75}$/day, in increments of 15g in experiment 2. The maximum DM intake for *Gliricidia* and the mixture *Gliricidia:Leucaena* were, in experiment 1:72.5 and 90 and, in experiment 2:55.5 and 63.4g/kg$^{0.75}$/day, respectively. In both experiments *Gliricidia:Leucaena* mixtures were more digestible than *Gliricidia* alone, the difference in the second experiment being larger (10.3 vs. 3.6 percentage units). Maximum weight gain for *Gliricidia* was 2g /kg$^{0.75}$/day and for the *Gliricidia:Leucaena* mixture was 8.2g /kg$^{0.75}$/day, obtained when offered at a level of 80 and 106g DM/kg$^{0.75}$/day respectively. These studies indicate that a mixture of fodder tree leaves can be used to increase productive parameters over those obtained when a single fodder trees species is used.

The effect of supplements of single fodder tree leaves on increasing intake was shown in 2.5. Research conducted with sheep and goats receiving *Panicum maximum ad libitum* and different rates of mixed *Leucaena:Gliricidia* supplements, showed that increasing levels of browse legume supplementation led to a decrease in intake of *Panicum*, an increase in intake of the legumes and an increase in total DM intake (Reynolds and Adediran, 1988).

Mixtures of fodder tree leaves also have been shown to have important effects on animal reproduction. Reynolds and Adeoye (1989) reported that supplementation of a basal diet of *Panicum* grass with a mixture of *Leucaena leucocephala:Gliricidia sepium* reduced the interval between parturitions. Reynolds and Adediran (1988) showed that supplementation of a grass and cassava peel basal diet with a mixed *Leucaena leucocephala:Gliricidia sepium* feed resulted in significant increases in both growth rate of lambs and their survival to 24 weeks.

Fodder tree leaves are chemically complex feedstuffs which gives them the potential for all kinds of interactive processes in the animal. The interplay, at the digestive level, of nutrients and antinutritional factors can have an important impact on animal productivity. It has been suggested that the deleterious effect of secondary compounds can be overcome by the simple approach of reducing toxicity by feeding the toxic plant in a mixture with other plants, thus diluting the effective level of each compound. The effect of condensed tannins can be overcome by complexing them with polyethylene-glycol (PEG). Lowry (1990) suggested that natural analogues (soluble, non-degradable polyhydroxy compounds) occur in plants, and there is the possibility of a positive interaction between tannin and PEG analogue when the two plants are fed together.

The concept of using mixtures of fodder plants with variable tannin levels to improve nitrogen utilization by ruminants (by reducing soluble protein degradation in the rumen) has been suggested (Barry, 1988; Lascano and Palacios, 1993; Waghorn *et al.*, 1987). Because of the property of binding protein at neutral pH and releasing it at low pH, tannins could be used to reduce the extent of soluble protein degradation in the rumen and thus increase the amount of non-ammonia nitrogen flow to the small intestines (Lascano and Palacios, 1993). This concept is being tested by using the legumes *Cratylia argentea* (tannin-free) and *Flemingia macrophylla* (25.1g CT/kgDM) (Fässler, 1993). Intake, digestibility and nitrogen retention were measured in sheep fed low quality grass (*Brachiaria dictyoneura*) alone, low quality grass (60%) supplemented with *Cratylia argentea* alone (40%) or mixed with *Flemingia macrophylla* at two levels. Results showed that, as the proportion of *Flemingia macrophylla* increased in the mixture, there was a greater faecal nitrogen excretion and reductions in dry matter and fibre digestibility. The positive effects found by a reduction in the amount of urea excreted in the urine were offset by the decline in digestibility. It was concluded that in formulating mixtures to supplement low quality forages it is important to consider not only tannin level but also the digestibility of the legumes used.

In another experiment, *Cratylia argentea* replaced with 0, 25, 50 or 100% *Flemingia macrophylla* was fed as 40% of the total ration as a supplement to *Brachiaria dictyoneura* offered to African Hair sheep

(Powell, *et al.*, 1995). As the intake of *Flemingia* increased, duodenal N flow (as proportion of nitrogen ingested) decreased. This was associated with decreasing rumen ammonia concentration; increasing proportion of nitrogen appearing in the urine, increasing loss of soluble condensed tannin and increasing protein-bound condensed tannins across the rumen. This suggests that N breakdown in the rumen was inhibited by the formation of undegradable protein-tannin complexes between feed protein and soluble tannins. There was an increment in the proportion of ingested nitrogen appearing in the faeces, indicating that post-ruminal digestion of nitrogen was inhibited. The authors concluded that, although there was no apparent benefit in terms of the overall nitrogen retention, tannins from one feed can affect the digestion of nitrogen from another feed.

2.7 AIM OF THE STUDY

This review shows that, given the diversity of fodder tree leaves and their complex chemistry, there is a considerable potential to develop feeding strategies based on strategic mixtures that result in 'enhanced' nutritive value. The interplay, at the digestible level, of nutrients and anti-nutritional factors can be the basis of this 'enhanced' nutritive value. Although associative effects between feedstuffs have been studied before, no study has been done on mixtures of fodder tree leaves. The suggested mechanisms of the associative effects reported in the literature may not be the same for fodder tree leaves as they are chemically more complex than hays and silages. The aim of this work is to begin to understand the factors that determine the associative effects of mixtures of fodder tree leaves and the interactions between tannins and other components of the feed.

Objective

The objective of this work is to develop the scientific knowledge of the mechanisms that govern the associative effects of combining tree leaves on gas production during *in vitro* incubation.

Structure of the thesis

The general objective was achieved by means of:

1) identifying which chemical components of plants have an important effect on the fermentation,
2) identifying plant material covering a range of these components,
3) studying the interactions between those components by modelling their fermentation kinetics using commercially available chemical compounds,
4) studying the interactions between the chemical compounds and the plant material, and
5) studying the interactions of mixtures of plant material from different species in the gas production.

Samples of leaves from 20 different species of fodder trees and shrubs were used as a source of a wide range of chemical components to study their effects on the fermentation (Chapter 4). From this set of samples, 5 different plant species with contrasting chemical composition were selected to study the interactions (Chapter 5 and 6). The effects on and interactions of tannins with the fermentation were studied by using purified tannins, and by means of a tannin binding agent on the selected plant material (Chapter 7). The scientific knowledge gained from these experiments was used to explain and to underline the factors that determine the associative effects of mixtures of fodder tree leaves (Chapter 8). Finally, the implications of the findings of this study in animal nutrition in the tropics, are discussed (Chapter 9).

Chapter 3

MATERIALS AND METHODS

3.1 INTRODUCTION

Samples of leaves from 20 different plant species were collected in Colombia. This diverse data set included trees and shrubs, all with rather different chemical characteristics. The replication refers only to repeats of the chemical techniques on the same samples and only one sample of each species/material is included in the analyses. Therefore the values expressed here are only useful for estimating the variation in these procedures and to compare between methods but are not by any means representative of a particular species. This set of samples was used purely as a source of a wide range of plant chemical components, to understand the importance of the chemical components in the fermentation and to study the associative effects of mixing fodder tree leaves with different chemical composition.

The plant material was analysed for soluble and crude protein, structural and non-structural carbohydrates, ether extract, ash and phenolic compounds. The methods used are routine methods of chemical analysis to determine feed composition and therefore well known. Because of this, only variations from the published method are discussed in the main text. The full detailed description of the methods is given in the appendix. The gas production technique is a relatively new method which was used as the means to achieve the objective of the thesis. For this method, a full description, including variations from the original method, is given in the main text. A comparison of different mathematical models to describe the gas kinetics is made. This chapter also describes the general methodology used to assess the associative effects.

3.2 MATERIALS

COLLECTION OF SAMPLES

Samples were collected in the Cauca valley in the south west of Colombia. This fertile valley is 1000 metres above sea level, with an average temperature of 23 °C. The annual rainfall is between 600 to 1200mm. Preliminary studies published just recently (Rosales *et al.*, 1992; Vargas, 1994) have shown that farmers in the valley region used over 32 different plant species a year to feed their animals. A list

of the 20 species with most potential was made, based on their use by the farmers, palatability and degradability studies. The sample collection was carried out in August 1992 (see Table 3.1).

Table 3.1: *List of the selected forage plants from Colombia for this study.*

Scientific name	Common name	Sort of Plant
Malvasrum sp.	Escoba Blanca	Shrub
Canavalia ensiformis (L.) DC.	Canavalia	Shrub
Dioclea sericea (Humb. Bonpl. & Kunth)	Abrecaminos	Shrub
Sapindus saponaria (L.)	Chambimbe	Shrub
Amaranthus dubius Mart.	Bledo	Shrub
Urera baccifera (L.) Gaud.	Pringamoza	Shrub
Heliconia sp.	Platanillo	Shrub
Tithonia diversifolia (Hemsl.) Gray	Botón de Oro	Shrub
Simphytum peregrinum Lebed.	Confrey	Herb
Bidens pilosa (L.)	Papunga	Shrub
Clitoria ternatea (L.)	Clitoria	Shrub
Inga sp.	Guamo	Tree
Enterolobium cyclocarpum (Jacq.) Griseb.	Orejero	Tree
Erythrina edulis Triana ex Micheli	Chachafruto	Tree
Erythrina poeppigiana (Walp.) O.F. Cook	Cachimbo	Tree
Pithecellobium dulce (Roxb.) Benth	Chiminango	Tree
Prosopis juliflora (Swartz.) DC.	Algarrobo	Tree
Leucaena leucocephala (Lam.) de Wit	Leucaena	Tree
Trichanthera gigantea (Humb. et Bonpl.) Nees.	Nacedero	Tree
Gliricidia sepium (Jacq.) Steud.	Matarratón	Tree

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

3.3 METHODS

For all methods of chemical composition, samples were analysed in duplicate with a maximum acceptable error of ±2%.

PROXIMATE ANALYSIS

The samples were analysed for their chemical composition in terms of dry matter, crude protein, ether extract, ash and organic matter according to conventional methods (MAFF, 1986) (See Appendices 1 to 4 for a description of the methods).

WATER SOLUBLE PROTEIN

The solubility of the protein was measured by suspending 0.5 g of the sample in 100 ml of water in a water bath (39 °C) for 1 hour without shaking, according to the method proposed by Wohlt *et al.*, 1973. The nitrogen content was measured in the liquid fraction obtained after centrifugation (2000 rpm x 10 minutes) by the Kjeldahl analysis (AOAC, 1984) and not in the dry residue after filtration as described in the original method (Appendix 5).

SOLUBLE CARBOHYDRATES

The soluble carbohydrates were extracted after suspending 0.2 g of the sample in 200 ml of water in a water bath at 25 °C with continuous agitation and the concentration of carbohydrates (expressed as glucose) was determined using a spectrophotometer according to the method proposed by Thomas (1977) (Appendix 6).

STARCH, REDUCING AND TOTAL SUGARS

These analyses were carried out by the laboratory of the Post Harvest Horticulture Group of the Natural Resources Institute, Chatham. The starch, reducing and total sugars were estimated by the spectrophotometric method according to AOAC (1984) (Appendix 7).

DETERGENT EXTRACTED FIBRE (ADF, NDF)

The crude lignin, cellulose and silica fractions of the plant material were estimated by measuring the acid detergent fibre (ADF) and the cell wall material (lignin, cellulose and hemicellulose) by measuring the neutral detergent fibre (NDF), as proposed by Van Soest (1975) (Appendices 8 and 9).

EXTRACTION OF THE PHENOLIC FRACTION

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

TOTAL PHENOLS AND CONDENSED TANNINS

The samples were also analysed for total phenols (TP) (low molecular weight phenolics as well as condensed and hydrolysable tannins) by the Prussian blue method (Price and Butler, 1977) (Appendix 10) and condensed tannins (CT) by the acid butanol method (Hagerman and Butler, 1989) (Appendix 11).

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

PROTEIN PRECIPITATION ACTIVITY (PPA)

The method used was derived from that of Hagerman (1987) which assesses the biological effect of the tannins in relation to their ability to precipitate proteins. It relies on the reaction (ring formation) of the plant phenolic extract with the protein present in agarose-haemoglobin plates.

One litre of a buffer solution of 50mM acetic acid and 60mM ascorbic acid was adjusted to pH 5.0 using sodium hydroxide pellets. A 1%W/V solution of agarose (Type 1, Sigma Chemicals Ltd.), was dissolved in the buffer, transferred to a water bath at 38°C and 0.1% haemoglobin was added. Using a pipette, 9.5ml batches of the agarose/protein solution were placed into the required number of 8.5ml

glass petri dishes and allowed to cool. A maximum of 8 wells were made per plate and cut with a cork borer of 4mm diameter. Samples were extracted in duplicate and each extract plated out in duplicate. 15µl of extract were transferred to each well using a Hamilton syringe. When fully loaded, the plates were left until the extracts had penetrated the gel. Plates were sealed with a strip of parafilm and incubated in the dark for 5 days at 30°C. The diameters of the rings of precipitated protein formed were measured using a Vernier scale. Each ring was measured twice, parallel to the circumference of the petri dish and then axially (see Appendix 12 for a detailed description of the method).

IN SACCO DEGRADABILITY

The *in sacco* DM degradability was determined by incubating 3g of dried sample (ground to 1mm) in nylon bags (Ørskov *et al.*, 1980) in the rumens of two Lucerna cows fitted with rumen cannulae. The animals were offered sugar cane tops *ad libitum*. The periods of incubation were 0, 12, 24, 48 and 72 hours after which, the bags were withdrawn, washed manually and dried at 60°C. Duplicate samples were used and incubations were repeated in each animal (n=4). See Appendix 13.

THEODOROU'S GAS PRODUCTION TECHNIQUE

Measurement of gas production based on head-space pressure (Theodorou *et al.*, 1994) is a simple *in vitro* procedure, not requiring expensive glass syringes like the comparable Menke gas production method. Theodorou's gas production technique provides precise data relating to the fermentation kinetics of ruminant feeds. This method has the advantage of enabling a large number of different substrates to be fermented for an extended time, which makes it ideal for comparative studies (Merry *et al.*, 1991).

Culture medium (reagents)

In an *in vitro* system, the first stage of the digestion is carried out by adding rumen liquor mixed with artificial saliva (culture medium). The artificial saliva has three purposes: 1) it acts as a buffer solution to maintain the pH level within the usual limits of digestion and to ensure that the final acid concentration does not exceed that found in the animal; 2) to provide the micro-organisms with macro and micro-mineral solutions to make sure that no mineral will become limiting during the fermentation; and 3) to provide rapidly fermentable nitrogen in the form of urea. The rumen liquid supplies the inoculum of micro-organisms as well as co-factors for efficient digestion, such as valeric acid, protein for bacterial growth and trace elements. To ensure the anaerobic conditions needed, the

medium is gassed with CO_2 and a small volume of reducing agent is added. A redox indicator is also used.

The composition of the culture medium was similar to the artificial saliva used in the Tilley and Terry (1963) procedure. The media for *in vitro* digestibility systems (Tilley and Terry, 1963; Van Soest, 1975; Czerkawski and Breckenridge, 1977; Menke *et al.*, 1979; Merry *et al.*, 1987) are based on the composition of the artificial saliva described by McDougall (1948). Theodorou *et al.*, (1994) used a different medium, the semi-defined Medium B, reported by Lowe *et al.*, (1985) to develop the method.

The medium used here was similar to the so-called Basal Medium D, described by Theodorou and Brooks (1990). The only difference was that trypticase peptone (also used by Van Soest, 1975) was not included. The tryptic (pancreatic) digestion of casein is used in some *in vitro* systems as a source of peptides. Many rumen bacteria need isovaleric, isobutyric and 2-methylbutyric acids for the synthesis of the amino acids leucine, valine and isoleucine respectively. Peptides can replace these requirements, although the reason is not entirely understood (Van Soest, 1975). In this case, as the fodder trees are a source of protein, it was considered that a better understanding of the fermentative potential of the leaves could be obtained if they were left to provide the medium with the only source of protein, peptides and amino acids.

Basal Medium D

This medium was prepared by mixing the following component solutions (in the order shown) to make about 900 ml of the medium. The same proportions were used when more buffer was required (which was always the case).

1. Microminerals	0.1ml
2. Buffer	200ml
3. Macrominerals	200ml
4. Resazurine (redox indicator)	1ml
5. Distilled water	500ml

The medium was saturated with CO_2, and a small volume of reducing agent (2ml per litre buffer) was added prior to bottling. The composition of the different component solutions can be seen in Table 3.2.

Table 3.2: *Component solutions for Theodorou's medium (made up to volume in distilled water)*

1. Micromineral solution (g per 100 ml)

This was made up in 100ml lots and stored at 4°C as a stock solution.

$CaCl_2.2H_2O$	13.2
$MnCl_2.4H_2O$	10.0
$CoCl_2.6H_2O$	1.0
$FeCl_3.6H_2O$	8.0

2. Buffer solution (g per l)

This was made up fresh in variable quantities according to the experiments, following the formulation below.

NH_4HCO_3	4
$NaHCO_3$	35

3. Macromineral solution (g per l)

This solution was also made up fresh in variable quantities according to what was needed for each experiment.

$Na_2HPO_4.12H_2O$	9.45
KH_2PO_4	6.20
$MgSO_4.7H_2O$	0.60

4. Resazurine solution (g per 100 ml)

Resazurine (fresh)	0.1

Theodorou's reducing agent

All the ingredients were added together (in the order specified) in a fume cupboard and stirred under a stream of N_2. The reducing agent was freshly prepared prior to use.

Cysteine $HCl.1H_2O$	625 mg
Distilled water	95 ml
1M NaOH	4 ml
$Na_2S.7H_2O$	625 mg

Procedure

Preparation of the sample

The ground sample was weighed. The amount of substrate depended on the experiment. Generally it was 0.5g total substrate (tolerance of ±0.001g), although 1g total substrate, weighed to tolerance of ±0.002g was used in some of the experiments.

One day before the preparation of the medium, the stock solutions for the medium were prepared according to formulations given above and the serum bottles were arranged in order, placing them on trays for easy handling.

Preparation of the medium

In the early morning, a suitable amount of medium was made up, stirred and gassed with CO_2 for about 2 to 3 hours, then a small volume of reducing agent (about 2 ml per litre buffer) was added. The medium was thoroughly saturated with CO_2 until it changed from a bluish to a pink colour (reduced).

The pre-reduced medium was dispensed (90 ml) into 125 ml serum bottles (Phase Separations Ltd, Clwyd, UK; actual capacity 160 ml, but sold as 125 ml bottles) using an automatic dispenser and gassing with CO_2. Spare bottles were made up to use in preparing the inoculum. The bottles were sealed with butyl rubber stoppers (Bellco Glass Inc., Vineland, NJ, USA), but they were not crimped and were stored at 4 °C to be filled with the substrates the following day.

Placing the samples into the bottles

A suitable amount of reducing agent was prepared in a fume cupboard, as described above, and stirred continuously. A small wide bore funnel was used to transfer the substrates into the bottles and 4ml of reducing agent were added to each. At this moment, any other substrate needed for the experiments (e.g. Casein) was added. Reducing agent was also added to the spare bottles. These additions were made always under a constant stream of CO_2. The rubber stoppers were replaced and they were crimped with aluminium caps. The bottles were replaced in the incubator at 4 °C which was programmed to switch to 39 °C at about 2 am.

Preparation of the inoculum

Rumen digesta was taken from 2 sheep fitted with a rumen fistula by means of a small vacuum pump (Vacuubrand ME2 80mbar) with a hose and a Büchner flask, Pyrex 2000ml, attached to it. The sheep were a Border Leicester crossbred and a Hampshire weighing 100 and 70kg respectively. They were fed with 1.2 and 1 kg/day respectively of meadow hay distributed in two meals. They had also *ad libitum* access to salt blocks and fresh water. The sample was taken from the donor sheep prior to the morning feed. Immediately after collection, the sample was transported in Thermos flasks to the laboratory (1 minute trip).

The fluid was filtered through 4 layers of course cotton muslin and collected in a beaker under an atmosphere of CO_2. The liquid was gently stirred through. The approximate volume of filtered liquid was noted. The solids were transferred to a blender and a volume of medium (using the spare bottles

prepared earlier) approximately equal to the volume of filtered liquid was added. This was blended for about 30 seconds and filtered again through muslin into the beaker with the filtered liquid to be pooled with the original filtered rumen fluid. This inoculum was kept under CO_2.

Inoculation of the bottles

Prior to inoculation, the serum bottles were adjusted to atmospheric pressure as described below, but the gas volumes produced were not noted. The bottles were returned to the incubator at 39°C.

Using a 10ml syringe and 21 gauge 1.5in (0.8 x 40 mm) needles, 5ml of inoculum were injected into each bottle. The bottles were shaken and returned to the incubator. Starting at 10 a.m., the bottles were readjusted to atmospheric pressure, shaken and returned to the incubator. This was taken as the starting point (time=0) of the experiment. The entire procedure is summarised in Table 3.3.

Table 3.3: *Protocol followed for the pre-inoculation and inoculation periods.*

Preparation of the sample	**Days before inoculation day**
Grind and weigh out substrates.	5
Prepare serum bottles.	5
Preparation of the medium	
Make up stock solutions for medium.	3
Make up suitable amount of medium.	2
Dispense medium into serum bottles, seal with butyl rubber stoppers, but do not crimp and store at 4°C.	2
Place the samples into the bottles	
Transfer substrates into bottles and add 4 ml reducing agent.	1
Reseal and crimp with aluminium caps, replace in incubator at 4°C and programme it to switch to 39°C at about 2 am.	1
Prepare inoculum	**Inoculation day (time a.m)**
Collect rumen fluid.	8:45
Filter fluid and prepare inoculum.	9:00
Inoculation of bottles	
Adjust to atmospheric pressure.	9:00
Inject inoculum, shake and return each bottle to the incubator.	9:45
Readjust to atmospheric pressure, shake and return bottles to the incubator.	10:00

Measuring gas production

A pressure transducer (Bailey and Mackey Ltd, Birmingham B42 1DE, UK) was used to measure the headspace pressure in the bottles. The transducer had a range of 0 - 25psi, accuracy of 0.1 ± 2%, and it was calibrated to read in units of psi. It was connected to a disposable Luer lock 3-way tap allowing a needle (23 gauge 1in, 0.6 x 25mm) and a syringe to be fitted to the other outlets.

The gas pressure was read by removing the bottles tray by tray from the incubator, inserting the needle through the butyl rubber stopper into the headspace above the culture medium. The pressure was noted. The pressure was then adjusted to atmospheric by removing gas into the syringe and the volume of gas removed (read from syringe) was noted (see Figure 3.1). When the readings for all of the bottles in the tray were taken, the bottles were shaken and returned to the incubator. Times at which the readings were taken are presented in Table 3.4.

Table 3.4: *Timetable for the gas production readings for a long term fermentation (166 hours).*

Time	Hours after start	Day
13.00	3	Day 1
16.00	6	
19.00	9	
22.00	12	
02.00	16	Day 2
06.00	20	
10.00	24	
14.00	28	
19.00	33	
01.00	39	Day 3
07.00	45	
14.00	52	
22.00	60	
08.00	70	Day 4
20.00	82	
08.00	94	Day 5
20.00	106	
08.00	118	Day 6
08.00	142	Day 7
08.00	166	Day 8

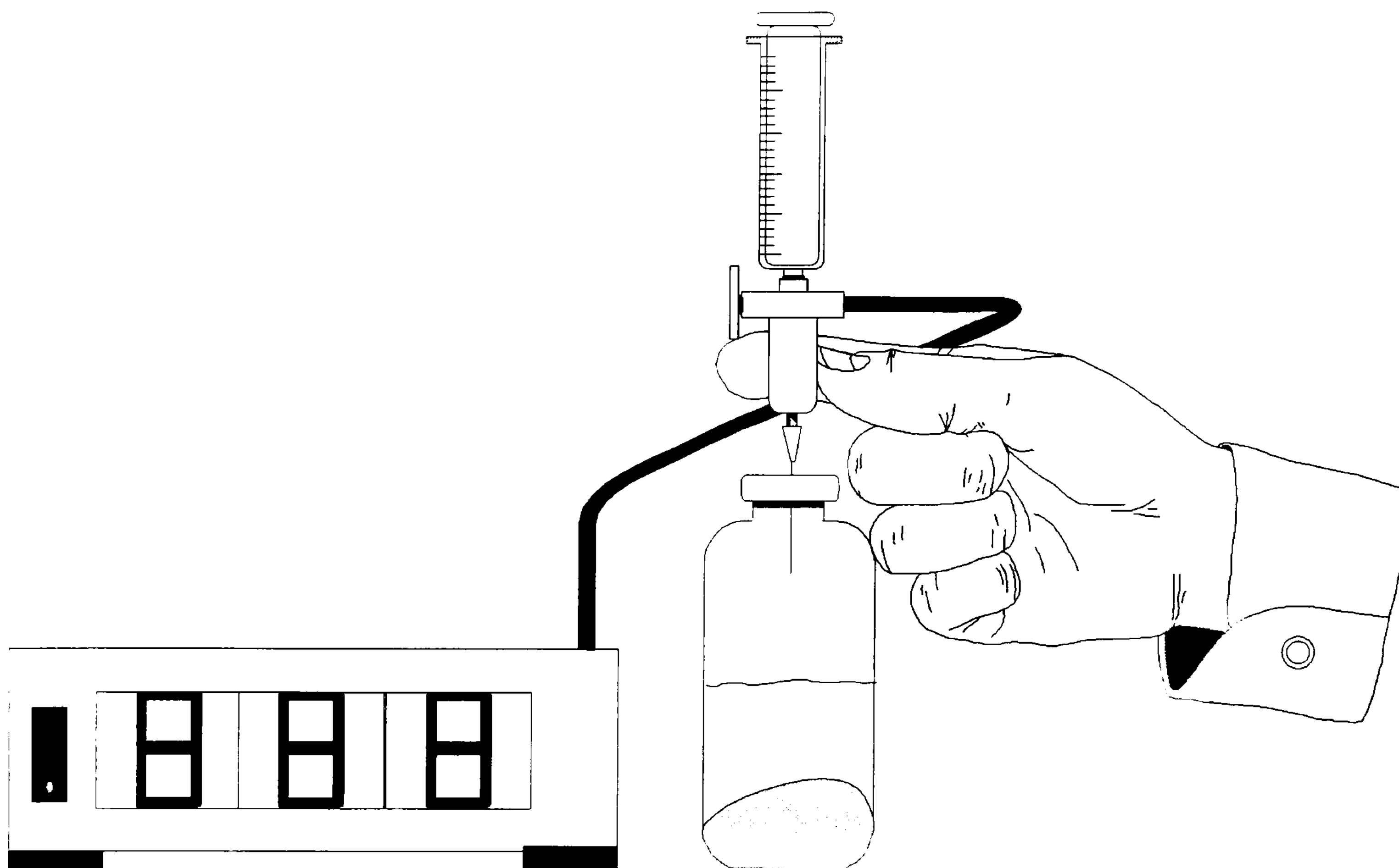


Figure 3.1: *The gas produced was recorded using a needle, syringe and pressure transducer fitted to a 3-way tap and LED digital readout ammeter. The gas was measured as the pressure value indicated on the digital display (psi) and the volume of gas collected in the syringe when readjusting to atmospheric pressure (ml).*

Determination of the dry matter disappearance (DMDp)

At the end of the incubation period, the substrate left in the bottles was recovered by vacuum filtration through pre-weighed filter crucibles (Sintaglass, porosity 1 - regraded P160). Water was used to wash the bottle to remove residues and to wash residues on the filter. The crucibles were oven-dried overnight at 105 °C then allowed to cool in a desiccator and weighed. The DMDp was expressed as a proportion of the initial dry matter in the substrate. After much use, the crucibles tend to become clogged with residual matter so, after each use, the following cleaning procedure was adopted. After recovering the residue, the crucibles were ashed at 500°C overnight. When cooled, water in reverse flow was forced through the filter plate and the crucibles were immersed overnight in a solution of 0.5% ethylene diamine tetra acetic acid (EDTA), then rinsed and washed as normal.

Calculations

Data handling

The gas volumes were expressed per unit dry matter weight according to the following formula.

$$\text{Cumulative Gas Volume (ml) (corrected)} = \frac{\text{Cumulative gas x Nominal Weight}}{\text{Weight of Substrate g x Dry matter (g/kg)/1000}}$$

Sometimes when reading the volume the needle can break or the syringe can be pulled off from the 3 way tap by accident. There were a few cases in which the gas volume could not be recorded at a given time. In these cases, the gas pressure was taken and the volume calculated from a regression equation. This equation was produced using data gathered at random from all fermentations. Figure 3.2 shows the relationship and the regression equation.

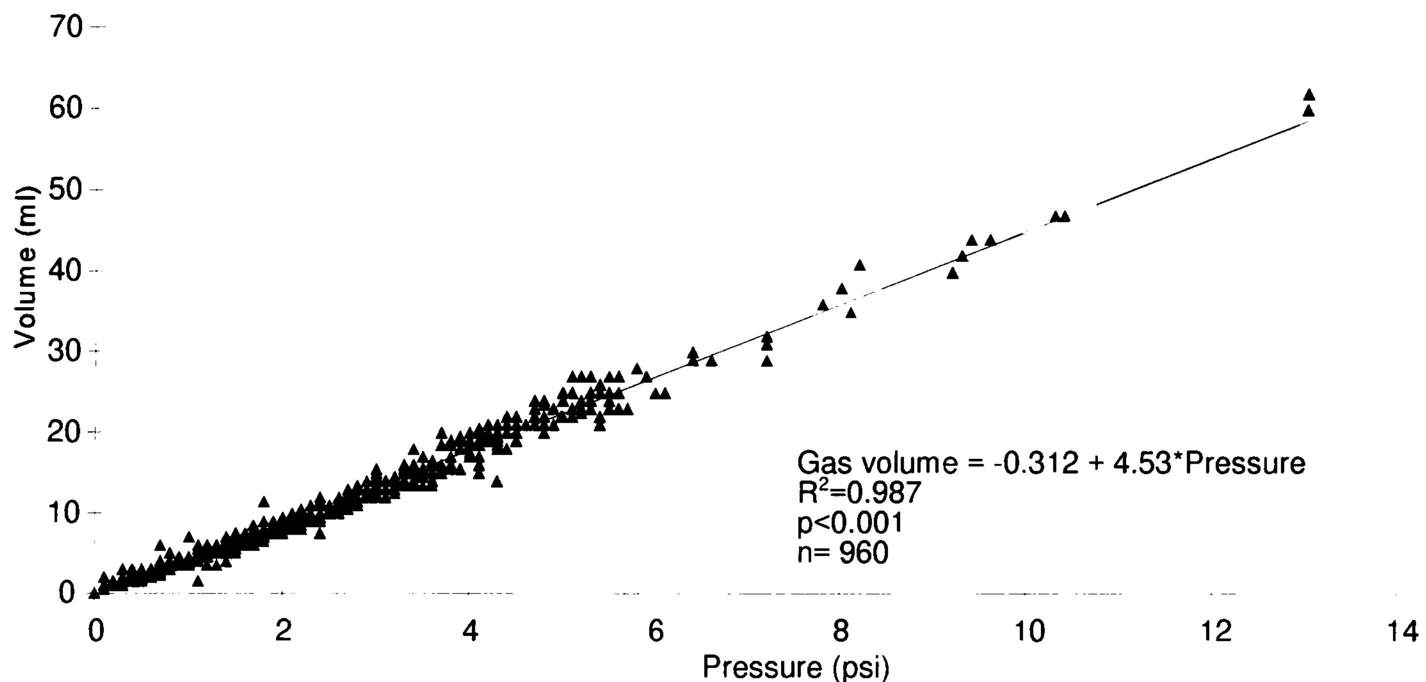


Figure 3.2: *Relationship between volume and pressure for the gas production technique.*

MODIFICATIONS OF THE THEODOROU'S GAS PRODUCTION TECHNIQUE WITH MENKE'S MEDIUM

Only two of all fermentations were carried out with Theodorou's medium. The rest were carried out, using the same gas production technique described above, but the medium was changed. The aim was to have a nitrogen-free medium in which to study the effects of different sources and levels of nitrogen and in order to characterise the tree leaves as sources of nitrogen. A full explanation of the reasons why the medium was changed is given and supported by data in Chapter 4.

The nitrogen-free Menke medium used here has the composition described by Menke *et al.*, (1979). In 1988, Menke and Steingass changed the original composition of the medium by changing the buffer solution from 39 g/l of $NaHCO_3$ to 35 g/l of $NaHCO_3$ plus 4 g/l of NH_4HCO_3 (identical to Theodorou's). However, the use of the first version of Menke was preferred as it contains no nitrogen at all.

Theodorou's medium is similar to Menke (1979 version) but it is less concentrated as more water is added. Apart from nitrogen concentration (Theodorou's medium has 165mgN/l), the main differences are in the macromineral and buffer solutions (as described above) and in the reducing agent.

Theodorou's reducing agent uses cysteine hydrochloric acid, $C_3H_7NO_2S.HCl$, but Menke's does not (to keep the medium free of nitrogen). Due to the absence of cysteine HCl, it takes longer for Menke's medium to be fully reduced. It is therefore necessary to add more reducing agent to speed up the process (Theodorou's uses 2ml of reducing agent per litre of medium whereas 3ml are used with Menke's medium). The composition of the Menke's medium is described below.

Menke's medium

This medium was prepared by mixing the following component solutions (in numerical order).

1. Microminerals	0.1 ml
2. Buffer	200 ml
3. Macrominerals	200 ml
4. Resazurine (redox indicator)	1 ml
5. Distilled water	400 ml

The medium was gassed with CO_2 and small volume of Menke reducing agent (3 ml per litre buffer), was added to reduce it prior to bottling, similar to the treatment of the Theodorou medium. Table 3.5 shows the component solutions for Menke's medium.

The ingredients were added together (in the order specified) in a fume cupboard and stirred but no N_2 was used. The reducing agent was freshly prepared prior to use. The fermentation procedure was followed exactly as described for the Theodorou's gas fermentation technique.

Table 3.5: *Component solutions for Menke's medium (made up to volume in distilled water)*

1. Micromineral solution (same as Theodorou's) (g per 100 ml)	
This was made up in 100ml lot and stored at 4°C as a stock solution.	
$CaCl_2.2H_2O$	13.2
$MnCl_2.4H_2O$	10.0
$CoCl_2.6H_2O$	1.0
$FeCl_3.6H_2O$	8.0
2. Buffer solution (g per l)	
This is made up fresh in variable quantities according to the experiments.	
$NaHCO_3$	39
3. Macromineral solution (g per l)	
This solution was also made up in variable quantities according to what was needed for each experiment.	
Na_2HPO_4 (anhydrous)	5.7
KH_2PO_4	6.2
$MgSO_4.7H_2O$	0.6
4. Resazurine solution (same as for Theodorou medium)(g per 100 ml)	
Resazurine	0.1
Menke reducing agent	
Distilled water	95 ml
1M NaOH	4 ml
$Na_2S.7H_2O$	625 mg

Mathematical models

According to Cheng *et al.* (1980), three phases can be distinguished in gas production curves: 1) the phase of slow or no gas production (initial phase or lag phase), 2) The phase of rapid gas production (exponential phase), and 3) the phase in which the rate of gas production slows and finally reaches zero (asymptotic phase). During the initial phase, hydration, attachment and colonization of insoluble substrate by rumen microbes take place. When the substrate is saturated with microbes or enzymes, the phase of exponential gas production is reached. During this phase the most easily degradable part of the insoluble substrate is degraded first, leaving an increasingly less digestible substrate. Finally, non-degradable material is left and the gas production rate reaches zero. The length and magnitude of each of the these phases depend upon the substrate. The description of such a dynamic system requires an appropriate mathematical model. Most models available to fit the curves produced by the fermentation of a substrate *in vitro* represent a sigmoidal shape, but it is necessary that the model allow for linear and exponential trends as well.

The model proposed for gas production (France *et al.*, 1993) has been criticised for being too complicated and difficult to fit (Huntington, 1995), and for lacking a clear biological basis (Schofield *et al.*, 1994). Recently, Adesogan *et al.*, (1995) tested different available models and suggested that it may be necessary to fit different models according to different substrates. The suitability of different models for fodder tree leaves has not been characterised. Because of these considerations and because of the modifications to the original method, it was necessary to find the appropriate model to describe the gas production curves under the conditions of this thesis.

Several models have been proposed for different gas production systems. The most frequently quoted in the literature for Menke's gas production is that of Ørskov and McDonald (1979), which was developed for the *in sacco* degradability. Other growth models have been tested for Menke's gas production. Beuvink and Kogut (1993) tested several models (exponential, logistic, Gompertz, Richards and Schnute) using grass silage and found that, although Gompertz and Schnute produced a good fit, the best description of the data was given by a modification of the Gompertz model.

The gas production of bacterial cellulose (Cellulon), was studied using the *in vitro* method of Goering and Van Soest (1970) modified to include computer-interfaced pressure sensors (Schofield *et al.*, 1994). Different models were tested (exponential with lag phase, logistic and Gompertz). In this case, the Gompertz and logistic models gave good fits, but the best description was given by a modification of the logistic model, proposed by the authors.

For Theodorou's gas production system, the exponential (Ørskov and McDonald, 1979), modified Gompertz (Beuvink and Kogut, 1993) and France models (France *et al.*, 1993) have been compared using whole-crop wheat (Adesogan *et al.*, 1995). Results showed that the Ørskov and McDonald (1979) model fitted the exponential part of the curve poorly, France's model underestimated the asymptotic phase, while the modified Gompertz gave the most accurate description of the data.

Ørskov and McDonald's (1979) is a single exponential (non-sigmoidal) model that has a single rate and has no lag phase term. This model clearly cannot described the three phases of the fermentation process as the assumption behind the model is that the substrate is fermented instantaneously at maximum rate. This model produced the worst fit in all comparisons previously mentioned. The model was modified by the inclusion of a lag phase term by McDonald (1981). This revised model is the same substrate limited exponential model proposed to describe digestion kinetics by Mertens and Loften (1980).

After considering the various alternatives, it was decided to test the following models: exponential with lag phase (McDonald, 1981), Gompertz, logistic, modified Gompertz (Beuvink and Kogut, 1993), modified logistic (Schofield *et al.*, 1994) and France (France *et al.*, 1993). Table 3.6 shows the mathematical description of the evaluated models.

Table 3.6: *Mathematical description of the evaluated models.*

McDonald	V=C*{1-exp[-B*(t-M)]}
Logistic	V=C/{1+exp[2+4*B1/C1*(M-t)]}
Modified Logistic	V={C1/[1+exp(2+4*B1/C1)*(M-t)]}+{C2/[1+exp(2+(4*B2/C2)*(M-t)]}
Gompertz	V=C*exp{-exp[-B*(t-M)]}
Modified Gompertz	$V=C*exp(-\mu_{0r}/D_r * exp(D_r*t)-\mu_{0s}/D_s *exp(-D_s*t)$
†	$V=C*exp(-B*exp(D_r*t)-C*exp(-D_s*t)$
France	V=C{1-exp[-b(t-M)-c(√t-√M)]}
††	$V=C(Q^t)(Z^{\sqrt{t}})$

V= Gas volume,
C= Gas Pool Size or Total gas production (represents the asymptote).
C1,C2= Specific gas pool size (dual pool model).
B= Gas Production Rate.
B1, B2= specific gas production rates (dual pool model).
M= Lag Phase.
t= Time.
μ_{0r}= Initial gas production rate for the rapidly fermentable fraction.
μ_{0s}= Initial gas production rate for the slowly fermentable fraction.
D_r= Decay constant for rapid gas production (early stages).
D_s= Decay constant for slow gas production (late stages).
b= Rate constant related to the substrate.
c= Rate constant of the lag phase.
†Modified Gompertz functional form.
††France functional form (see main text), where $Q=e^{-b}$ and $Z=e^{-c}$.

MacDonald's model was described previously. The logistic is a very common model also known as the autocatalytic growth function. This is a sigmoidal model which is symmetric about its point of inflection. The modified logistic is a dual pool model which adds two single logistic equations together. It has two gas pool size terms and two rates, but the two lag phases are considered identical. It attempts to explain the fermentation kinetics obtained from mixed substrates (e.g., starch plus cellulose). The Gompertz model is a widely used model in many areas of science. It is similar to the logistic but the difference is in the substrate dependence term; the logistic model assumes a linear and the Gompertz a logarithmic dependence (i.e., rate of growth is proportional to cell mass and growth

rate decays with time due to inactivation of the bacteria).The modified Gompertz is a more elaborate model than the modified logistic. It is a single pool model that divides gas production into two parts, one for the rapid early gas production rates and one for the slower gas production rates during the later stages of fermentation. In the modified Gompertz, the lag phase, maximum gas production rate and time at which gas production rate is maximal can be calculated from the resultant parameters. France's model describes an initial microbe-limiting phase and a secondary substrate-limiting phase. The rate constant of the lag time (C) allows the model to describe exponential and sigmoidal trends. A negative rate may occur during optimization and as the equation does not permit a negative term, the authors suggested fitting the functional form of the model (see Table 3.6). All models have been criticised at some time for lacking precision and/or biological bases.

Comparison of models

Curve fitting was done using the statistical program Genstat 5 Release 3.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, 1993). Up to 100 iterations were used to fit an equation. The statistics used to evaluate a fit were: the residual mean square (RMS), coefficient of determination (R^2) and the standard error (SE). The R^2 was close to unity in all cases and was insensitive to small variations between the models. Therefore it was used as an overall measure of the fit rather than as a basis for choosing between models. The model that gives the most adequate description of the data has the lowest RMS. Strictly speaking, there is not an overall "best" model. Because gas profiles can have different trends, different models may be preferred for different substrates. However, it was necessary to choose one model to compare between experiments. The most suitable model is that which gives a good fit to the data for the majority of substrates under all conditions. Data from different experiments were used to test the suitability of the different models. The kinetics of each fermentation will be discussed in the respective chapters. The models were tested for their suitability for long and short-term fermentations (166 and 70 hours) and for nitrogen content in the medium (0 and 165mgN/l).

Gas production kinetics of fodder tree leaves for a long term fermentation

Samples of 19 fodder tree leaves were fermented with Theodorou's medium for 166 hours and the fermentation kinetics described for all models. Table 3.7 shows the RMS of the different models for each of the forages. The model that had the lowest RMS values was the modified Gompertz, followed by France, McDonald, modified logistic, Gompertz and logistic. These results are in agreement with those found by Adesogan *et al.*(1995). It was concluded that the modified Gompertz was the best model to describe the fermentation kinetics of the gas production technique under these conditions.

Table 3.7: *Residual mean squares of gas production curves of fodder tree leaves fitted using 6 mathematical models. Incubation was carried out for 166 hours with Theodorou's medium.*

	McDonald	France	Gompertz	Modified Gompertz	Modified logistic	logistic
Amaranthus dubius	14.65	15.22	58.89	1.25	18.46	112.7
Malvasrum sp.	10.71	11.18	41.65	0.77	8.81	81.82
Bidens pilosa	27.31	23.23	98.12	0.59	17.25	174.3
Dioclea sericea	1.22	1.22	17.48	2.17	11.93	45.87
Simphytum peregrinum	12.97	13.26	54.42	5	20.61	106.3
Urera baccifera	5.5	4.2	45.26	0.92	10.59	92.43
Canavalia ensiformis	11.7	11.7	36.8	0.47	10.74	78.42
Sapindus saponaria	11.34	10.64	36.98	3.48	16.87	67.58
Tithonia diversifolia	16.09	14.76	46.19	0.55	9.38	103.1
Clitoria ternatea	24.24	18.43	100.8	4.49	31.82	180.4
Erythrina edulis	11.21	11.75	45.7	1.23	14.97	90.63
Enterolobium cyclocarpum	1.96	1.75	11.33	2.04	6.59	23.45
Pithecellobium dulce	7.52	3.23	31.14	0.3	4.3	54.91
Leucaena leucocephala	1.87	1.98	33.35	2.14	15.29	79.17
Trichanthera gigantea	27.08	9.62	10.37	4.65	9.84	35.67
Inga sp.	1.86	0.62	9.39	1.22	3.45	17.49
Erythrina poeppigiana	8.85	2.44	27.54	1.45	7.91	45.02
Gliricidia sepium	21.34	22.04	53.78	0.55	14.32	108.5
Prosopis juliflora	7.03	5.72	7.75	6.26	9.86	18.39

Gas production kinetics of fodder tree leaves for a short term fermentation

The samples were fermented for 70 hours with Theodorou's medium. The RMS of the different fitted equations can be seen in Table 3.8. The model that had the lowest RMS values was France followed by McDonald, Gompertz and logistic. Modified Gompertz and modified logistic could not be fitted to most of the curves. Just 5 curves were fitted by the modified Gompertz and 6 by the modified logistic. These two models rely on the data reaching an asymptote and most of the fodder tree leaves curves did not reach this condition in 70 hours. It was concluded that France was a better model for fodder tree leaves in a short term fermentation with Theodorou's medium.

The same samples were fermented with Menke's medium (no nitrogen) for 70 hours. The fits provided by the different models can be seen in Table 3.9. Under these conditions, (short term fermentation with no nitrogen), the fermentation profiles for fodder tree leaves were lower than previous examples, with increased lag phases and some curves tended towards linearity. The modified versions of Gompertz and logistic were not able to describe the fermentation of these samples. The model with the lowest RMS was France followed by Gompertz, McDonald and logistic. It was decided that France was the most adequate model for these conditions.

Table 3.8: *Residual mean squares of gas production curves of fodder tree leaves fitted by 6 mathematical models. Incubation was carried out for 70 hours with Theodorou's medium.*

	McDonald	France	Gompertz	Modified Gompertz	Modified Logistic	Logistic
Amaranthus dubius	10.21	2.28	8.73	0.95	3.84	29.98
Malvasrum sp.	10.13	2.84	6.66	1.36	------	23.31
Bidens pilosa	14.23	3.01	19.71	------	------	59.37
Dioclea sericea	8.17	5.45	3.04	------	------	6.22
Simphytum peregrinum	13.26	3.55	14.76	1.90	------	43.28
Urera baccifera	12.18	1.47	2.15	------	3.30	10.78
Canavalia ensiformis	10.44	0.66	3.88	0.57	------	21.30
Sapindus saponaria	4.73	2.78	7.84	------	8.41	22.79
Tithonia diversifolia		2.09	2.05	------	------	7.07
Clitoria ternatea	18.76	1.21	13.28	------	------	49.66
Erythrina edulis	2.59	1.38	13.90	------	------	37.89
Enterolobium cyclocarpum	0.83	0.32	4.36	------	------	12.65
Pithecellobium dulce	1.22	1.33	11.64	------	------	25.46
Leucaena leucocephala	3.70	2.60	15.27	------	------	41.26
Trichanthera gigantea	14.05	10.72	8.77	------	------	19.07
Inga sp.	0.59	0.21	2.24	------	0.13	4.03
Erythrina poeppigiana	0.19	0.21	4.35	------	0.20	10.22
Gliricidia sepium	11.23	1.60	17.43	0.37	10.82	59.14
Prosopis juliflora	3.66	3.02	13.67	------	------	33.25

------ The model did not fit the data.

Table 3.9: *Residual mean squares of gas production curves of fodder tree leaves fitted by 4 mathematical models. Incubation was carried out for 70 hours with Menke's medium.*

	McDonald	France	Gompertz	Logistic
Amaranthus dubius	27.32	2.80	4.38	20.21
Malvasrum sp.	0.66	0.71	8.49	20.34
Bidens pilosa	44.83	4.61	7.32	34.56
Dioclea sericea	13.02	1.78	2.01	5.18
Simphytum peregrinum	10.81	1.78	9.36	33.08
Urera baccifera	8.89	1.52	4.41	17.18
Canavalia ensiformis	9.89	7.33	0.92	3.12
Sapindus saponaria	17.95	12.44	7.37	9.42
Tithonia diversifolia	74.06	2.32	2.01	1.76
Clitoria ternatea	9.85	7.09	14.07	28.56
Erythrina edulis	2.89	1.42	9.94	29.53
Enterolobium cyclocarpum	1.26	0.56	2.93	9.05
Pithecellobium dulce	0.61	0.67	8.57	20.32
Leucaena leucocephala	2.83	2.01	10.61	30.16
Trichanthera gigantea	9.42	6.68	6.49	17.70
Inga sp.	0.17	0.10	0.43	0.71
Erythrina poeppigiana	0.21	0.20	2.04	4.44
Gliricidia sepium	7.04	1.04	5.50	19.79
Prosopis juliflora	3.23	1.39	4.90	15.02

Discussion

The fit at 166 hours was better with exponential models than with Gompertz or Logistic. The two latter models are sigmoidal and the long incubation time and high level of nitrogen favour an exponential response. Short term fermentations and low levels of nitrogen increased lag phases and curves tended to have a sigmoidal or almost linear response, especially with slowly fermentable substrates (starch, cellulose and fodder tree leaves high in tannins). Gompertz and logistic equations could be fitted to this type of response. The logistic always gave the worst fit in all cases. This is due to its symmetrical point of inflexion. It will be shown that this is not the case in this fermentation system where the point of inflexion varies according to the substrate.

Initially, it was decided to use France's equation to fit all the data in this thesis as most of the fermentations were carried out for 70 hours. This equation fitted data for the fermentation of glucose at 45 hours extremely well. However it could not be fitted to some of the data for starch and cellulose up to 70 hours, especially with low levels of nitrogen. The reason for this is that the incubations with starch had a very long lag phase (starch exhibits a characteristic "S" shape curve) and the functional form chosen to fit this model does not allow for a lag phase (it may be calculated from acid pepsin digestion of the residue after fermentation). Treatments with cellulose showed curves that approach linearity, and this non-linear model could not be fitted. Other models were also tested including single and modified Gompertz and logistic. The only model that could fit all the curves was the standard Gompertz equation with the constant set to zero. It is important to have the best fit to describe the fermentation kinetics but at the same time it is also important to be able to compare the data across different fermentations. Therefore it was decided to use the modified Gompertz for the 166 hours and France for the 70 hours data (Chapter 4); and Gompertz was chosen as the main model for the experimental part of the thesis (Chapter 5 onwards).

The Gompertz model

The Gompertz model assumes that the microbial growth rate is proportional to the microbial mass M and to a function of the digestible substrate concentration S, i.e.,

$$\frac{dM}{dt} = \mu M F(S)$$

Where μ is a rate constant. The integration of this rate produces the Gompertz model (see Schofield *et al.*, 1994):

$$V=C*exp\{-exp[-B*(t-M)]\}$$

Where:

V= Cumulative gas production

t = Time

C = Gas pool size

B = Rate constant

M = Lag time

Although this curve is sigmoidal like the logistic, it is not symmetrical about its point of inflexion. The time of inflexion occurs when the second derivative of the rate is equal to 0, i.e.;

$$ti = \frac{(Log\ C)}{B}$$

The gas produced at the time of inflexion (C_{ti}) can be calculated by replacing its value in the equation. A complete evaluation of the fermentation kinetics can be achieved by the parameters obtained from the Gompertz model. These parameters are: length of the initial phase (M), rate constant (B), total gas production (C), time at which the rate is maximal (ti) and gas produced at the time of inflection (C_{ti}).

The rate profiles were used to understand the dynamics and fluctuations between the treatments. The rate equation was derived from the Gompertz model (full derivation is shown in Appendix 14):

$$Rate = C*B*exp(-exp(-B(X-M)) * exp(-B(X-M))$$

3.4 ASSOCIATIVE EFFECTS

Theodorou's gas production has proved to be useful for investigating associative effects (Prasad *et al.*, 1994). To study the associative effects it is necessary to test additivity. The method chosen was similar to that followed by Brown and Pitman (1991) to study mixtures of grasses and legumes *in vitro*. In this thesis, all the mixtures were restricted to a 50:50 ratio to obtain the observed values. The expected values were calculated from the values obtained from the fermentation of the individual components of the mixture (100:0 and 0:100 values).

Student's *t* tests were used to test for associative effects on the cumulative gas production from fermentation of the different mixtures at 12, 24, 45 and 70 hours. The null hypothesis was that gas production of a mixture was equal to the sum of the gas production of its individual components

(H_0 {GasAB-GasA-GasB=0}) (testing additivity). The cumulative gas production at a given time of each individual component of the mixture was halved and their means were compared against the observed value as explained below. The variances of each halved individual component and the observed mixtures were determined and a pooled variance was calculated as:

$$\sigma^2 = \frac{(n_{AB}-1)\sigma^2_{AB} + (n_A-1)\sigma^2_A + (n_B-1)\sigma^2_B}{(n_{AB}-1)+(n_A-1)+(n_B-1)}$$

The pooled standard error of ($\bar{x}_{AB}-\bar{x}_A-\bar{x}_B$) was then calculated as:

$$S.E = \sqrt{S^2\left(\frac{1}{n_{AB}}+\frac{1}{n_A}+\frac{1}{n_B}\right)}$$

The value of t for testing the null hypothesis was calculated as the difference between the expected values of the individual components and the observed values of the mixture divided by the standard error {$t_{12}=(\bar{x}_{AB}-\bar{x}_A-\bar{x}_B)/S.E$}; and it was compared against a tabulated point of the t-distribution (5%) with $(n_{AB}-1)+(n_A-1)+(n_B-1)$ degrees of freedom.

Associative effects were tested at a given time as explained before. To compare the fitted parameters, it is necessary to calculate the expected response of a particular mixture, as the sum of its individual components. However, despite the fact that there were enough replicates to calculate the expected response, replicates of one component cannot be assigned to the replicates of the other component, as there they are not true replicates of each other. That is to say, replicate A of individual component 1 cannot be added to replicate A of component 2, to obtain replicate A of the mixture 1-2. Because of this, the curve parameters of the expected profile cannot be tested statistically. However, parameters of the observed value can be obtained and tested.

Chapter 4

THE IMPORTANCE OF CHEMICAL CONSTITUENTS OF FODDER TREE LEAVES FOR THE FERMENTATION

4.1 INTRODUCTION

Chemical analysis of feeds is used routinely to predict digestibility and to assess the most appropriate use of forages as components of diets for ruminants. Prediction of digestibility from chemical composition is based on statistical association between the chemical components and feeding value. Because a single chemical component cannot predict the nutritive value, it is necessary to include several components to improve the prediction and to use multivariate statistical analyses.

Chemical components of tropical feeds have been found to be poorly related to the *in sacco* DMD (Vadiveloo and Fadel, 1992). There is even less certainty about the relationship between chemical composition and the fermentability of feeds using newer methods like Theodorou's gas production.

This chapter studies the chemical components of tree leaves and their contribution to gas production as a way to characterise the plant material and to better understand the fermentation process *in vitro*. In addition, two main modifications of the original gas production method are proposed: the reduction of the incubation period and the change to a nitrogen-free medium. The contribution of the chemical composition to gas production and the time at which this contribution is more important are also studied under the proposed modifications.

4.2 THE RELATIONSHIP BETWEEN GAS PRODUCTION AND CHEMICAL COMPOSITION OF FODDER TREE LEAVES

OBJECTIVES

The objectives of this section are to:

- characterise the plant material according to its chemical composition;
- identify the chemical components that most affect the fermentation and;
- according to the previous results, select a smaller number of tree species for further research.

MATERIALS AND METHODS

Samples of leaves from twenty different plant species from Colombia (see Table 3.1) were dried, ground and analysed for their chemical composition, and *in sacco* DM degradability and *in vitro* fermentability were assessed according to the methods described in Chapter 3. The gas production was carried out for 166 hours with Theodorou's medium. Samples were run in triplicate. All results are expressed on a dry matter basis.

The relationship between mean values for condensed tannins, total phenols and protein precipitation activity were established by linear regressions. This approach was also used to compare the calculated values for the rate constant and degradability from the *in vitro* and *in sacco* methods and to establish the relationship between them.

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

The final model for each time was tested by multiple regression analysis and possible interactions between the chemical components were tested by the General Linear Model (GLM) procedure. Statistical analyses were carried out using the MINITAB statistical package Release 10.1 (1994). The McDonald model and the modified Gompertz equation were fitted to *in sacco* DMD and *in vitro* gas production data respectively using Genstat 5 Release 3.1 (1993).

RESULTS AND DISCUSSION

Chemical composition

Crude and soluble protein

Table 4.1 shows the values of crude and soluble protein for the plant material. The values for crude protein ranged widely from 123 to 303g/kg (*Dioclea sericea* and *Gliricidia sepium* respectively). High values were found for *Leucaena leucocephala, Prosopis julifora*, *Inga* sp., and both *Erythrina* species. Devendra (1992) reported protein values for tree leaves that varied from 140 to 366g/kg for 12 different species. That report, which included data gathered from several references, showed average

crude protein values of 147g/kg for *Gliricidia sepium*, 222g/kg for *Leucaena leucocephala*, and 258g/kg for *Erythrina fusca*. Benavides (1994) found a range of crude protein from 109 to 424g/kg in a data set comprising 24 tree and 22 shrub species.

Table 4.1: *Crude and soluble protein contents of forage plants (on a dry matter basis).*

Plants	Crude protein g/kg	Soluble protein g/kg	Soluble protein as % of crude protein
Shrubs			
Amaranthus dubius	189.0	37.3	19.7
Malvasrum sp.	124.9	13.5	10.8
Bidens pilosa	196.2	57.9	29.5
Dioclea sericea	123.3	13.5	10.9
Simphytum peregrinum	165.3	42.9	26.0
Urera baccifera	216.3	42.3	19.6
Canavalia ensiformis	227.7	45.2	19.8
Sapindus saponaria	237.0	88.4	37.3
Heliconia sp.	223.7	na	na
Tithonia diversifolia	242.7	40.2	16.6
Clitoria ternatea	294.1	74.7	25.4
Trees			
Erythrina edulis	256.2	53.3	20.8
Enterolobium cyclocarpum	156.2	14.9	9.5
Pithecellobium dulce	178.3	37.6	21.1
Leucaena leucocephala	284.1	42.0	14.8
Trichanthera gigantea	178.2	35.4	19.8
Inga sp.	225.8	30.1	13.3
Erythrina poeppigiana	214.7	47.0	21.9
Gliricidia sepium	303.3	129.4	42.7
Prosopis juliflora	234.4	59.1	25.2

Values for soluble protein ranged from 13 to 129g/kg, again being the lowest for *Dioclea sericea* and the highest for *Gliricidia sepium*. This last plant, which is one of the most widely used fodder trees, had a value for soluble protein that equals almost half of its crude protein content. As well as *Dioclea sericea*, *Malvasrum* sp. also had a very low value for protein solubility. Both species also had high values for condensed tannins (see Table 4.5). Other species with high concentrations of condensed tannins such as *Enterolobium cyclocarpum*, *Inga* sp. and *Leucaena leucocephala* also had a very low protein solubility. Pezo *et al.* (1990) reported a very high protein content of 320g/kg for *Erythrina poeppigiana* and a soluble protein content of 49g/kg, contrasting with the 214g/kg and 47g/kg respectively found for this species in this work. The large differences between the samples are not surprising due to the wide variability among plants.

Waldo and Goering (1979) measured the solubility of protein in water for 15 feedstuffs, obtaining values from 460 to 922g/kg. These high values were obtained for corn gluten feed and corn gluten meal respectively. No fodder plants were evaluated. Other values of nitrogen soluble in artificial saliva (Burroughs medium) from 28 to 930 g/kg have been reported for 33 protein supplements (Wohlt *et al.*, 1972). Similar values were obtained by Stern and Satter (1984), ranging from 43 to 512 g/kg in 34 feedstuffs used as primary nitrogen sources. Sehgal and Makkar (1994) reported similar variation from 12 to 411g/kg in 9 protein supplements. Protein solubility was also reported for 17 tropical grasses and 5 legumes by Aii and Stobbs (1980). A range of 186 to 434g/kg in the grasses and from 76 to 447g/kg in the legumes was found. The tannin contents of the legumes were not reported.

Non-structural carbohydrates

The composition of non-structural carbohydrates for the studied plants is shown in Table 4.2. Values for water soluble carbohydrates were, in general, very low. They ranged from 3 to 43g/kg, with the lowest value for *Inga* sp. and the highest for *Trichanthera gigantea.*

The starch content varied from 105.1g/kg for *Erythrina poeppigiana* to 305.6g/kg for *Bidens pilosa.* Apart from *Erythrina poeppigiana*, other fodder trees like *Gliricidia sepium* and *Prosopis julifora* also showed low values for starch. Tree leaves are not thought of as sources of starch. However, *Trichanthera gigantea* showed the highest starch contents among the tree leaves.

With respect to total sugars, the highest value was obtained for *Trichanthera gigantea* and the lowest value for *Amaranthus dubius.* The reducing sugar contents fluctuated from 18.2g/kg and 19.7g/kg, for *Erythrina edulis* and *Amaranthus dubius* respectively, to 95.2 and 91.6g/kg for *Leucaena leucocephala* and *Trichanthera gigantea.* Concentrations of total sugars of 122.9 and 140.8g/kg have been reported for *Leucaena leucocephala* and *Gliricidia sepium* (Vadiveloo and Fadel, 1992).

The content of non-structural carbohydrates, as described here, is not normally reported in the literature. This fraction is usually reported as nitrogen free extract (NFE) which also includes several other components (see literature review). Values of NFE for some of the species (*Canavalia ensiformis,* 364g/kg; *Gliricidia sepium,* 436g/kg; *Leucaena leucocephala,* 460g/kg and *Prosopis juliflora*, 216g/kg) were reported by FAO (1993).

Table 4.2: *Non-structural carbohydrate components of forage plants (on a dry matter basis).*

Plants	Total water soluble carbohydrates g/kg	Starch g/kg	Total sugars g/kg	Reducing sugars g/kg
Shrubs				
Amaranthus dubius	9.1	209.1	31.7	19.7
Malvasrum sp.	13.6	269.2	93.1	64.0
Bidens pilosa	11.0	305.6	42.6	32.7
Dioclea sericea	10.1	194.2	53.2	39.1
Simphytum peregrinum	20.9	174.4	77.3	67.3
Urera baccifera	17.3	171.5	44.3	35.5
Canavalia ensiformis	7.0	125.3	15.2*	30.6*
Sapindus saponaria	22.3	170.6	66.5	37.2
Heliconia sp.	34.3	205.9	120.0	64.1
Tithonia diversifolia	7.6	172.7	39.8	35.0
Clitoria ternatea	15.4	254.3	91.7	75.6
Trees				
Erythrina edulis	12.6	204.8	70.9	18.2
Enterolobium cyclocarpum	15.1	193.4	107.9	71.2
Pithecellobium dulce	10.7	223.2	81.6	48.1
Leucaena leucocephala	18.4	155.9	99.1	95.2
Trichanthera gigantea	43.2	248.2	170.1	91.6
Inga sp.	3.3	192.5	84.4	59.5
Erythrina poeppigiana	12.2	105.1	42.9*	56.3*
Gliricidia sepium	20.7	109.5	88.8	63.2
Prosopis juliflora	11.0	113.2	69.1	10.9

* Considered erratic values - see page 74.

Structural carbohydrates

The composition of the plants in terms of structural carbohydrates is shown in Table 4.3. The cell wall content (NDF) varied from 282g/kg for *Simphytum peregrinum* to 725g/kg for *Dioclea sericea*. As far as trees are concerned, high values were found for *Erythrina edulis* and *Inga* sp. and low values for *Leucaena leucocephala*, *Trichanthera gigantea* and *Gliricidia sepium*.

The lignocellulose (ADF) contents ranged from 218g/kg for *Trichanthera gigantea* to 620 and 628g/kg for *Inga* sp. and *Dioclea sericea*. Low values were found for *Gliricidia sepium* and *Erythrina edulis*. The low values for *Gliricidia sepium* are similar to those cited by Norton (1994) which ranged from 216 to 279g/kg. The same author also reported values from 226 to 234g/kg for *Leucaena leucocephala*, which are not dissimilar from that found in this work.

Table 4.3: *Cell walls and lignocellulose contents of forage plants (on a dry matter basis).*

Plants	Cell Walls g/kg (NDF)	Lignocellulose g/kg (ADF)
Shrubs		
Amaranthus dubius	550.5	368.8
Malvasrum sp.	570.3	468.1
Bidens pilosa	517.3	421.2
Dioclea sericea	724.9	628.2
Simphytum peregrinum	281.8*	344.8*
Urera baccifera	345.7	285.4
Canavalia ensiformis	382.8	376.9
Sapindus saponaria	432.8	344.0
Heliconia sp.	514.6	283.7
Tithonia diversifolia	353.3	304.8
Clitoria ternatea	448.3	346.7
Trees		
Erythrina edulis	612.6	264.2
Enterolobium cyclocarpum	459.2	443.9
Pithecellobium dulce	566.2	457.2
Leucaena leucocephala	308.1	247.5
Trichanthera gigantea	294.1	217.6
Inga sp.	630.8	620.0
Erythrina poeppigiana	455.5	405.0
Gliricidia sepium	298.4	220.2
Prosopis juliflora	318.4*	388.2*

* Considered erratic values - see text.

Vadiveloo and Fadel (1992) found values for NDF of 391 and 341g/kg and for ADF of 242 and 152g/kg, in *Gliricidia sepium* and *Leucaena leucocephala* respectively. These do not differ much from those found here.

In two cases, the value of ADF was higher than the value of NDF (*Simphytum peregrinum* and *Prosopis julifora*). According to Van Soest and Robertson (1985) these apparently erratic ADF values may indicate problems with a particular sample but not necessarily an error. The causes for this are: very badly heat-damaged samples (Malliard products may be partially soluble in neutral detergent), and plants with high biogenic silica and low hemicellulose (biogenic silica is recovered in ADF but largely dissolved in neutral detergent). In any case, the higher figure must be regarded as the true value.

Ether extract and organic matter

Contents of organic matter and ether extract are shown in Table 4.4. Ether extract ranged from 3.9 to 58g/kg. These results are similar to those reported for different species by FAO (1993), ranging from 21 to 65g/kg, and by Norton (1994) from 14 to 60g/kg. Some of the values reported by FAO (1993) for selected species are: *Canavalia ensiformis* 21, *Enterolobium cyclocarpum* 53, *Gliricidia sepium* 43, *Leucaena leucocephala* 20, *Prosopis juliflora* 29, *Simphytum peregrinum* 29 and 33g/kg. In general, fat is low in fodder plants.

Table 4.4: *Ether extract and organic matter contents of forage plants (on a dry matter basis)*

Plants	Ether Extract g/kg	Organic Matter g/kg
Shrubs		
Amaranthus dubius	3.9	811.9
Malvasrum sp.	17.9	934.9
Bidens pilosa	4.6	848.6
Dioclea sericea	18.8	941.2
Simphytum peregrinum	22.0	758.8
Urera baccifera	15.1	730.1
Canavalia ensiformis	24.3	817.1
Sapindus saponaria	10.1	865.8
Heliconia sp.	58.0	892.2
Tithonia diversifolia	14.0	785.9
Clitoria ternatea	13.4	913.6
Trees		
Erythrina edulis	23.6	891.7
Enterolobium cyclocarpum	40.3	884.4
Pithecellobium dulce	9.1	898.2
Leucaena leucocephala	32.2	887.1
Trichanthera gigantea	31.2	804.1
Inga sp.	8.5	909.1
Erythrina poeppigiana	30.7	825.3
Gliricidia sepium	22.6	878.8
Prosopis juliflora	18.1	870.5

Organic matter contents ranged from 730 to 941g/kg for *Urera baccifera* to *Dioclea sericea* respectively. Low contents were found for *Urera baccifera*, *Simphytum peregrinum*, *Tithonia diversifolia* and *Trichanthera gigantea* and high for *Dioclea sericea*, *Malvasrum* sp., and *Clitoria ternatea*. The organic matter contents of *Canavalia ensiformis*, *Gliricidia sepium*, *Leucaena leucocephala*, *Prosopis juliflora* and *Simphytum peregrinum* are not far from those values reported by FAO (1993), of 884, 920, 872, 915 and 731g/kg respectively.

Phenolic compounds

Contents of phenolic compounds such as condensed tannins and total phenols and their capacity to precipitate protein, are shown in Table 4.5. Phenols were present in all plant material and their contents varied widely from 1.7 to 208 optical density (OD)/g (*Urera baccifera* and *Trichanthera gigantea* respectively). Other plants species with low values were the shrubs *Amaranthus dubius* and *Tithonia diversifolia*. High values were found for the shrub *Malvasrum* sp. and for the trees *Leucaena leucocephala* and *Inga* sp.

Table 4.5: *Radial diffusion and phenolic components of forage plants (on a dry matter basis).*

Plants	Protein precipitation activity cm^2/g	Condensed tannins OD/g*	Total phenols OD/g
Shrubs			
Amaranthus dubius	82.9	0.0	10.0
Malvasrum sp.	256.9	254.1	122.6
Bidens pilosa	112.9	0.0	35.0
Dioclea sericea	155.9	222.5	68.8
Simphytum peregrinum	0.0	0.0	71.0
Urera baccifera	0.0	0.0	1.7
Canavalia ensiformis	71.6	0.0	23.3
Sapindus saponaria	72.7	0.0	29.6
Heliconia sp.	50.6	0.0	42.5
Tithonia diversifolia	0.0	0.0	12.3
Clitoria ternatea	80.3	0.0	39.7
Trees			
Erythrina edulis	132.8	0.0	38.6
Enterolobium cyclocarpum	180.6	251.2	87.9
Pithecellobium dulce	114.5	182.3	67.3
Leucaena leucocephala	244.1	284.2	111.2
Trichanthera gigantea	323.5	0.0	208.8
Inga sp.	529.0	595.4	151.9
Erythrina poeppigiana	128.6	0.0	40.3
Gliricidia sepium	163.6	0.0	39.2
Prosopis juliflora	68.3	0.0	49.1

*OD: Optical Density

Unlike total phenols, condensed tannins were found in very few of the samples. The highest value was found for *Inga* sp. (595.4 OD/g) and the lowest for *Pithecellobium dulce* (182.3 OD/g). Valerio (1994) reported a value of condensed tannins by the acid butanol method for *Inga* sp. of 615 OD/g, which is very close to that found in this study.

The main problem when comparing tannin contents with values reported in the literature is that different methods of analysis have been used. Generally tree and shrub leaves contain both types of tannins, but one type is predominant. Condensed tannins have been detected for *Leucaena leucocephala* and *Inga spectabilis* (Rosales *et al.*, 1992), and small amounts in *Gliricidia sepium* (Norton, 1994).

Extracts of most of the forages studied were able to precipitate protein. Three species showed no activity: *Simphytum peregrinum*, *Urera baccifera* and *Tithonia diversifolia*. The highest activity was found in *Inga* sp. All fodder trees showed capacity to precipitate protein, with *Prosopis juliflora* having the lowest activity and *Inga* sp. the highest. *Trichanthera gigantea* showed one of the highest activities.

All plants with condensed tannins showed high protein precipitation activity (PPA), but this was also the case in plants with moderate levels of total phenols. This may indicate that the capacity to precipitate protein is not a property exclusive to condensed tannins or that there are other phenolic compounds that are not detected by the acid butanol method but which have the capacity to bind proteins.

The relationship between protein precipitation activity, condensed tannins (CT) and total phenols (TP) was examined by regression analysis. The capacity to precipitate protein can be partially explained by the condensed tannin content. The regression equation is:

$$PPA = 82.6 + 0.624\ CT$$

$R^2 = 62.6\%$

$p < 0.001$

Total phenol content can also explain the protein precipitation activity. The regression equation is:

$$PPA = 14.3 + 1.99\ TP$$

$R^2 = 67.0\%$

$p < 0.001$

A multiple regression analysis was carried out to establish the effect of both condensed tannins and total phenols on the precipitation of protein. The equation was:

$$PPA = 22.4 + 0.377\,CT + 1.32\,TP$$

$R^2 = 82.3\%$

$p < 0.001$

This suggests that phenolic compounds detected by both methods have the capacity to precipitate protein as discussed earlier or in other words that the radial diffusion method is a good technique for estimating the activity of both type of compounds. The multiple regression model confirms that 82% of the protein reaction can be explained by these two methods.

***In sacco* degradability**

The rumen degradabilities of the samples estimated by the *in sacco* method are shown in Table 4.6. The *in sacco* degradability of the leaves at 72 hours varied from 36 to 96% . The lowest degradability was found for *Inga* sp., as expected from previous experience with this species where its digestibility has been found to be very low (Rosales *et al.*, 1992). Here, its degradability was consistently low (about 32%) from 12 to 72 hours. This forage has the highest amount of condensed tannins and a high NDF. Another forage with low degradability at 72 hours, *Enterolobium cyclocarpum*, was also high in condensed tannins but very low in NDF.

The highest degradabilities were found for *Trichanthera gigantea*, *Tithonia diversifolia* and *Urera baccifera*. In the case of *Trichanthera gigantea*, degradability goes rapidly up to 77% at 12 hours. *Urera baccifera* had a similar pattern of degradability. *Trichanthera gigantea* had very low NDF and ADF and high soluble carbohydrates whereas *Urera baccifera* had medium values for NDF, low ADF and low soluble carbohydrates. None of these plants has condensed tannins. This shows that plants with similar degradability patterns can have very different compositions.

Some data on degradability of fodder tree species indicate a range from 38 to 78% (Skarpe and Bergstrom, 1986). Similar findings were reported by Walker (1980). According to Veereswara *et al.*, (1993), leaves of *Leucaena leucocephala* and *Gliricidia sepium* exhibited a low *in sacco* degradability at 48 hours of 64.5 and 55.9% compared to 73% for *Sesbania grandiflora* at 24 hours. Values reported by FAO (1993) for the *in sacco* degradability at 48 hours of *Leucaena leucocephala*, *Gliricidia sepium* and *Enterolobium cyclocarpum* were 79, 82.1 and 87.6%.

Table 4.6: Rumen dry matter degradability (%) of fodder plants†.

	Incubation Time (hours)				
	0	**12**	**24**	**48**	**72**
Shrubs					
Amaranthus dubius	25.33	45.17	61.25	76.25	78.25
Malvasrum sp.	15.33	47.92	60.92	79.00	82.33
Bidens pilosa	32.33	48.50	68.50	83.83	84.33
Dioclea sericea	26.83	29.92	42.00	57.83	62.50
Simphytum peregrinum	20.67	44.00	56.09	82.00	85.42
Urera baccifera	40.83	72.17	89.25	95.92	95.17
Canavalia ensiformis	26.00	38.50	60.17	78.25	80.92
Sapindus saponaria	43.50	60.17	62.25	71.67	69.33
Tithonia diversifolia	33.00	50.75	83.25	90.17	92.75
Clitoria ternatea	32.67	56.50	70.83	81.00	81.83
Trees					
Erythrina edulis	28.50	36.08	52.08	60.75	63.17
Enterolobium cyclocarpum	27.00	36.75	36.37	43.46	42.88
Pithecellobium dulce	29.33	40.67	49.83	59.17	59.67
Leucaena leucocephala	35.33	47.58	60.17	77.25	82.08
Trichanthera gigantea	47.33	76.83	88.00	93.17	94.50
Inga sp.	18.33	29.50	32.00	34.00	35.33
Erythrina poeppigiana	38.33	50.17	51.42	55.08	56.17
Gliricidia sepium	46.67	67.17	80.17	84.50	86.00
Prosopis juliflora	32.50	63.50	68.42	74.00	73.75

†Average on dry matter basis from two replicates per time in two animals (n=4).

The *in vitro* gas production

The rate and extent of the gas produced by anaerobic rumen bacteria when degrading the fodder tree leaves are shown in Table 4.7. The cumulative gas values were obtained after a period of incubation of 166 hours and fitted by the modified Gompertz equation. The model fitted the data very well. The average R^2 was 99.9%. The cumulative gas production at 166 hours ranged from 81ml to 230ml. The lowest gas production was found for *Inga* sp. which also has the highest NDF and condensed tannin contents. The highest was found for *Clitoria ternatea*, which has the second highest protein content and no condensed tannins. The fermentation of *Bidens pilosa, Trichanthera gigantea*, and *Gliricidia sepium* were among the highest. *Trichanthera gigantea*, as discussed earlier, has very low NDF and high soluble carbohydrates. Whereas *Bidens pilosa* has a medium NDF value, *Gliricidia sepium* with very low NDF has the highest value for soluble protein. None of these plants had condensed tannins.

The forages with the highest fermentation rate for the rapidly fermentable fraction were *Bidens pilosa* and *Simphytum peregrinum* and the lowest for *Dioclea sericea* and *Prosopis juliflora*. The highest fermentation rate for the slowly fermentable fraction were those of *Dioclea sericea* and *Leucaena leucocephala* and the lowest was found for *Gliricidia sepium* and *Sapindus saponaria*.

Table 4.7: *Gas production kinetics of fodder plants.*

Scientific Name	Cumulative Gas 166 h	Gas Pool Size ml	Rate (h^{-1})		*In vitro* Dry Matter Disappearance %
			Rapidly Fermentable	Slowly Fermentable	
Shrubs					
Amaranthus dubius	180.3	186.2	3.88	0.77	70.8
Malvasrum sp.	162.8	166.2	3.39	0.79	52.0
Bidens pilosa	216.9	219.1	4.12	0.82	76.3
Dioclea sericea	175.6	173.7	1.82	1.74	45.2
Simphytum peregrinum	209.1	206.4	3.98	0.81	74.5
Urera baccifera	183.7	184.3	2.91	1.07	62.5
Canavalia ensiformis	173.4	175.3	3.79	0.63	51.2
Sapindus saponaria	177.7	179.5	2.92	0.42	55.5
Tithonia diversifolia	195.4	198.6	3.66	0.76	69.6
Clitoria ternatea	230.0	229.1	3.98	0.90	74.6
Trees					
Erythrina edulis	182.1	183.0	3.53	0.68	60.9
Enterolobium cyclocarpum	117.0	117.4	2.14	0.65	47.5
Pithecellobium dulce	125.6	128.6	2.95	1.04	44.7
Leucaena leucocephala	190.7	189.4	2.74	1.26	58.2
Trichanthera gigantea	206.5	218.6	2.83	0.20	60.2
Inga sp.	80.9	82.6	2.12	1.19	30.7
Erythrina poeppigiana	119.3	120.4	2.79	0.72	41.7
Gliricidia sepium	217.3	219.4	3.55	0.49	69.3
Prosopis juliflora	126.5	127.8	1.97	0.70	47.3

Wood *et al.*(1993) reported a cumulative gas production from 38 to 223ml at 166 hours for leaves of 19 species of Bolivian plants. The values found for *Leucaena leucocephala* (179ml) and *Gliricidia sepium* (193ml) were lower than those found in this work. The gas produced by *Inga marginata* and *Inga ingoides* (82 and 63ml) agree closely with the value reported here for *Inga* sp. Nsahlai *et al.*, (1994), using the *in vitro* Menke's Gas Production technique (Menke *et al.*, 1979), reported values from 21.2 to 64.5ml per 200 mg of dry matter (106 to 322.5 ml per gramme), for 23 accessions of *Sesbania*. Although the technique is different, the results are close to those reported here.

***In vitro* DM disappearance**

The *in vitro* dry matter disappearance varied from 29.7 to 75.2%, with *Inga* sp. having the lowest and *Bidens pilosa* the highest (see Table 4.7). A range from 35.6 to 66% has been reported for other tropical plants (Wood *et al.*, 1993). A wide range of *in vitro* dry matter digestibility from 16.9 to 66.9% has been reported for fodder trees (Vercoe, 1987).

There was a highly significant correlation between the gas pool size and the dry matter disappearance measured by filtration of the residue (R^2=80.5%, $p<0.001$). However a higher coefficient of determination was expected. This relatively poor fit could be due to the presence of soluble material which is not digested by rumen microorganisms or the production of soluble end products rather than gas. Soluble, indigestible material would not be retained by filtration and hence would be considered to be digested even though the gas production method indicates otherwise. These observations agree with those reported by Wood *et al.* (1993).

Relationship between *in sacco* degradability and *in vitro* fermentability.

Figure 4.1 illustrates the correlation between the *in vitro* gas pool size and the *in sacco* degradability. This demonstrates that these two techniques give broadly similar values. Part of the difference may be attributed to the fact that the animals used as donors for the rumen liquid were not the same as those used for the *in sacco* degradability. Other factors that could have an effect are: basal diet, rate of passage and particle size. The *in vitro* dry matter disappearance (IVDMD) at 166 hours did not correlate as well as the gas production with the *in sacco* degradability (R^2 = 50%, $p<0.001$). This could be due to the presence of soluble but unfermentable material as discussed earlier.

Blümmel and Ørskov (1993) used the McDonald (1981) model to describe both the Menke's gas production and the *in sacco* degradability and they related it to intake, digestible dry matter intake and growth rate. The results showed a good correlation between the *in vitro* (ml per 100 mg) and *in sacco* (mg per 100 mg) methods. The coefficients of determination were 93% at 8 hours and 97% at 24, 48 and 72 hours. However, these results are affected by the fact that the *in sacco* degradability was measured at 7 hours. Another important factor is that the experiments were done with barley and wheat straws, as opposed to this work where condensed tannins play an important role in reducing the degradability and fermentability.

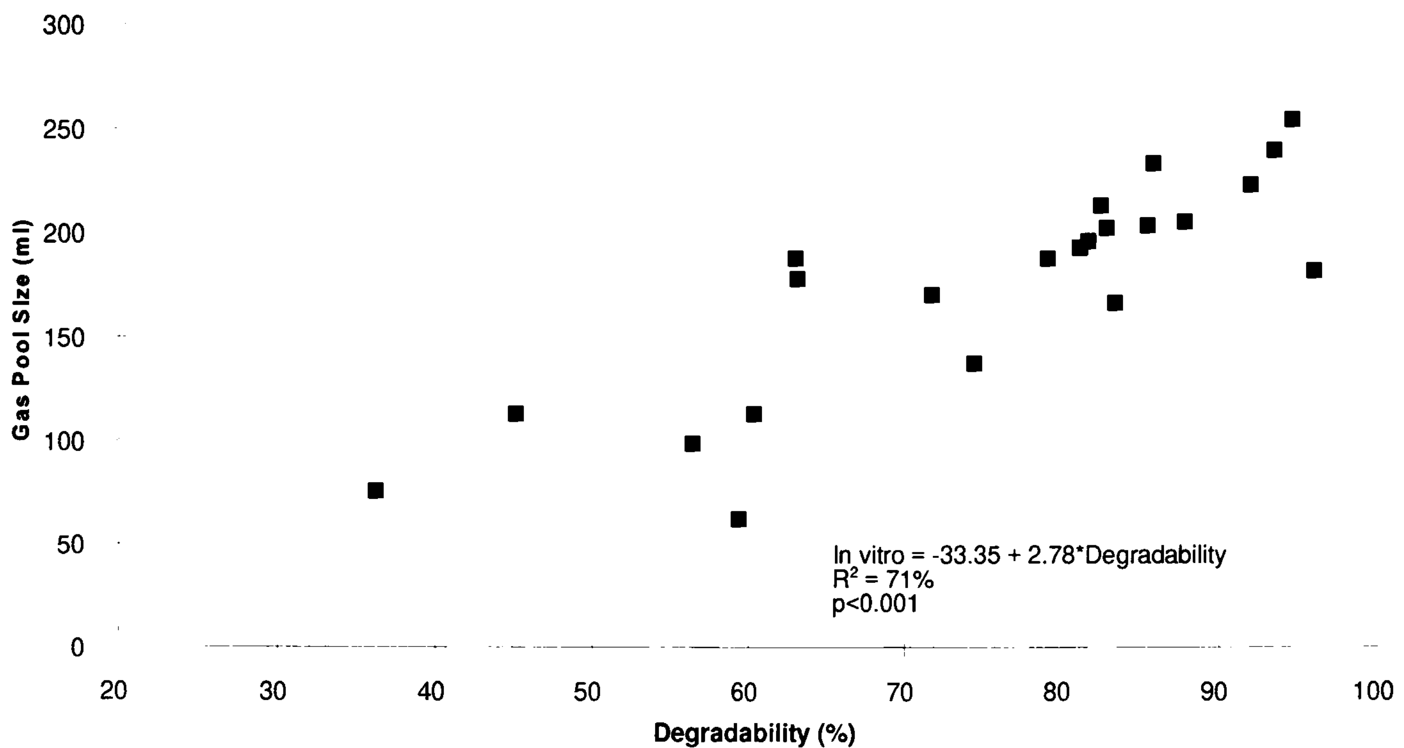


Figure 4.1: *Correlation between in vitro gas production (166 hours) and in sacco degradability (72 hours).*

Kibon and Ørskov (1993) calculated the correlation coefficients between dry matter loss *in sacco* and *in vitro* gas production (Menke) at 6, 24 and 48 hours and found them to be 80, 83 and 90% respectively for 5 tropical browse species, but the correlation (R^2= 58%) was low at 96 hours. The rate constant for gas production was moderately correlated (R^2= 68%) with the rate constant of dry matter degradation obtained by the nylon bag technique. The same exponential equation was used to describe both degradabilities. No correlation between the rates was found in this study (R^2= 1%). In two isolated works but using the same sample material, Soebarinoto *et al.* (1993a) and Soebarinoto *et al.* (1993b) compared the *in vitro* gas production (Menke) and the *in sacco* degradability of ten rice varieties with voluntary intake and *in vivo* organic matter digestibility. The accuracy of the predictions was low (R^2= 40% and 36% for *in vitro* and 52 and 62% for *in sacco*). The two methods were not compared to each other.

Chemical components affecting the fermentation

A data set was prepared to examine the effects of the chemical components on the fermentation. Non-cumulative gas production values were used as the aim here was to know the effects at each particular time. A correlation matrix of the chemical composition was constructed to find highly correlated variables (Table 4.8). The correlation coefficients between all protein measurements were high, so they were divided into soluble and insoluble fractions by subtracting the values of the water soluble protein from those of crude protein. Soluble protein as percentage of crude protein was excluded from the data set.

Table 4.8: *Correlation matrix (Pearson) for chemical components.*

	Prot	SolProt	SolProt%	SolCho	Starch	TotSug	RxSug	EE	Ash	OM	PPA	CTan	TotPhe	NDF
SolProt	0.746													
SolProt%	0.527	0.936												
SolCho	0.015	0.205	0.277											
Starch	-0.382	-0.321	-0.219	0.146										
TotSug	-0.036	-0.038	-0.077	0.757	0.297									
RxSug	0.035	-0.068	-0.149	0.546	0.163	0.741								
EE	0.02	-0.156	-0.26	0.578	-0.224	0.514	0.466							
Ash	0.019	0.038	0.169	0.154	-0.241	-0.288	-0.157	-0.087						
OM	-0.019	-0.038	-0.169	-0.154	0.241	0.288	0.157	0.087	-1					
PPA	-0.094	-0.248	-0.345	-0.002	0.191	0.465	0.44	-0.01	-0.459	0.459				
Ctannins	-0.235	-0.477	-0.579	-0.338	0.121	0.209	0.323	-0.069	-0.52	0.52	0.791			
TotPhe	-0.305	-0.378	-0.38	0.381	0.293	0.755	0.659	0.202	-0.28	0.28	0.819	0.577		
NDF	-0.411	-0.417	-0.406	-0.412	0.425	-0.168	-0.279	-0.196	-0.613	0.613	0.296	0.449	0.016	
ADF	-0.564	-0.527	-0.469	-0.597	0.172	-0.26	-0.181	-0.33	-0.454	0.454	0.373	0.655	0.165	0.711

Where:

Prot: Protein, SolProt: Soluble protein, SolProt%: Soluble protein as percentage of crude protein, SolCho: Soluble carbohydrates, Starch, TotSug: Total sugars, RxSug: Reducing sugars, EE: Ether extract, Ash, OM: Organic matter, PPA: Protein precipitation activity, CTan: Condensed tannins, TotPhe: Total phenols, NDF: Neutral detergent fibre, ADF: Acid detergent fibre.

Total sugars were also not included as they are well related to soluble carbohydrates and to reducing sugars. Some relationship between soluble carbohydrates and total sugars is expected as for both, the soluble components are extracted first (water and 85% ethanol respectively) and treated with acid before the concentration of carbohydrates is determined as glucose. Also, both reducing sugars and soluble carbohydrates are part of the total sugars of the sample. Total sugars and reducing sugars were also highly related to total phenols. This may be a casual relationship or may be due to the fact that some toxic phenols are bound to sugars to prevent self toxicity in the plant. In the cases of *Canavalia ensiformis* and *Erythrina poeppigiana*, the values obtained for reducing sugars were higher than the values for total sugars. These values were considered erratic and therefore excluded from the data set.

PPA values were not included in the data set as they are closely related to condensed tannins and total phenols and because protein precipitation is an effect and not a chemical entity. Condensed tannins and total phenols were not highly related to each other.

Organic matter values were excluded from the analysis due to collinearity. Ether extract and ash were excluded from the data set as, in initial attempts, they were shown to have little effect on the gas production and caused noise in the analysis. Ash is regarded as a measure of the minerals in a feedstuff. However, the medium used for the gas production is sufficient in minerals. Ash was not included in the data set as the contribution from the minerals of the leaves was small or none in preliminary attempts and also caused noise.

Rather than predict the gas production completely from the chemical components, the objective of this analysis was to identify which were the main chemical entities contributing to gas production and the time at which such contributions were more important. The multiple regressions at the different times were identified by stepwise regression analysis.

Results are shown in Table 4.9. The table shows the coefficients of the regression equations at different times. It was possible to fit significant regression equations from 3 to 39 hours. From 45 to 60 hours and at 94 and 166 hours, no a single chemical component could significantly predict the gas production. After 60 hours, although the regressions were significant, they were contributing little to the gas produced. The best prediction equation for the gas produced from the chemical composition was from 0 to 3 hours and used three predictors. That was the maximum number of predictors used in any equation.

Table 4.9: *Coefficients for multiple regression equations for predicting non-cumulative gas production from the chemical composition at different incubation periods.*

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

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

In general, it was not possible with this data set to predict confidently the gas production from the chemical composition from 6 hours onwards, except from 24 to 28 hours when ADF and condensed tannins converged together to decrease the production of gas. Despite the extensive chemical analyses of the leaves, it was not possible to predict the gas production accurately using more than three chemical components at a time. An attempt was made to increase the precision of the prediction by including all chemical components in the data set. The best regression equations were calculated by the stepwise regression analysis. The best prediction was achieved for the gas produced between 0 and

3 hours by the equation:

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

$R^2 = 80.4\%$

$p<0.001$

Where:

SolProt: Soluble Protein

SolCho: Soluble Carbohydrates

PPA: Protein Precipitation Activity

ADF: Acid Detergent Fibre

The second best prediction was found for the gas produced between 12 and 16 hours by the following equation:

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

$R^2=74.1\%$

$p<0.001$

Where:

SolProt: Soluble Protein

TotSug: Total Sugars

ADF: Acid Detergent Fibre

Other equations for different times were identified, but they were considered artificial as they included highly correlated variables for the prediction (e.g., total sugars and reducing sugars). These results show that, it is possible to predict gas production reasonably from the chemical composition in the early stages. It is in the first hours that all chemical components have most effect on the fermentation. However, a prediction equation for the early stages cannot itself predict the extent of the fermentation process.

CONCLUSIONS

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

The selected samples of trees and shrubs showed a wide range of chemical characteristics which was similar to the variability found in the literature. Five tree species were selected for further research.

- *Trichanthera gigantea* as a source of sugars and starch. It is also high in total phenols and has no tannins.
- *Inga* sp. as a source of tannins. It is also high in starch and ADF.
- *Erythrina edulis* as a source of starch and ADF but no condensed tannins.
- *Leucaena leucocephala* as source of protein. It is also high in condensed tannins and total phenols.
- *Gliricida sepium* as a source of protein (soluble and insoluble). It has no condensed tannins and is very low in total phenols.

The proposed modifications, 70 hours incubation period with a nitrogen-free medium, are tested in the next section of this chapter.

4.3 THE RELATIONSHIP BETWEEN GAS PRODUCTION AND CHEMICAL COMPOSITION OF FODDER TREE LEAVES UNDER NITROGEN FREE CONDITIONS.

OBJECTIVES

The objectives of this section are to:

- characterise the plant material according to its response to a nitrogen-free medium for an incubation period of 70 hours and;
- identify changes in the contribution of the chemical components to gas production under the above modifications.

MATERIALS AND METHODS

Duplicates of the 19 tree leaves samples were fermented with two different media: Theodorou's (165mg N/l) and Menke's (N free). Fermentations were carried out according to the protocol described in Chapter 3, but the incubation period was reduced to 70 hours. The sample of *Heliconia* sp. was excluded as there was not enough plant material to carry out the determinations.

The difference in cumulative gas production from the two media was tested at 12, 24, 45 and 70 hours by one-way analysis of variance. These times were selected to resemble those from the *in sacco* DMD. The chemical components involved in the response of gas production to the different media were identified by multiple regression analysis.

Initially France's model was used to fit the response curves. However, it was later changed for the Gompertz model for reasons given in the text. Each replicate was fitted separately and the equation parameters were analysed for significant differences by means of one-way analysis of variance.

The effect of the chemical components on the gas production under nitrogen free conditions was examined by stepwise regression analysis. The chemical composition data set was the same as that used in section 4.2 and the gas values corresponded to the non-cumulative gas production of the plant material with Menke's medium.

RESULTS AND DISCUSSION

The response of the fermentation of tree leaves to the nitrogen content in the medium

There was a significant increment in gas production with Theodorou's medium at 12 ($p<0.001$), 24 ($p<0.001$) and 45 hours ($p<0.05$). The difference in gas production at 70 hours was not significant. The response, expressed as percentage difference between the two media, varied widely from 2.8 to 75% and there was a negative response from *Urera baccifera* after 24 hours. The highest response corresponded to that of *Gliricidia sepium*. In the five selected species, the response to the medium was high for *Inga* sp. and low for *Leucaena leucocephala* and *Erythrina edulis*. The response of *Trichanthera gigantea* was among the lowest. The results are summarised in Table 4.10

Table 4.10: *Response of gas production (%) on the fermentation of fodder tree leaves with Theodorou and Menke media.*†

	Time of response (hours)			
	12	**24**	**45**	**70**
Shrubs				
Amaranthus dubius	40.3	18.0	6.0	4.4
Malvasrum sp.	2.8	24.1	19.6	11.0
Bidens pilosa	42.3	18.3	5.6	3.0
Dioclea sericea	50.8	48.3	42.1	27.5
Simphytum peregrinum	39.7	24.3	10.6	2.1
Urera baccifera	13.3	12.9	-1.3	-11.0
Canavalia ensiformis	61.0	59.6	41.9	28.8
Sapindus saponaria	43.3	41.8	21.7	18.1
Tithonia diversifolia	11.0	50.0	46.7	19.3
Clitoria ternatea	41.4	43.8	29.0	15.6
Trees				
Erythrina edulis	8.7	7.1	4.6	1.4
Enterolobium cyclocarpum	12.9	15.2	6.5	4.3
Pithecellobium dulce	21.8	13.8	9.7	6.8
Leucaena leucocephala	18.8	16.1	11.9	7.7
Trichanthera gigantea	3.9	3.5	4.7	4.4
Inga sp.	59.0	59.9	59.1	57.3
Erythrina poeppigiana	48.9	47.5	41.6	32.3
Gliricidia sepium	75.7	66.5	52.1	40.9
Prosopis juliflora	43.4	26.8	26.3	19.1

† Response calculated as: (Theodorou-Menke)/Theodoroux100

As the main difference between the two media is the nitrogen content the response difference can be described as the response of the microbes to nitrogen from the plant material. The response to nitrogen from the different media cannot be attributed to the amount of carbohydrates in the sample. In the case of *Trichanthera gigantea* for example, the response to nitrogen from the medium was only 4% on

average. This plant material had the highest contents of total sugars, soluble carbohydrates and starch. This may indicate that the plant has sufficient nitrogen to ferment its carbohydrates. In other words, it may indicate an adequate nitrogen:energy balance. In the case of *Inga* sp. the response to nitrogen in the medium was among the highest (60%). According to the chemical composition, this plant material is very high in starch and crude protein. It is more balanced than *Trichanthera gigantea* in terms of the ratio 'crude protein:(starch + total sugars)' (0.81 *Inga* sp. vs 0.42 *Trichanthera gigantea*). *Inga* sp. also has the highest condensed tannin content and the highest protein precipitation activity. So despite having enough protein, it may not be available for the fermentation of carbohydrates. This means that the response to nitrogen in the medium may also indicate, at least partially, the availability of the nitrogen from the plant material. This can also be illustrated with the case of *Gliricidia sepium*, which has a ratio of 'protein:(starch + total sugars)' of 1.5, yet the response to nitrogen was still high (65% from 0 to 45 hours).

A multiple regression analysis between the response to nitrogen and the chemical composition was carried out. The results show that not only carbohydrates are implicated in this response. Soluble protein is also an important contributor to the response to nitrogen. The regression equation for the response at 12 hours was:

$$Response_{12h} = -25.1 + 0.631\ SolPro - 0.126\ Starch + 0.139\ ADF$$

$R^2 = 76.1\%$
$p < 0.001$

Where:
SolPro = Soluble Protein
ADF = Acid Detergent Fibre.

Considering that Theodorou's medium is high in nitrogen, the contribution from soluble protein to the prediction supports the hypothesis that the response to nitrogen from the medium can partially illustrate nitrogen availability of the plant material. The regression equations for the response at 24 and 45 hours support this.

$$Response_{24h} = -4.9 + 0.431\ SolPro - 0.144\ Starch + 0.115\ ADF$$

$R^2 = 60.5\%$
$p < 0.01$

$$Response_{45h} = -3.3 + 0.301\ SolPro - 0.152\ Starch + 0.108\ ADF$$

R2= 53.5

p<0.01

Where:

SolPro = Soluble Protein

ADF = Acid Detergent Fibre.

France's model was fitted to the data and the parameters: gas pool size and rates of increase and decrease were obtained. There were no significant differences between the estimated parameters of gas production with the two media. Although the differences in cumulative gas production were considerable from 0 to 45 hours, this model was unable to show these differences. The Gompertz model was fitted to the data and the rate constant, lag phase and gas pool size were estimated. Results are presented in Table 4.11. The model fitted the data very well. The average R^2 was 99.4% for both media.

Table 4.11: *Fermentation kinetics of fodder tree leaves under two nitrogen contrasting media.*

Scientific Name	Theodorou's medium			Menke's medium		
	Rate (h^{-1})	Lag (h)	Gas Pool Size (ml)	Rate (h^{-1})	Lag (h)	Gas Pool Size (ml)
Shrubs						
Amaranthus dubius	0.074	17.4	125.4	0.076	20.3	120.1
Malvasrum sp.	0.068	18.2	122.4	0.046	22.3	119.1
Bidens pilosa	0.075	18.3	152.7	0.072	22.0	151.6
Dioclea sericea	0.043	27.4	127.8	0.033	39.6	111.8
Simphytum peregrinum	0.081	17.3	133.2	0.059	22.7	140.0
Urera baccifera	0.085	17.3	92.3	0.068	20.8	103.7
Canavalia ensiformis	0.072	17.5	134.9	0.044	31.2	116.3
Sapindus saponaria	0.064	18.0	129.8	0.052	25.6	117.8
Tithonia diversifolia	0.047	33.4	100.0	0.017	97.6	335.0
Clitoria ternatea	0.076	18.4	156.5	0.044	29.3	157.0
Trees						
Erythrina edulis	0.071	16.0	135.0	0.063	17.2	135.3
Enterolobium cyclocarpum	0.073	15.4	80.2	0.065	17.5	78.8
Pithecellobium dulce	0.067	17.4	88.2	0.068	14.9	94.1
Leucaena leucocephala	0.062	17.7	158.7	0.054	20.3	151.2
Trichanthera gigantea	0.044	27.1	181.5	0.044	26.4	171.0
Inga sp.	0.054	13.7	47.2	0.048	15.1	20.6
Erythrina poeppigiana	0.065	16.3	66.4	0.047	23.1	48.2
Gliricidia sepium	0.085	14.5	178.1	0.055	26.0	113.4
Prosopis juliflora	0.056	21.4	134.8	0.047	26.2	116.9

The analysis of variance showed highly significant differences ($p<0.001$) between the fermentation rates of the plant material with the two media. The lag phases were also significantly different ($p<0.01$). There were no differences among the gas pool sizes due to the treatment. The effect of changing to Menke's medium was a decrease in the fermentation rate and an increase in the lag phase. This is expected as, in a nitrogen-rich medium, the fermentation may be faster and start earlier than in conditions lacking nitrogen. This shows that the Gompertz equation can provide a better biological description of the fermentation than France's model.

Effect of chemical components on gas production under nitrogen free conditions

The results of the multiple regression analysis are shown in Table 4.12. It was possible to fit significant regression equations to every period of time. The best prediction equation for the gas produced from the chemical composition was from 39 to 45 hours with four predictors. Soluble protein reduced gas production from 3 to 6 hours. Insoluble protein positively affected gas production from 39 to 45 hours. The soluble carbohydrates increased the fermentation from 33 to 45 hours, whereas starch contributed largely to gas production from 12 to 33 hours and at 45 and 60 hours. Reducing sugars increased the fermentation from 6 to 9 hours. The effect of condensed tannins was a reduction in fermentation from 33 to 52 and at 70 hours, whereas the effect of phenolic compounds was an increase in gas production in the early stages and from 48 to 52 hours. NDF contributed to gas production at 3, 9 and 60 hours. The effect of ADF was a constant reduction in gas production from 0 to 33 hours.

Although significant, it was not possible to give a biological interpretation of the negative effect of soluble protein from 3 to 6 hours. It may be a casual relationship due to the lag phase. The positive effect of phenolic compounds is not surprising since it has been found that phenols can be fermented by rumen bacteria. Polyphenols like quercetin, rutin and gallic acid, present in browse plants, have been shown to be degraded by rumen bacteria (Parrinder, *et al.*, 1991).

A pattern can also be identified with Menke medium. After the first 6 hours, where ADF is reducing the fermentation, the carbohydrate components: reducing sugars, starch and ADF, mainly affect the fermentation from 9 to 33 hours. After this time, other fractions become more important. This is the case with condensed tannins (from 39 to 52 hours). With Menke's medium, the insoluble protein from the forages becomes an important predictor of gas production at 45 hours. The number of predictors and the R^2 value are reduced from 60 to 70 hours. There were no significant interactions between chemical components at any time.

Table 4.12: *Coefficients for multiple regression equations for predicting non-cumulative gas production from the chemical composition at different incubation periods (Menke's medium).*

Predictors	Non-cumulative gas production between times n (hours)														
	3	**6**	**9**	**12**	**16**	**20**	**24**	**28**	**33**	**39**	**45**	**48**	**52**	**60**	**70**
Constant	1.51	10.8	4.33	7.93	8.66	8.14	6.33	7.7	12.1	7.28	-5.06	4.47	4.89	9.34	8.98
Soluble Protein		-0.050													
Insoluble Protein											0.0387				
Soluble Carbohydrates										0.194	0.166				
Starch				0.0212	0.040	0.034	0.0394	0.0436	0.0261		0.0308			0.0252	
Reducing Sugars			0.0556												
Condensed Tannins										-0.011	-0.011	-0.010	-0.010		-0.011
Total Phenols	0.0161											0.021	0.0238		
NDF	0.0088		0.0172											-0.015	
ADF	-0.011	-0.011	-0.025	-0.014	-0.02	-0.017	-0.017	-0.016	-0.021						
R2 (%)	43.8	30.5	51.2	50.2	47	38.4	38.3	37.1	49.2	49.1	68.1	52.2	45	34.7	25.6
p	0.03	0.05	0.01	0.004	0.01	0.02	0.02	0.02	0.004	0.01	0.002	0.003	0.01	0.03	0.03

As for the Theodorou's medium, an attempt to increase the precision of the prediction was made with the full data set. In this case, the best predictions were obtained in the later stages of the fermentation. The best equation was found for the gas produced between 45 and 48 hours and included 5 predictors.

$$Gas_{48h} = -1.20 + 0.0191\ NoSolPro - 0.0333\ NoRxSug - 0.0187\ CT + 0.0393\ TP + 0.00873\ ADF$$

$R^2 = 82.2\%$

$p<0.001$

Where:

NoSolPro= Non-Soluble Protein

NoRxSug= Non-Reducing Sugars

CT= Condensed Tannins

TP = Total Phenols

ADF= Acid Detergent Fibre

The second best prediction equation was found for gas production in the 52 to 60 hours period.

$$Gas_{60h} = 9.08 - 0.102\ NoRxSug + 0.0508\ TP - 0.0206\ CT$$

Where:

NoRxSug= Non-Reducing Sugars

CT= Condensed Tannins

TP = Total Phenols

In general, the predictors for the fermentation with both Theodorou and Menke's medium were the same, but their effects were different. With Theodorou's medium, the soluble components were more important during the first 12 hours, after which ADF and phenolic components became the major predictors for the gas production. Chemical composition best predicted the gas production during the first few hours. This is biologically feasible as, in the Theodorou's medium, the nitrogen may be used to ferment the soluble fractions. This would provide the initial nutrients to the bacteria. In Menke's medium, soluble components are less important at the beginning. After a period of 6 hours (which may represent the lag phase) the fermentation was heavily influenced by the amount of carbohydrates, and ADF becomes the major constraint. After this period (from 33 hours onwards), other components become more important. An important observation is the coincidence of both protein and condensed

tannins at 45 hours. In the case of Theodorou's medium, the best prediction equations were found in the early stages whereas, with Menke's medium, they appeared towards the end of the fermentation.

The relationship between cumulative gas production with a N-free medium and *in sacco* DMD was also investigated. The regression equation was:

$$In\ Vitro\ Gas_{70h} = 3.6 + 1.36\ In\ Sacco_{72h}$$

$R^2 = 42.4\%$

$p < 0.01$

The lower R^2 value compared to the Theodorou's fit may be explained by the fact that the *in sacco* DMD was performed in an environment richer in nitrogen than in the case of the fermentation with Menke's medium.

CONCLUSIONS

There was a positive response to the different media up to 45 hours. It varied largely across plant species and it was related to soluble protein, starch and ADF. In the five selected plant species, the response was greatest in *Gliricidia sepium* followed by *Inga* sp., *Leucaena leucocephala*, *Erythrina edulis* and lowest in *Trichanthera gigantea*. The effect of the N-free medium was a decrease in the rate of fermentation and an increase in the lag phase.

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

Chapter 5

FERMENTATION KINETICS OF CHEMICAL ENTITIES

5.1 INTRODUCTION

According to the results in the previous chapter, the three main types of carbohydrates and two types of protein in the tree leaves were shown to have the greatest influence on gas production. Soluble components (carbohydrates and protein) were important during the first stages of fermentation especially with a high nitrogen medium. With a nitrogen-free medium, insoluble protein and ADF were shown to have important effects. Starch had a significant effect on the fermentation with both media, but it was more important with the nitrogen-free medium. It was concluded that the nitrogen-free medium was more suitable for this study.

The purpose of this chapter is to isolate the individual effects of these chemical components in order to understand their fermentation kinetics and to identify how these effects change in mixtures. This will provide the basis for interpreting associative effects of mixtures of feeds of known chemical composition.

OBJECTIVES

The objectives of this chapter are to:

- measure the time course of gas production from different chemical entities incubated with media of different nitrogen content;
- establish minimum levels of nitrogen for the maximum fermentation of individual chemical components;
- describe the fermentation dynamics of mixtures of chemical entities as a model for the fermentation patterns of a more complex substrate; and
- measure associative effects between mixtures of chemical entities.

To accomplish these objectives, the effects of two proteins of different solubility on the fermentation of different structural and non-structural carbohydrates were measured within a nitrogen-free basal medium (Menke's medium). These fermentations were carried out with different levels of the two proteins to establish the response to nitrogen. The minimum level of nitrogen which produced a significant response would be used subsequently (Chapter 7) to study the effects of tannins.

The fermentation dynamics of pure chemical entities, mixtures and associative effects were studied at the highest level of nitrogen and these were compared with the response under nitrogen-free conditions.

5.2 EFFECTS OF TWO PROTEINS ON THE FERMENTATION OF GLUCOSE

MATERIALS AND METHODS

The effect of different proteins on the gas production of rapidly fermentable carbohydrates was assessed by fermenting D-glucose (BDH AnalaR 10117-4Y) in the presence of bovine serum albumin (BSA) (SIGMA A-7906) and casein (SIGMA C-5890). BSA was chosen as a "soluble protein" with casein as an "insoluble protein". Total and soluble nitrogen contents of the two proteins were determined according to the methods described in section 3.3. Results showed that BSA had 144g/kgN and 90% of the nitrogen was soluble in water, whereas casein had a nitrogen content of 128g/kgN of which 0.17% was soluble in water.

For all fermentations, the amount of initial substrate was 0.5g since initial trials with 1g of glucose showed that the gas produced could build up pressure and slow down or stop the fermentation of purified components especially at high levels of nitrogen.

High levels of carbohydrates could change the pH. This was therefore checked at the end of every batch and found not to change in any of the fermentations in this study. Any pH effects on the fermentation (see Mould *et al.*, 1983b) were therefore excluded.

To enable direct comparison between runs, the gas production of the non-substrate control was subtracted from all measurements on each fermentation in this study.

Initially, it was necessary to carry out a preliminary screening for an adequate range of levels of nitrogen over which to conduct the subsequent experiments. The initial levels were decided after calculating that 165mg/l was the amount of nitrogen present in Theodorou's medium. In an initial experiment, 0.5 g of glucose was fermented with 10 different levels of nitrogen (0, 2.5, 5, 10, 20, 40, 80, 120, 165, 200 mgN/l) from BSA. Both BSA and glucose were dissolved in distilled water and dispensed as liquids. In a separate fermentation run, 0.5 g of glucose was fermented with 8 different levels of nitrogen from casein (0, 10, 20, 40, 80, 120, 165, 200 mgN/l). Both casein and glucose were weighed and dispensed as solids. Both fermentations were carried out for 45 hours. Initial results from

these fermentations suggested that, despite being less soluble, casein fermented faster and produced more gas than BSA. It was concluded that a smaller range (from 0 to 120 mg N/l) was more appropriate to determine the response of glucose to the protein. After these preliminary findings, it was decided to study the response of glucose to both proteins in the same fermentation run.

For this experiment, glucose was fermented with 8 different levels of nitrogen: 0, 5, 10, 20, 40, 60, 80 and 120mgN/l from two sources, BSA and casein, in the same fermentation batch. The experiment was carried out in triplicate for 45 hours. None of the compounds were dissolved before being placed in the bottles.

Statistical analysis

The effects of the increasing levels of BSA and casein on the cumulative gas production at 45 hours of glucose were analysed by one-way analysis of variance. Differences at 12, 24 and 45 hours between the response to BSA and casein at the highest level on nitrogen were also tested by one-way analysis of variance.

To study the fermentation kinetics, the Gompertz equation was fitted to each replicate at the highest level of nitrogen and the parameters: rate, lag phase and gas pool size were tested by one-way analysis of variance. Rate profiles were obtained by replacing the curve parameters in the rate equation of Gompertz model (see section 3.3).

RESULTS AND DISCUSSION

The response curves can be seen in Figure 5.1. There was a significant response ($p<0.001$) of the fermentation of glucose to increasing levels of nitrogen from both BSA and casein. There were no significant differences between the last two levels (80 and 120mgN/l). The response to BSA showed an early peak at 60mgN/l and then started to decline. Comparing the responses to the proteins at the same level, there were no significant differences at 5 and 10mgN/l. An adequate minimum level of nitrogen from both proteins for the fermentation of glucose was calculated to be 20mgN/l.

The dynamics of the fermentation of BSA and casein at the highest level of nitrogen can be seen in Figure 5.2. There were significant differences at each of the incubation times tested (12, 24 and 45 hours). The figure shows that, after a short lag phase, glucose with 120mgN/l ferments faster and produces more gas with casein than with BSA.

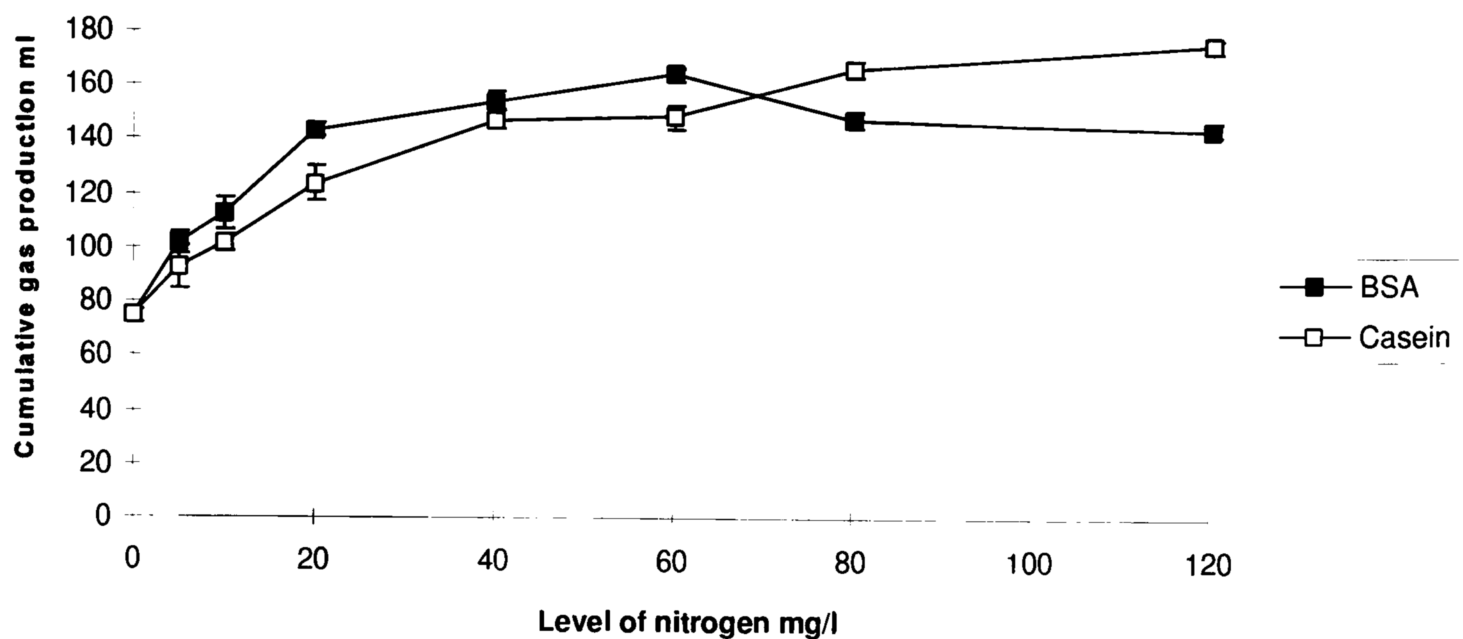


Figure 5.1: *Effect of the level of nitrogen from two sources, BSA and casein on the fermentation of glucose at 45 hours.*

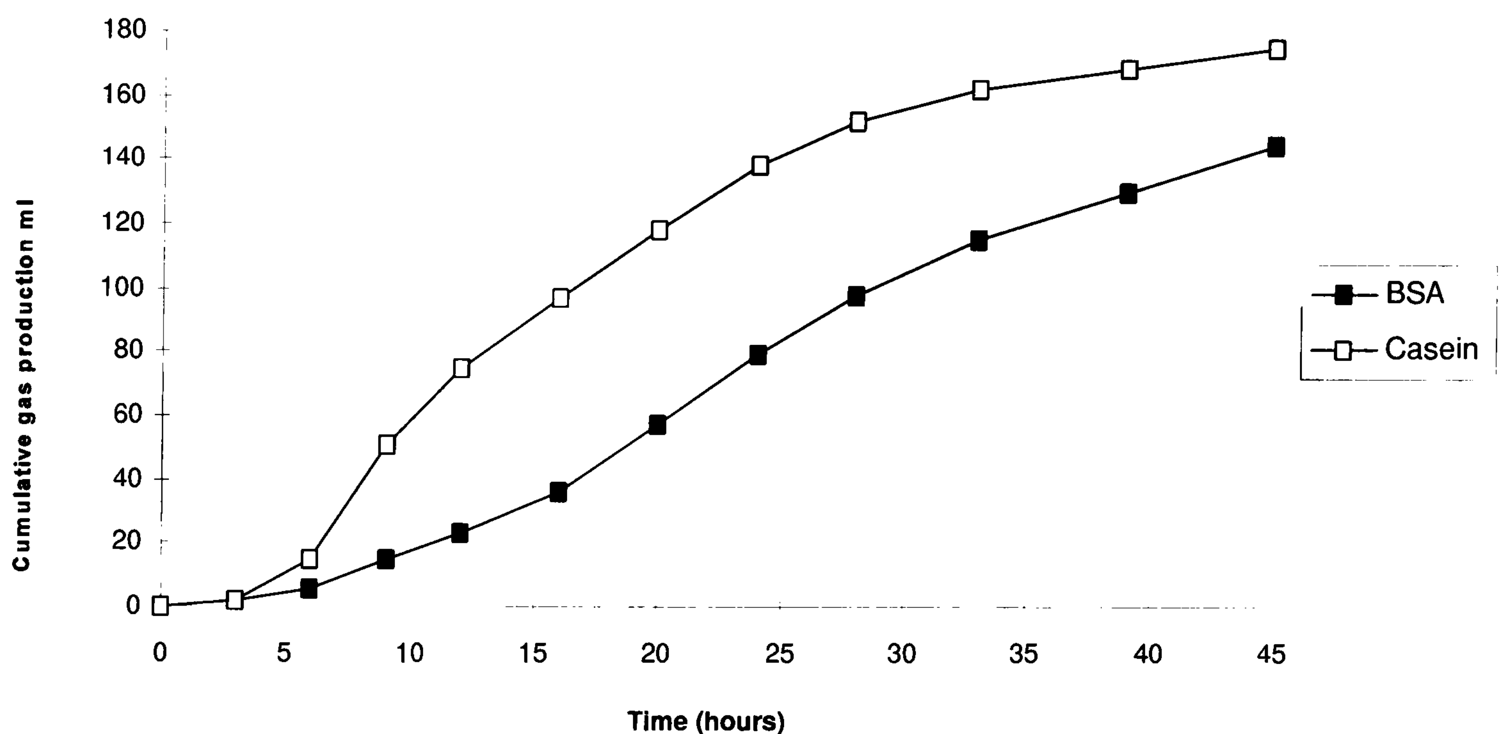


Figure 5.2: *Fermentation profiles of glucose when fermented at a level of 120mgN/l from two proteins*

This shows that casein is a more fermentable protein than BSA. This is despite the extremely low (0.17%) solubility of casein. As glucose is a rapidly fermentable and available material for bacteria, the results illustrate that the nitrogen from casein is more rapidly available for the fermentation of glucose than BSA. Table 5.1 shows the estimated parameters for both responses. The model fitted the data very well ($R^2 > 99\%$). The results confirm that casein ferments faster and with a shorter lag phase than BSA.

Table 5.1: *Gompertz curve parameters for the fermentation profiles of glucose with two proteins at a level of 120mgN/l.*

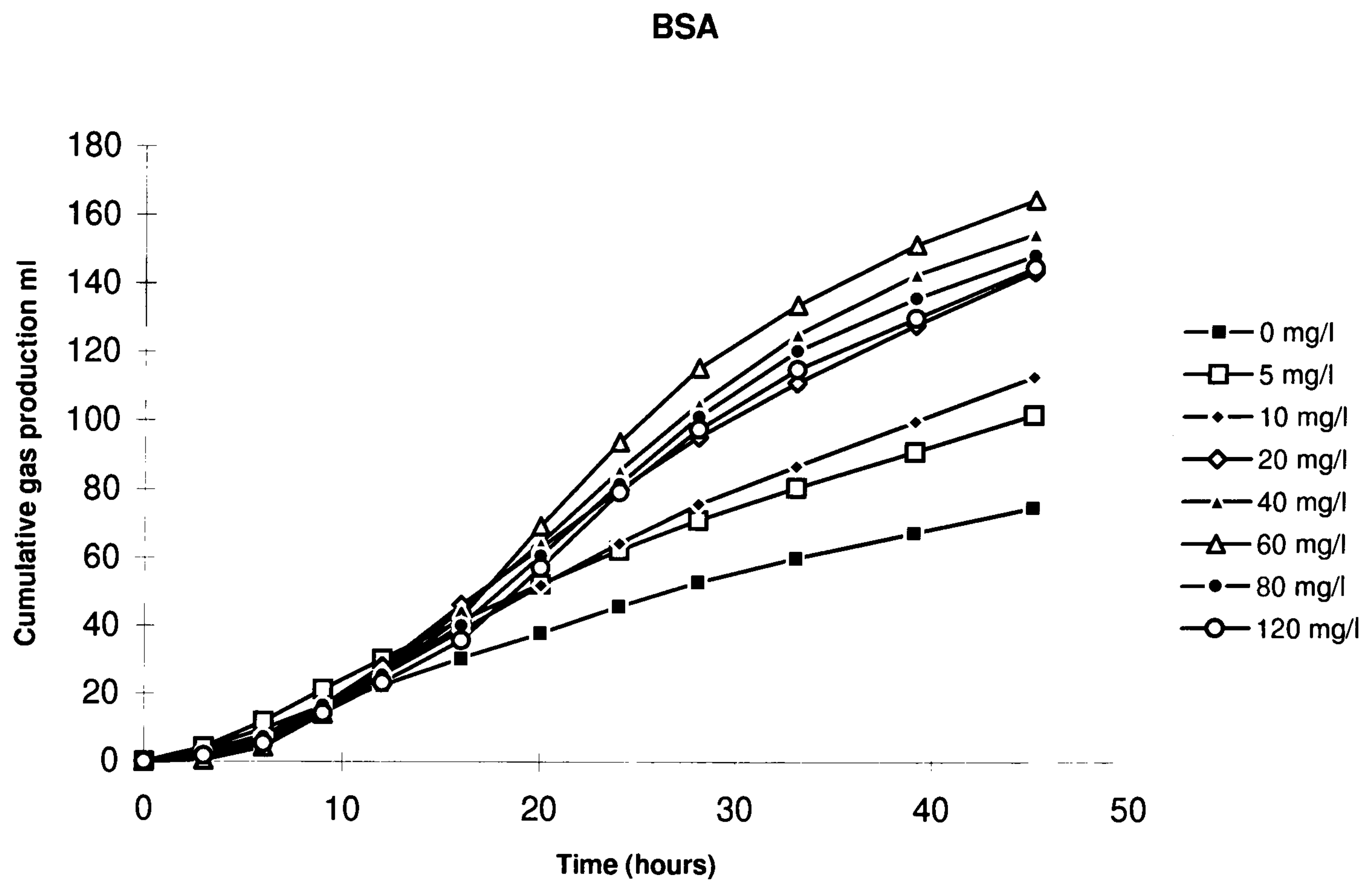
	BSA	Casein	S.E.D (4 df_{error})
Rate (h^{-1})	0.083	0.125	0.00627
Lag phase (h)	20.4	11.6	0.55
Gas pool size (ml)	163.8	174.5	5.64

The same type of response was obtained across all levels. Figure 5.3 shows the time course of gas produced from fermentation of glucose at all levels of BSA and casein. Response curves to BSA show no noticeable differences between levels from 0 to 18 hours whereas, for casein, this period is shorter (from 0 to 6 hours). This suggests that during each of these periods the availability of nitrogen is the same at all levels. In other words, this indicates that BSA releases nitrogen more slowly than casein and this is independent of the level of BSA.

This can be supported by studying the profiles of the fermentation rates. Figure 5.4 shows the fermentation rates of the glucose with each protein at the highest level of nitrogen. When glucose was fermented in the presence of BSA, the maximum rate was obtained at 20 hours. When fermented with casein, the fermentation rate curve peaked at 12 hours, that is 8 hours earlier than with BSA. As glucose is a rapidly fermentable substrate, this difference appears to be due exclusively to the availability of nitrogen from each protein.

BSA produced more gas between 0 and 60mgN/l than casein, whereas this situation was reversed between 60 and 120mgN/l (see Figure 5.1). This indicates that at the lowest levels (up to 60mgN/l), casein is less fermentable than BSA, despite releasing nitrogen more quickly. At the higher levels, the greater gas production from casein may be due to the fact that more nitrogen is present for fermentation than with BSA and not that it is more fermentable.

The general view that soluble proteins are rapidly fermented is not completely supported by these findings. BSA is more soluble than casein, and yet it appears to release nitrogen more slowly. However, this released nitrogen is more fermentable than that from casein. The results also show that it is possible to manipulate the fermentation of glucose by adding proteins of different characteristics.



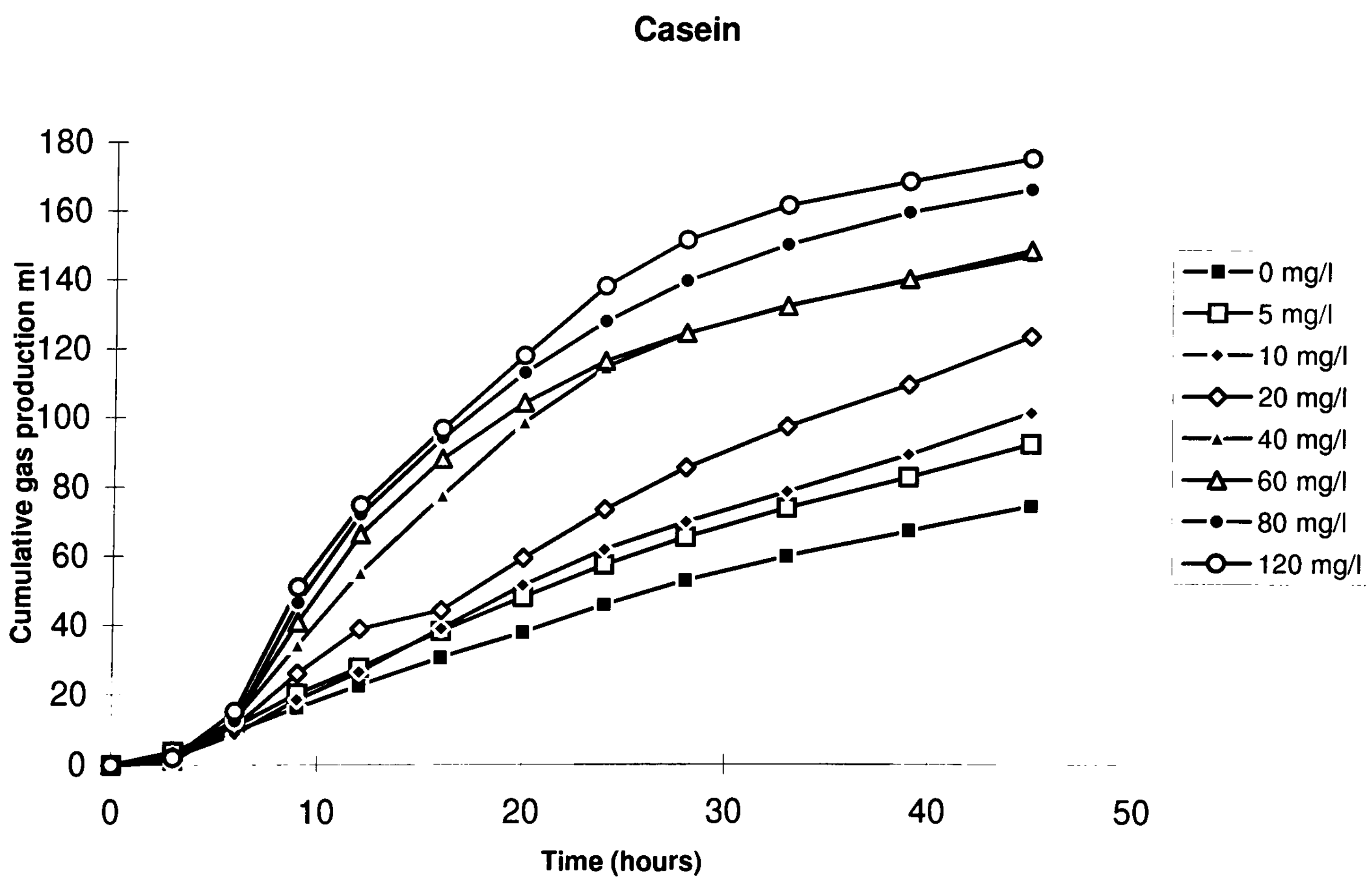


Figure 5.3: *Response of glucose to increasing levels of nitrogen from BSA and casein at 45 hours.*

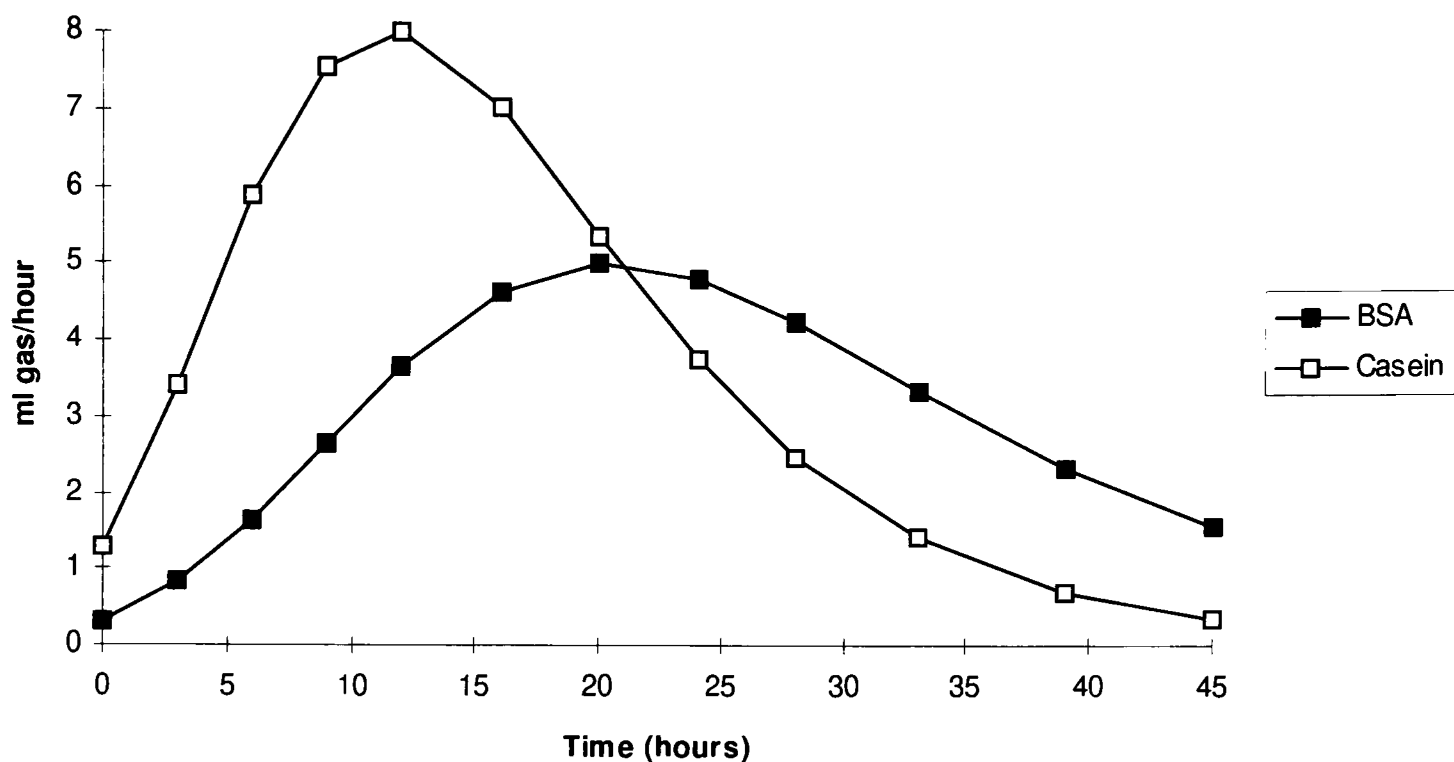


Figure 5.4: *Gas production rates (ml/h) of glucose fermented with BSA and casein at 120mgN/l.*

5.3 EFFECTS OF TWO PROTEINS ON THE FERMENTATION OF STARCH

MATERIALS AND METHODS

The following experiments with starch and cellulose were carried out with a 70 hour incubation period as these substrates were expected to be less fermentable. It was decided to have a maximum level of 80mgN/l as in the previous experiment with glucose there were no significant effects of increasing the nitrogen concentration beyond this point. Cassava starch (0.5g) was fermented separately with different levels of BSA and casein (0, 5, 10, 20, 40, and 80mg N/l) for a period of 70 hours in Menke's medium. This type of starch is widely available in the tropics. It was freshly extracted by the Post Harvest Horticulture Group of the Natural Resources Institute.

Statistical analysis

The effects of the increasing levels of nitrogen on the cumulative gas at 70 hours were analysed by one-way analysis of variance. The same analysis was carried out to test differences at 12, 24, 48 and 70 hours between the responses to BSA and casein at the highest level of nitrogen. Curve parameters were obtained and tested as in the previous experiment with glucose.

RESULTS AND DISCUSSION

There was a significant response ($p<0.001$) in the fermentation of starch to the increasing levels of nitrogen from both BSA and casein (Figure 5.5). There were no significant differences for the increments between the levels 0 and 20mgN/l for each protein but the differences were significant after that point. Therefore it was decided that the adequate minimum level of nitrogen from both proteins for the fermentation of starch was 30mgN/l.

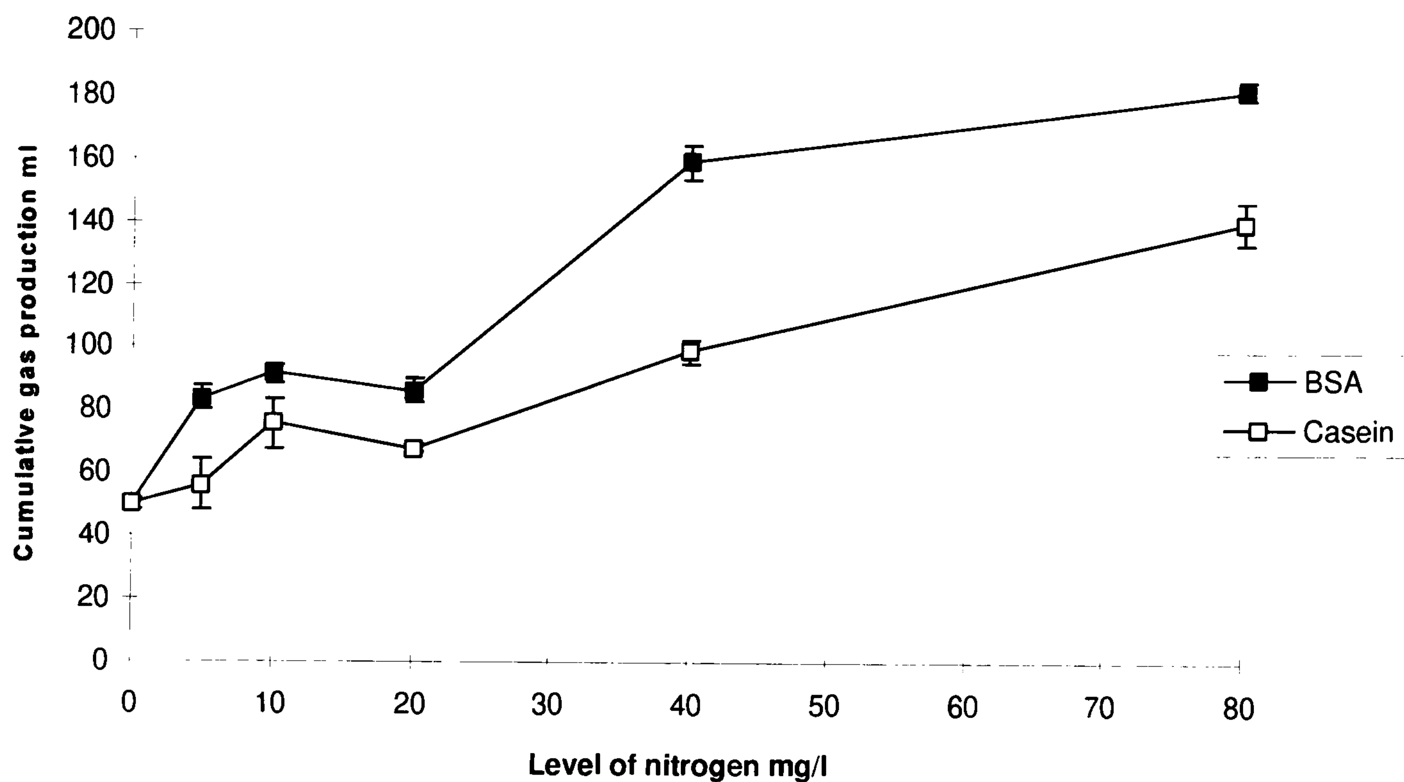


Figure 5.5: *Response at 70 hours of starch to increasing levels of nitrogen from two proteins.*

Contrary to the case of glucose, the cumulative gas production from starch when fermented in the presence of BSA was higher than that with casein (see Figure 5.6). There were significant differences in cumulative gas production at 45 and 70 hours ($p<0.01$). It was a similar situation at all levels of nitrogen. Figure 5.6 shows that after a similar lag phase the fermentation of starch in the presence of BSA increases more rapidly than in the presence of casein. This is supported by the rate and lag phase parameters (see Table 5.2).

Table 5.2: *Gompertz curve parameters for the fermentation profiles of starch with two proteins at a level of 80mgN/l.*

	BSA	Casein	S.E.D (2 df_{error})
Rate (h^{-1})	0.111	0.065	0.00279
Lag phase (h)	25.3	27	8.49
Gas pool size (ml)	182.9	143.6	0.82

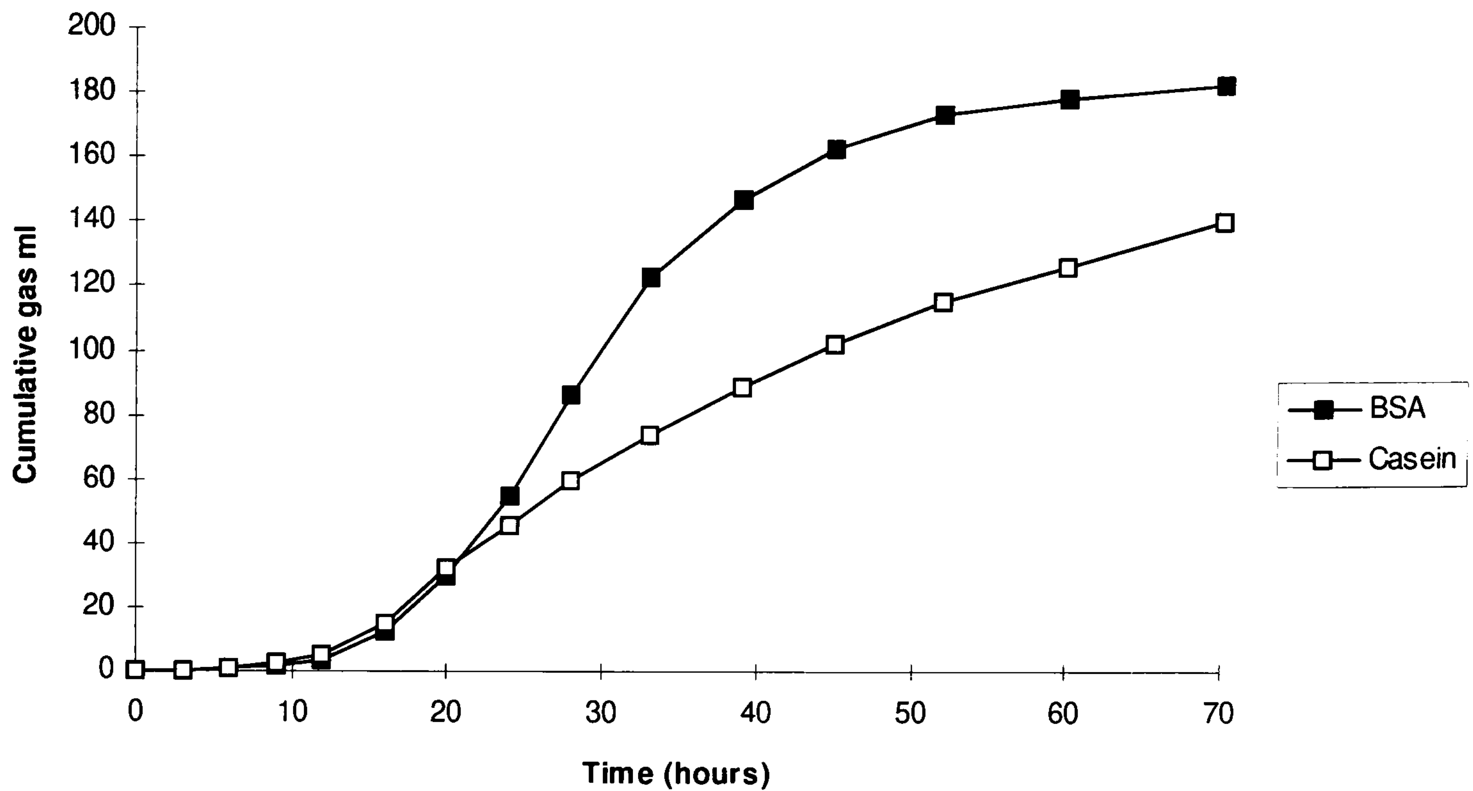


Figure 5.6: *Fermentation profiles of starch when fermented at a level of 80mgN/l from two proteins.*

The model fitted the data very well (R^2>99%). The results confirm that starch when fermented with BSA has a faster fermentation rate and produces more gas than with casein. Figure 5.7 shows the gas production rates of starch for the highest level of nitrogen. The rate of gas production is maximum at 24 hours when fermented with BSA and at 28 hours with casein. The maximum rate is higher with BSA. The implications of fermenting other carbohydrates with these two proteins will be discussed later in this chapter.

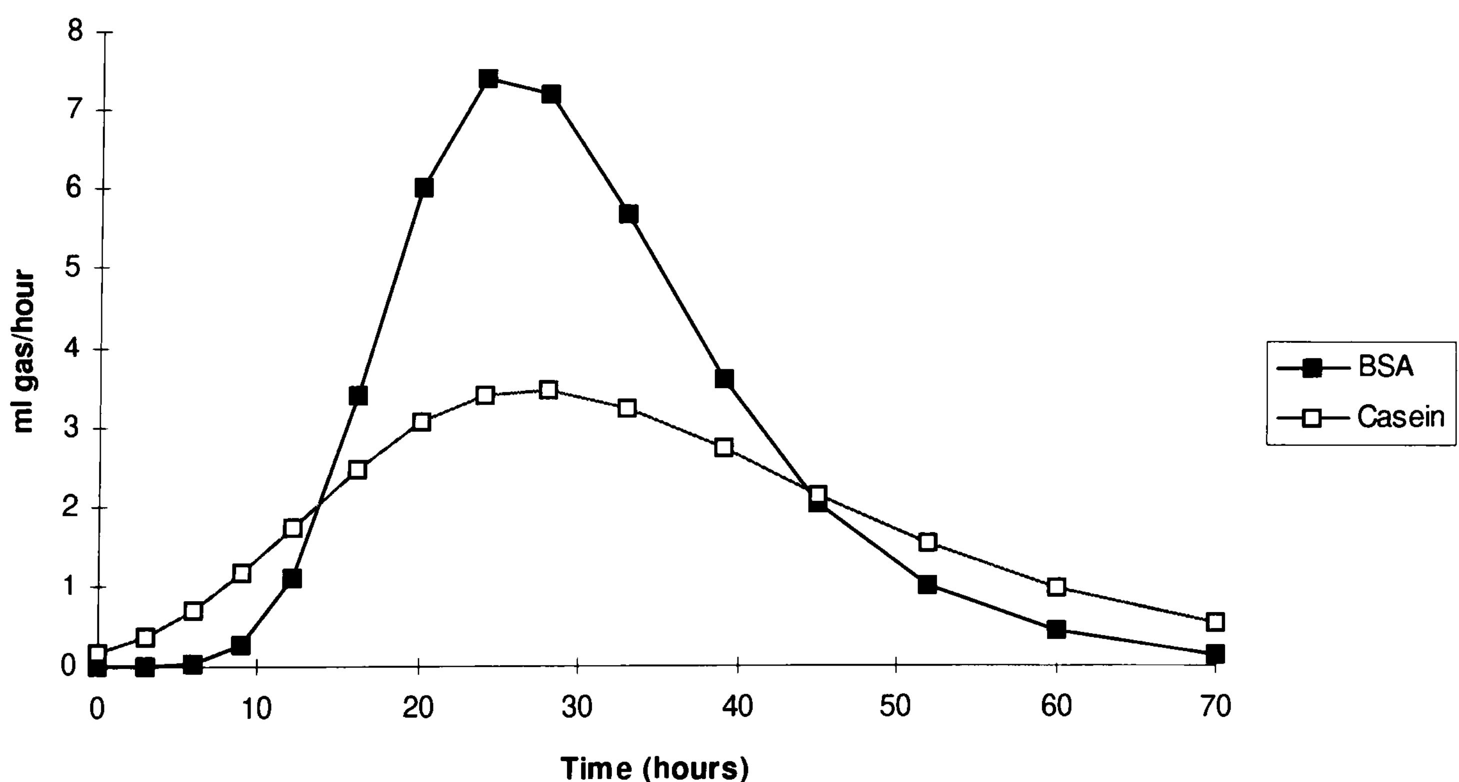


Figure 5.7: Gas production rates (ml/h) of starch fermented with BSA and casein at 80mgN/l.

5.4 EFFECTS OF TWO PROTEINS ON THE FERMENTATION OF CELLULOSE

MATERIALS AND METHODS

0.5 g of α-cellulose (SIGMA C-8002) were fermented with the same levels of BSA and casein as in the previous experiment for 70 hours. This experiment was carried out in the same fermentation batch as the starch experiment. Statistical analysis was carried out in the same way as in previous experiments.

RESULTS AND DISCUSSION

The response of cellulose to the different levels of proteins can be seen in Figure 5.8. There was a linear response to the addition of both proteins ($p<0.001$). There were no significant differences between the levels 5 and 10mgN/l. According to these results, a minimum level of nitrogen was 20mgN/l; however, to standardise the level with starch, the adequate minimum level of nitrogen was taken as 30mgN/l.

The cumulative gas profiles at the highest level of nitrogen can be seen in Figure 5.9. Gas production was significantly different at the times analysed (12, 24, 45 and 70 hours). The gas production at the highest level of nitrogen was 25% higher with casein than with BSA. The curve parameters (Table 5.3) show that the fermentation of cellulose with casein had a larger gas pool size and a shorter lag phase than with BSA. Cellulose was fermented at the same rate with both proteins.

Table *5.3:* *Gompertz curve parameters for the fermentation profiles of cellulose with two proteins at a level of 80mgN/l.*

	BSA	**Casein**	S.E.D (2 df_{error})
Rate (h^{-1})	0.04	0.041	0.00192
Lag phase (h)	40.2	32	1.19
Gas pool size (ml)	117.6	212.2	7.32

This situation is contrary to the case of starch and can be explained by studying the fermentation rates. Figure 5.10 shows the fermentation rates of cellulose at the highest level of nitrogen. The rates were low for both proteins compared to glucose and starch and reached a maximum at 33 hours for casein and 39 hours for BSA. This may be due to the low fermentability of the cellulose. The main difference between the two rates is during the first 42 hours. The combination of a low fermentable substrate with a slow release of nitrogen from BSA may have established a period of low availability of fermentable

nutrients during the first hours of fermentation. During this period, the microbial population may have been affected by the lack of nitrogen and energy to an extent that, by the time that more nitrogen was available, it could not recover. The greater availability of casein at the beginning of the fermentation may have been responsible for the overall greater gas production.

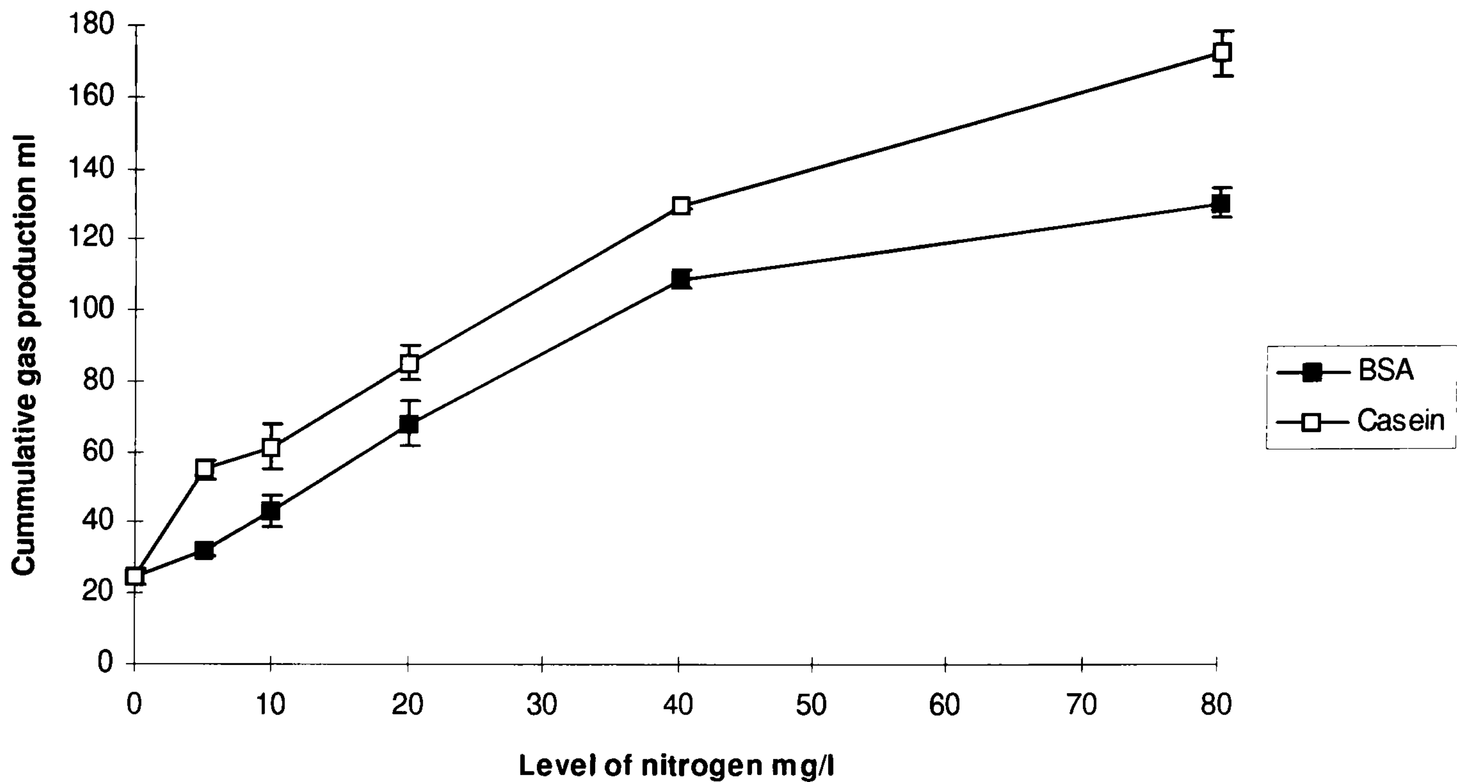


Figure 5.8: *Response at 70 hours of cellulose to increasing levels of nitrogen from two proteins.*

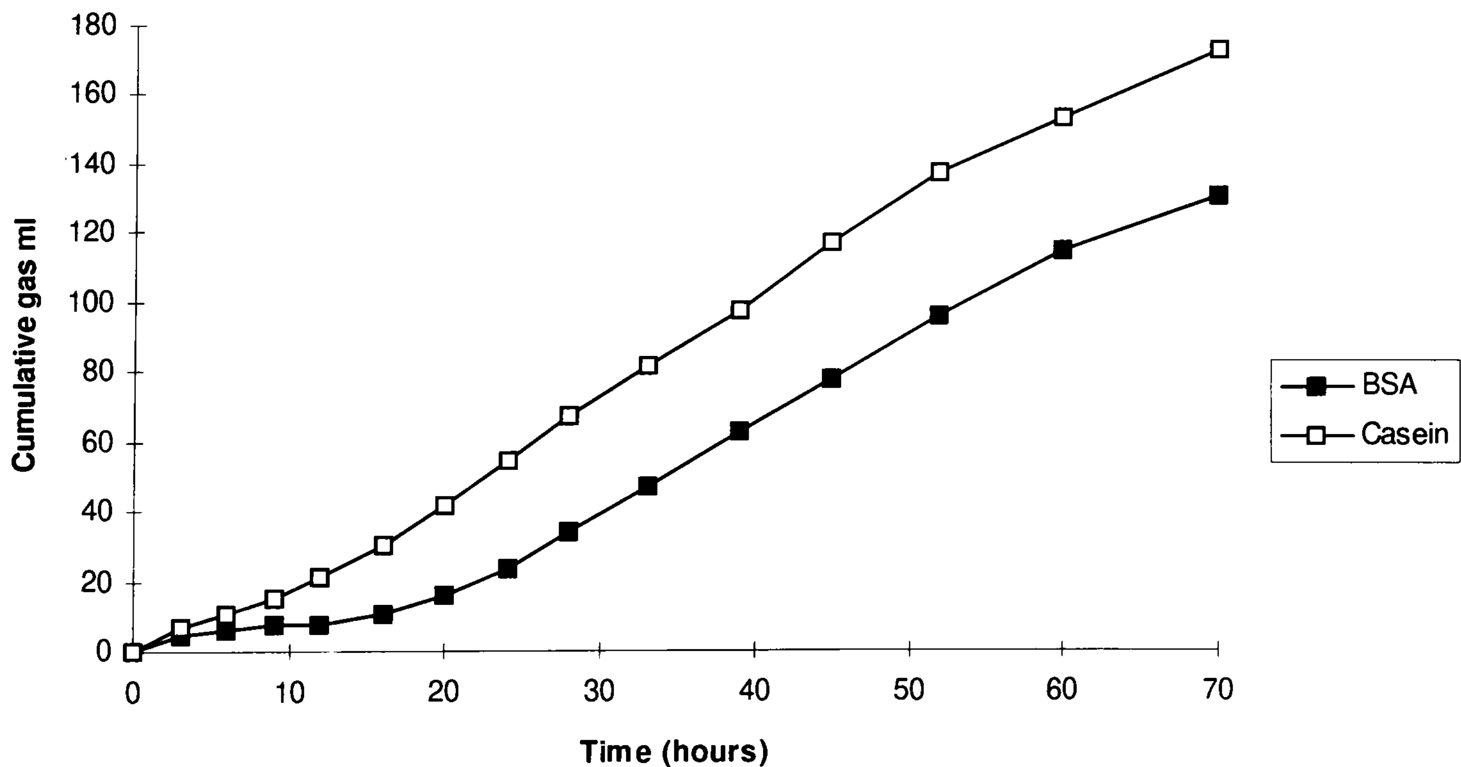


Figure 5.9: *Fermentation profiles of cellulose when fermented at a level of 80mgN/l from two proteins.*

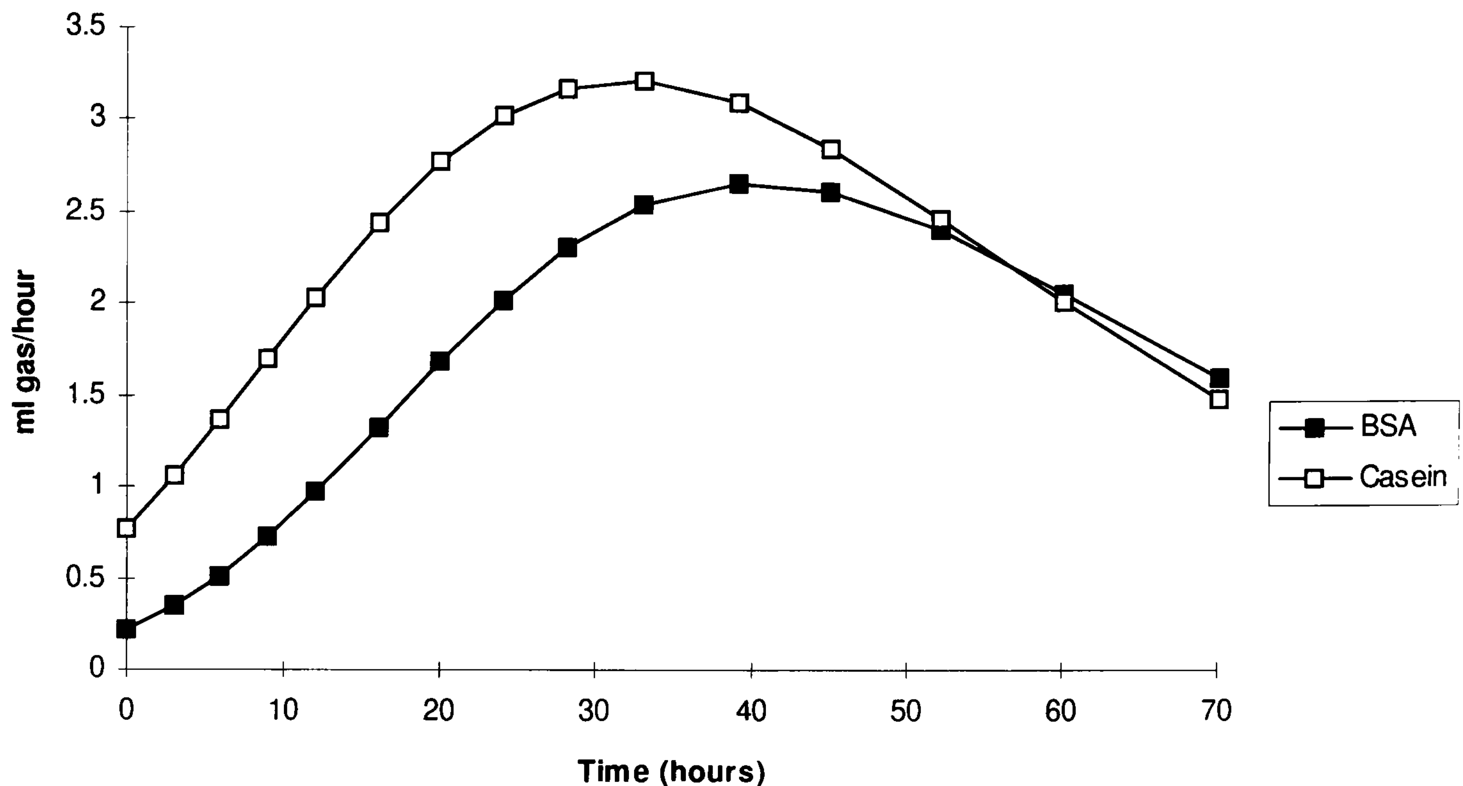


Figure 5.10: *Gas production rates (ml/h) of glucose fermented with BSA and casein at 80mgN/l.*

5.5 FERMENTATION KINETICS OF INDIVIDUAL CHEMICAL ENTITIES (GENERAL DISCUSSION)

To study the response of the carbohydrates to nitrogen, it is necessary to characterize their response under nitrogen free conditions. Figure 5.11 shows these responses. Glucose shows an exponential type of response whereas starch has a characteristic sigmoidal response. Its initial gas production (from 0 to 18 hours) is even lower than that from cellulose but, from 20 hours onwards, it recovers. Cellulose exhibited a more linear response. The end points confirm these carbohydrates as high, medium and low fermentable material respectively. This is in accordance with the general understanding of the fermentation of these chemicals in the rumen (Johnson, 1976., Smith, 1973., Van Soest, 1994).

The experiments have shown that casein (less soluble protein than BSA) releases nitrogen more quickly, but it is less fermentable than BSA. On the contrary, BSA is more soluble, releases nitrogen more slowly but the nitrogen is more fermentable than that from casein. This is very important when fermenting different types of carbohydrates. Figure 5.12 summarises the effects of proteins on the different carbohydrates. The results are examined at 45 hours to allow direct comparison with glucose.

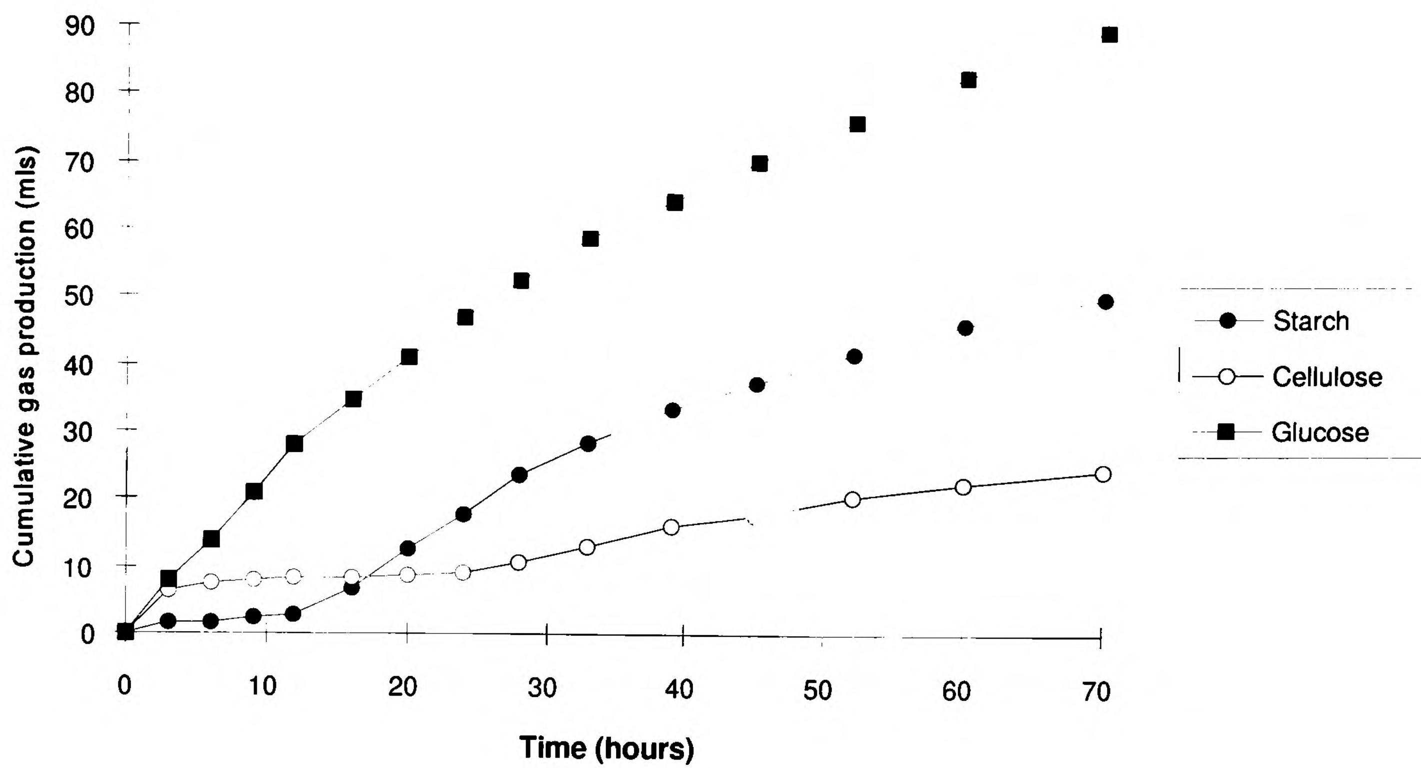


Figure 5.11: *Fermentation profiles of carbohydrates under nitrogen-free conditions.*

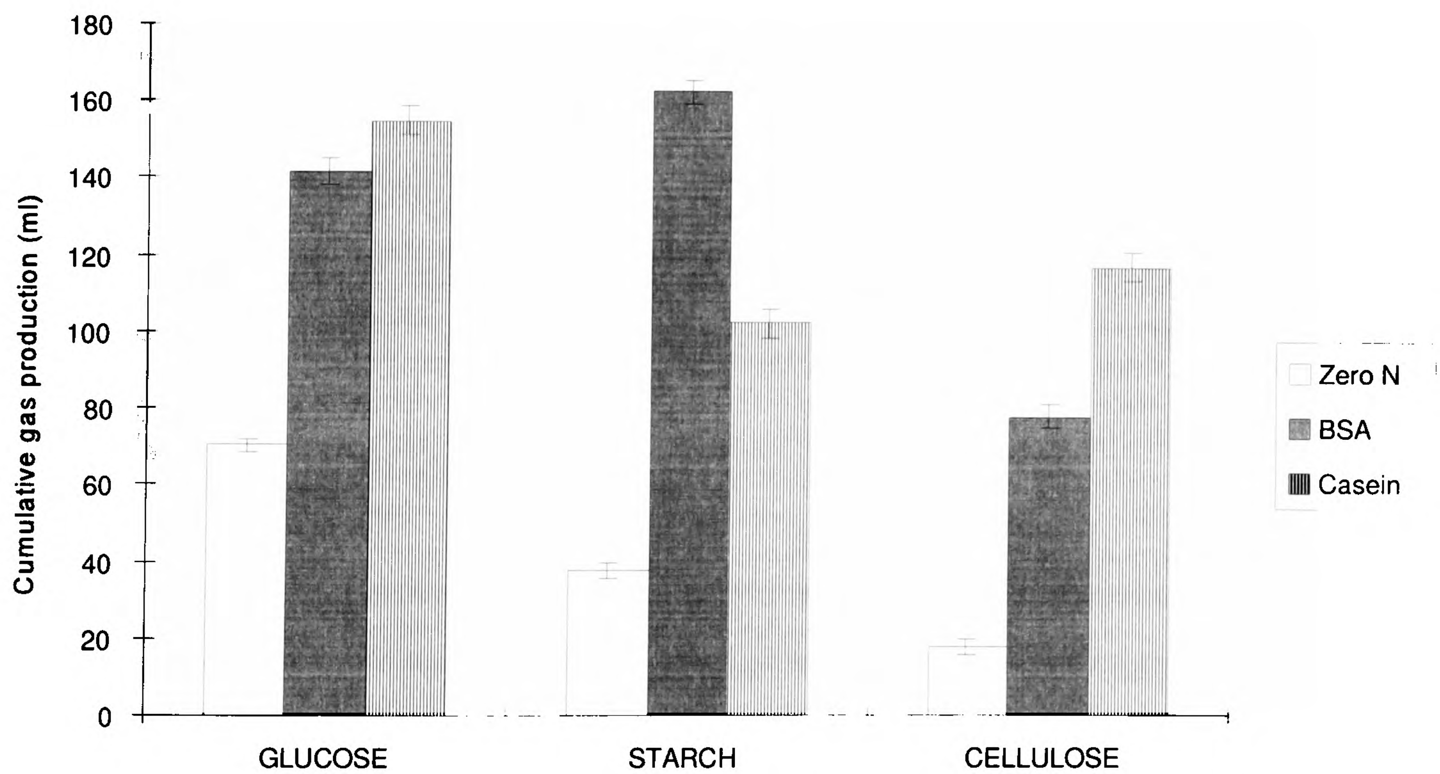


Figure 5.12: *Cumulative gas production at 45 hours of chemical entities as response to two proteins at 80mgN/l.*

These results show that, at 45 hours, casein is a better protein to ferment glucose and cellulose. In the case of glucose, early availability of nitrogen (even if is not rapidly fermentable) may boost the fermentation rapidly from the early stages. In the case of cellulose, this early availability of nitrogen may give more chance to cellulolytic bacteria to develop at early stages. On the other hand, BSA is

better for fermenting starch (it is also the best combination). As BSA releases nitrogen more slowly and starch has this initial period of low fermentability, the synchronisation of the release of fermentable material appears to be responsible for high gas production. This "*late*" synchrony is possible with starch and not with cellulose. When cellulose is fermented with BSA, the lack of fermentable material at the beginning may not provide the right conditions for the development of the bacterial population appropriate for that substrate.

5.6 FERMENTATION KINETICS OF MIXTURES OF CHEMICAL ENTITIES

MATERIALS AND METHODS

Mixtures of carbohydrates (starch:glucose, starch:cellulose, glucose:cellulose) were fermented in the presence of BSA and casein, at the maximum level of 80 mgN/l. To keep the maximum level of 0.5g, the individual weight of each of the carbohydrates in the mixture of carbohydrates was set at 0.25g. The fermentation was carried out in duplicate for 70 hours.

Statistical analysis

The Gompertz equation was fitted to all curves, each replicate separately and the resultant parameters were analysed by one-way analysis of variance. The responses at 12, 24, 48 and 70 hours were also tested by analysis of variance.

RESULTS AND DISCUSSION

There was a good fit for all curves. The average coefficient of determination was 98.8%. Table 5.4 shows the fitted parameters. There were significant differences ($p<0.001$) between the mixtures for the rate, lag phase and gas pool size.

The fastest fermentation rate was found for the mixture starch:glucose when casein was the source of nitrogen. This effect may be explained as the combination of rapidly fermentable material. It was shown earlier that glucose ferments faster with casein than with BSA.

The shortest lag phases were found for the mixtures of starch:glucose and glucose:cellulose when fermented with casein. These results support the previous discussion about the importance of the appropriate combination of fermentable material.

Table 5.4: *Fermentation kinetics of mixtures of carbohydrates at a level of nitrogen of 80mg/l from two proteins.*

Substrate	Rate(h^{-1})	Lag phase (h)	Gas pool size (ml)
BSA			
Starch:Glucose	0.038	27.3	213.5
Starch:Cellulose	0.044	32.7	230.5
Glucose:Cellulose	0.054	15.6	126.8
Casein			
Starch:Glucose	0.083	11.8	187.2
Starch:Cellulose	0.046	30.0	228.2
Glucose:Cellulose	0.060	13.6	182.3
S.E.D(6 df_{error})	0.0025	1.2	6.9

The mixtures of starch:cellulose showed the longest lag phase and the highest gas pool size independently of the source of nitrogen. This is an interesting finding as it indicates that, in this *in vitro* system, mixtures of less fermentable material like cellulose and starch (long lag phase which may indicate later availability) may have provided a better mix of substrates for bacteria than a more fermentable and/or available substrate like glucose. The fact that the lowest gas pool size was found for the mixture glucose:cellulose fermented with BSA supports this hypothesis.

Table 5.5 shows the cumulative gas production of the different mixtures. There were significant differences ($p<0.001$) in the gas produced by the mixtures at 12, 24, 45 and 70 hours.

Table 5.5: *Cumulative gas production at four times of mixtures of carbohydrates with two proteins at a level of 80mgN/l.*

	Time (hours)			
Substrate	**12**	**24**	**45**	**70**
BSA				
Starch:Glucose	37.3	72.7	123.5	177.1
Starch:Cellulose	17.9	53.7	127.9	190.8
Glucose:Cellulose	40.3	71.4	96.1	130.2
Casein				
Starch:Glucose	75.8	129.7	170.1	194.3
Starch:Cellulose	22.5	61.9	138.0	195.4
Glucose:Cellulose	70.5	109.8	149.6	188.4
S.E.D (6 df_{error})	2.04	4.07	5.95	6.11

From 12 to 45 hours, the highest gas production was found for the mixture starch:glucose fermented with casein. The mixture glucose:cellulose in the presence of casein was the second highest during the

same period. Mixtures of starch:cellulose showed the lowest gas production in the early stages but their responses were the highest at the end of the incubation.

These results confirm the findings in Chapter 4 relating to the importance of the components on the fermentability of the substrates according to the nitrogen in the medium. In this case, a greater gas production is achieved in the early stages (0 to 9 hours) by combining a rapidly fermentable carbohydrate with a rapidly fermentable protein. Also in Chapter 4, starch and ADF were the main contributors to gas production between 12 and 33 hours in a nitrogen-free medium and between 16 and 28 for starch and 70 hours for ADF in a medium high in nitrogen. Under these conditions, the combination of these carbohydrates was more important from 45 hours onwards. The combination of slowly fermentable material, like the mixture starch:cellulose with BSA, showed low gas production from 0 to 24 hours but, after a lag period of 32 hours, the gas production was among the highest.

At the level of nitrogen used in this experiment, the response of the mixtures of carbohydrates with casein was generally greater than the response to BSA. These results also highlight the importance of the fermentability of the source of nitrogen and the importance of having an appropriate mixture. Mixtures of rapidly fermentable material were shown to be more important at early hours. Mixtures of slowly fermentable material were, in turn, more important at the end of the fermentation.

5.7 ASSOCIATIVE EFFECTS OF MIXTURES OF CHEMICAL ENTITIES

MATERIALS AND METHODS

Mixtures of chemical entities (starch:glucose, starch:cellulose, glucose:cellulose), fermented in the presence of 80mgN/l from BSA or casein, were tested for associative effects. The individual weight of each carbohydrate was 0.25g. Student's *t* tests were used to test additivity as explained in section 3.4. The observed values of a mixture of carbohydrates were compared against the sum of half the gas produced by the single carbohydrates fermented at the same level of nitrogen. Associative effects were tested at 12, 24 and 45 hours. The observed values of the mixtures correspond to those in section 5.6. Fermentations of single components (0.5g) were also carried out in the same batch as the mixtures except for glucose. The single values of glucose correspond to those in section 5.2. Because the fermentation of glucose was carried out in a different batch, glucose values were corrected by the amount of gas produced by glucose only without nitrogen on the two different batches. There was a very small variation and the correction factor was 0.94 (calculated from the difference of both fermentations at 45 hours).

RESULTS AND DISCUSSION

Student's *t* tests showed significant differences between the sum of the gas production of the single components and the fermentation of the mixtures at certain times. In these cases, the null hypothesis that gas production of the mixture was equal to the sum of gas production of the individual components was rejected. This means that there were associative effects on gas production of the mixtures of chemical entities.

Associative effects of mixtures with BSA at 80mgN/l are shown in Figure 5.13. There was a positive associative effect of mixing starch and cellulose which was greatest during the first stages ($p<0.001$). The effect of the mixture of starch and glucose was in two parts. There was a positive associative effect ($p<0.001$) over the first 25 hours that changed to negative effect ($p<0.01$) after that time. In the case of the mixture of glucose and cellulose, there was first a positive associative effect ($p<0.001$) up to 33 hours and then a negative associative effect ($p<0.01$). These changes indicate that associative effects may change with time.

Figure 5.14 shows the associative effects of mixtures of carbohydrates when fermented with casein. There were positive associative effects of all mixtures of carbohydrates with this protein ($p<0.001$).

In general, two type of responses in the associative effects can be identified. The first response is sigmoidal and characteristic of when BSA is the source of nitrogen. In this case, a lower gas production was expected during the first part of the fermentation. The greater observed gas production may be explained if the mixture of carbohydrates of different fermentabilities and characteristics favours better bacterial growth (or better composition) than that obtained from the fermentation of single substrates. During the second part, the observed response was lower than expected. From the sum of the single components, a late boost in the fermentation was expected. The negative associative effect is similar to those commonly observed as the depression in fibre digestibility when rapidly fermentable carbohydrates are offered as part of the ration (Blaxter, 1962; Garnsworthy and Cole, 1990; Chesson and Forsberg, 1989; Oldham, 1984). The general explanation is that the "soluble" nitrogen is depleted by the rapid growth of amylolytic microorganisms. This seems to be the case in this study, except that this effect was due to the "fermentable" protein.

The second response is exponential and positive all the time. In all cases with casein, less gas production was expected from the mixtures. The greater gas production observed may have been because a mixture of carbohydrates favours a better bacterial growth compared to the single substrate.

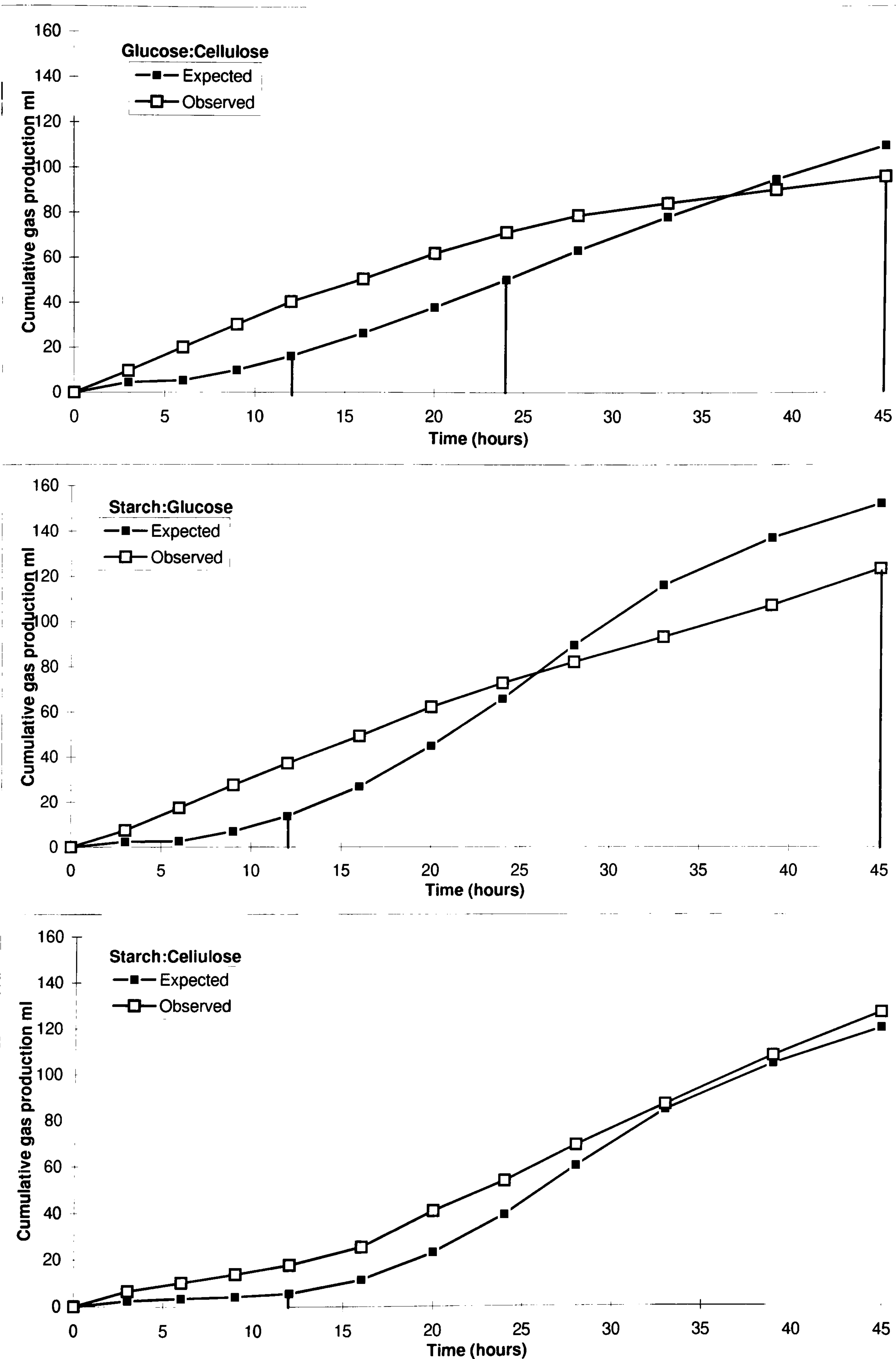


Figure 5.13: *Associative effects of mixtures of carbohydrates at a level of nitrogen of 80mgN/l from BSA. Associative effects were tested at 12, 24, 45 and 70 hours. Vertical lines show the times at which significant differences were found.*

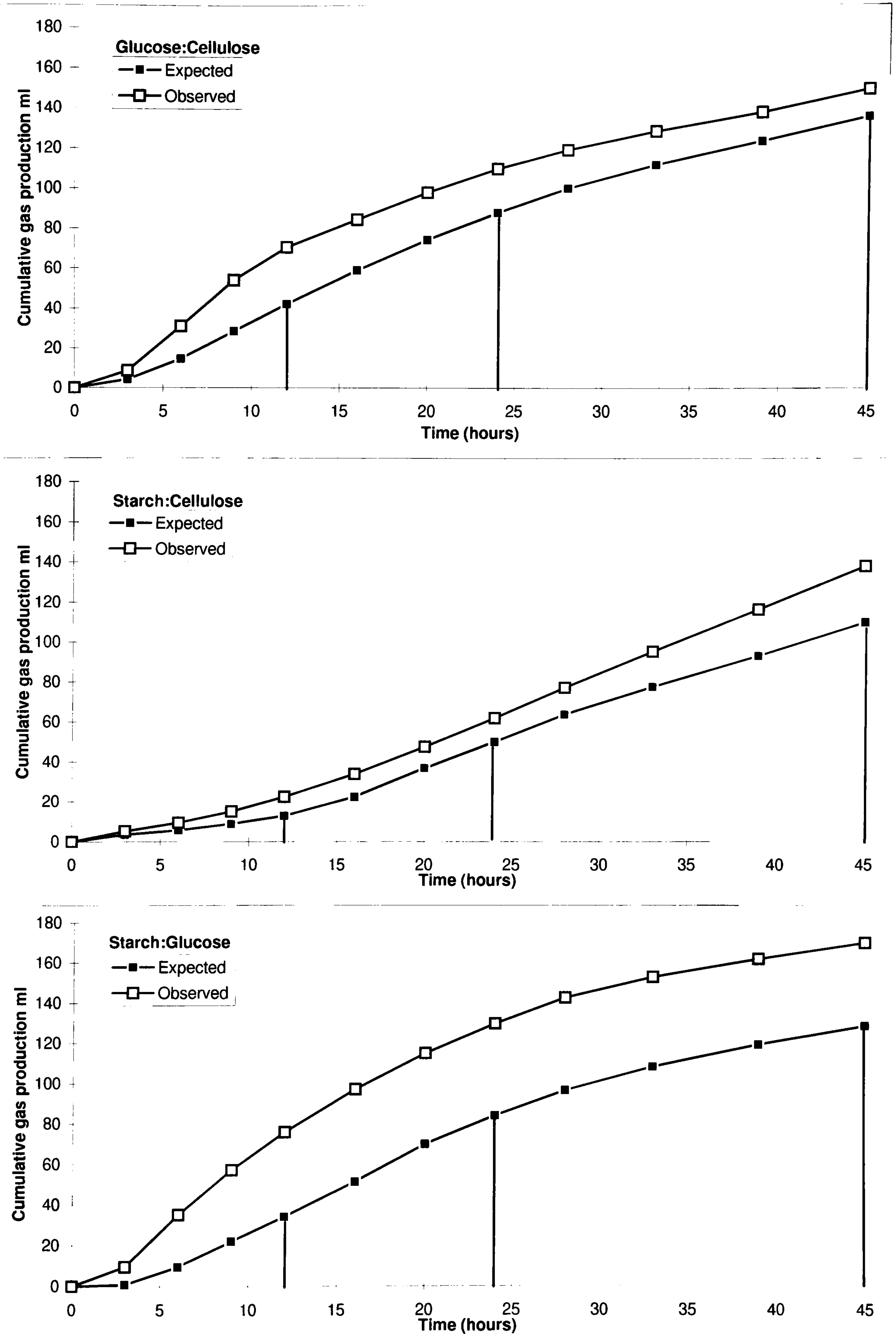


Figure 5.14: *Associative effects of mixtures of carbohydrates at a level of nitrogen of 80mgN/l from casein. Associative effects were tested at 12, 24, 45 and 70 hours. Vertical lines show the times at which significant differences were found.*

The importance of the form of fermentable carbohydrate for associative effects was highlighted by Chesson and Forsberg (1989). The findings in this study highlight the importance of the fermentability of the source of nitrogen. With a more rapidly available/fermentable protein like casein, associative effects were positive all the time. If a source of rapidly available nitrogen is provided (like casein), the combined effect of carbohydrate and protein produce a positive associative effect (early synchronisation). In the case of BSA, which is a less available/fermentable protein, there is an initial effect of the carbohydrates, but when the nitrogen is available at a later stage, a negative associative effect is shown. Positive associative effects occur *in vivo*, as a response to protein supplementation. It has been proposed that a reasonable explanation for these effects probably lies in the manner in which the rumen microbial population responsible for carbohydrate fermentation varies according to the form of the food eaten and the balance of nutrients available. Increasing the supply of amino acids and peptides may stimulate the growth of cellulolytic bacteria in the rumen (Huhtanen, 1991).The importance of the amount and form of dietary crude protein has been highlighted by Oldham (1984). Table 5.6 shows the associative effects expressed as a percentage of the observed response.

Table 5.6: *Associative effects (%) on the fermentation of mixtures of chemical entities†.*

	Time (hours)		
	12	24	45
BSA			
Starch:Glucose	63.0***	9.5n.s	-23.2***
Starch:Cellulose	68.3***	27.0n.s	5.4n.s
Glucose:Cellulose	60.2***	29.5***	-14.3**
Casein			
Starch:Glucose	54.9***	35.3***	24.4**
Starch:Cellulose	42.0***	19.2*	20.4*
Glucose:Cellulose	40.3***	19.9**	9.1*

†Calculated as: (observed-expected/observed)x100.
n.s Not significant.
* Significant at 5% level.
** Significant at 1% level.
*** Significant at 0.1% level.

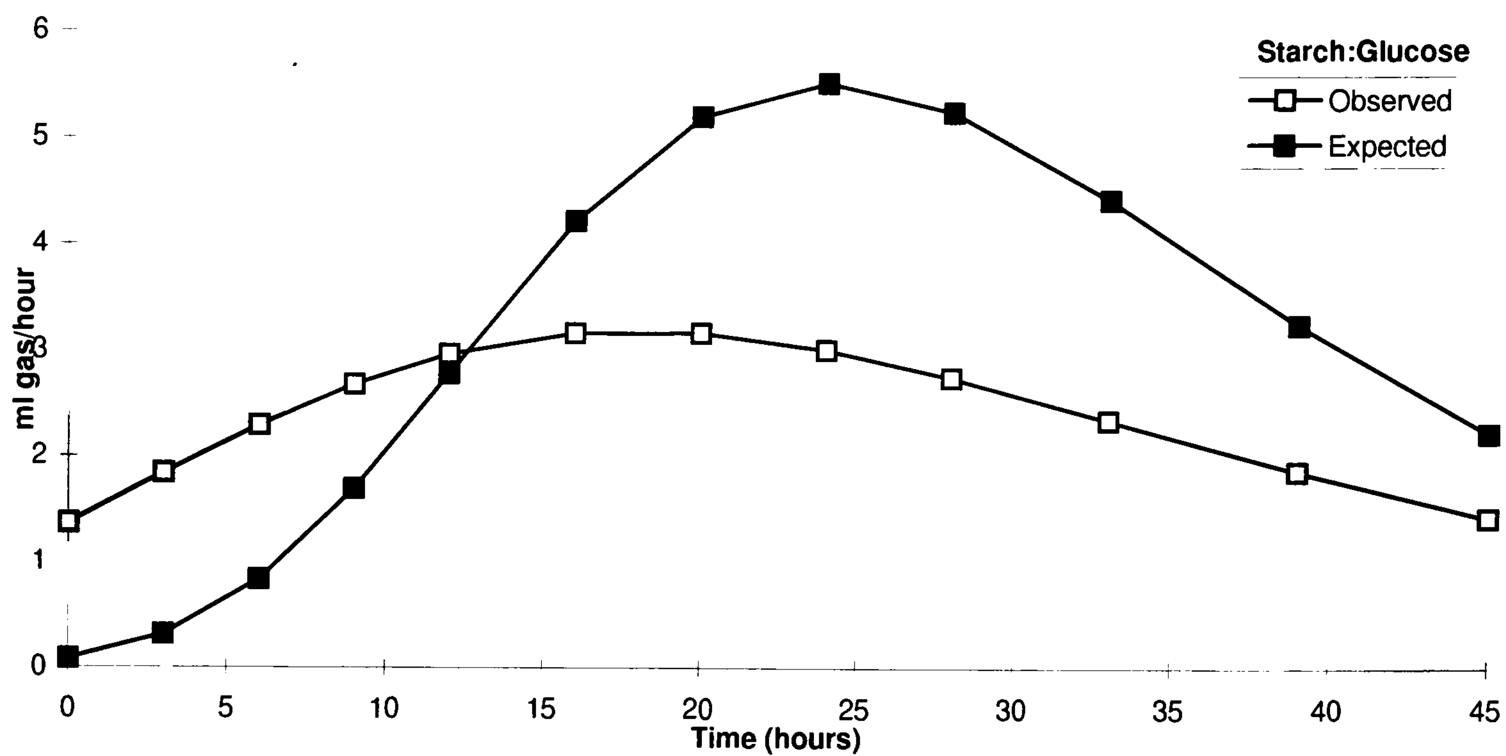
The first conclusion to draw from this table is that a protein that releases nitrogen slowly does not favour positive effects in the late stages of fermentation (at least in two cases) whereas a protein like casein, that releases its nitrogen more quickly, does. Also, greater associative effects in the early stages are found with this protein. The release of nitrogen within the system and in the presence of the appropriate carbohydrate has important consequences. In the case of the negative effects, glucose appears to have an important influence as previously discussed.

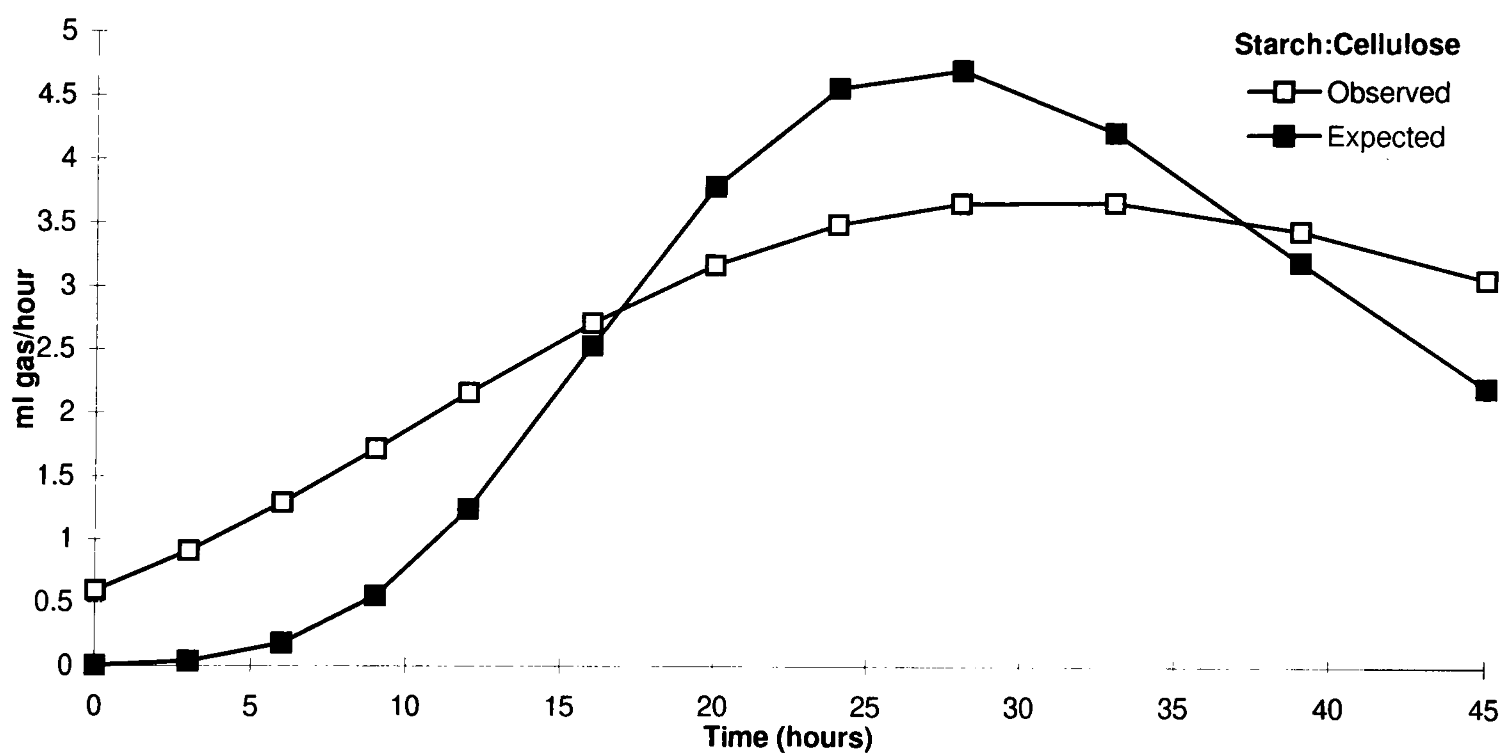
When BSA is combined with starch and cellulose, there is a "*synchronisation*" of the fermentable material (the three have *'medium to slow'* fermentation rates). This combination produced the highest associative effects in gas production. The slow release of nitrogen favours the medium and slow fermentability of the carbohydrates, presumably starch (BSA is a better protein to ferment starch). This may also explain the more steady fermentation rate of this particular combination (see Figure 5.15). Casein, on the other hand, showed a high positive associative effect when fermented with glucose:starch and with starch:cellulose. The mixture glucose:starch showed the highest associative effect when fermented with casein (the three have *'fast to medium'* fermentation rates). This suggests a synchronisation of the fermentability of the substrates. Synchronisation of the rate of supply of nitrogen and energy-yielding substrates to the rumen micro-organisms has been shown to improve the efficiency of microbial protein synthesis and optimise microbial growth rate (Johnson, 1976; Sinclair *et al.*, 1993; Garnsworthy *et al.*, 1995). This seems to be the case in this study.

Figures 5.15 and 5.16 show the rate profiles of the observed and expected values of the different mixtures. In the case of the mixture starch:glucose and glucose:cellulose, the observed rate was earlier than expected when fermented with BSA (the observed rates peaked 6 and 12 hours earlier than the expected, respectively). In the case of casein, the expected rate was always lower than the observed. The time at which the rate is maximum may illustrate the time at which the availability of nutrients from the different components of the mixture was maximum. This point of synchronisation occurred earlier for the mixtures with casein than with the analogous mixtures with BSA, except for the mixture glucose:cellulose. Chemical entities have been used to study the effects of synchronisation in the rumen. Rooke *et al.*,(1987) found that a continuous intraruminal infusion of sucrose stimulated microbial protein synthesis. They concluded that this effect was probably due to a better synchronisation of energy and nitrogen release. In a subsequent trial, the effect was enhanced with the continuous infusion of casein (which is rapidly degradable in the rumen) along with the sucrose, which resulted in a synchronisation of nitrogen and energy supply (Rooke and Armstrong, 1989). This study shows that a synchronisation on the release of energy and nitrogen can be achieved with appropriate combinations of the carbohydrate and protein sources.

5.8 CONCLUSIONS

This chapter illustrated the fermentation dynamics of chemical entities by incubating them at different levels of nitrogen and using the knowledge gained on their own kinetics to understand the complex associative effects of their mixtures. The carbohydrates glucose, starch and cellulose were characterised as of fast, medium and slow fermentability respectively. Their profiles were also identified as exponential, sigmoidal and linear.





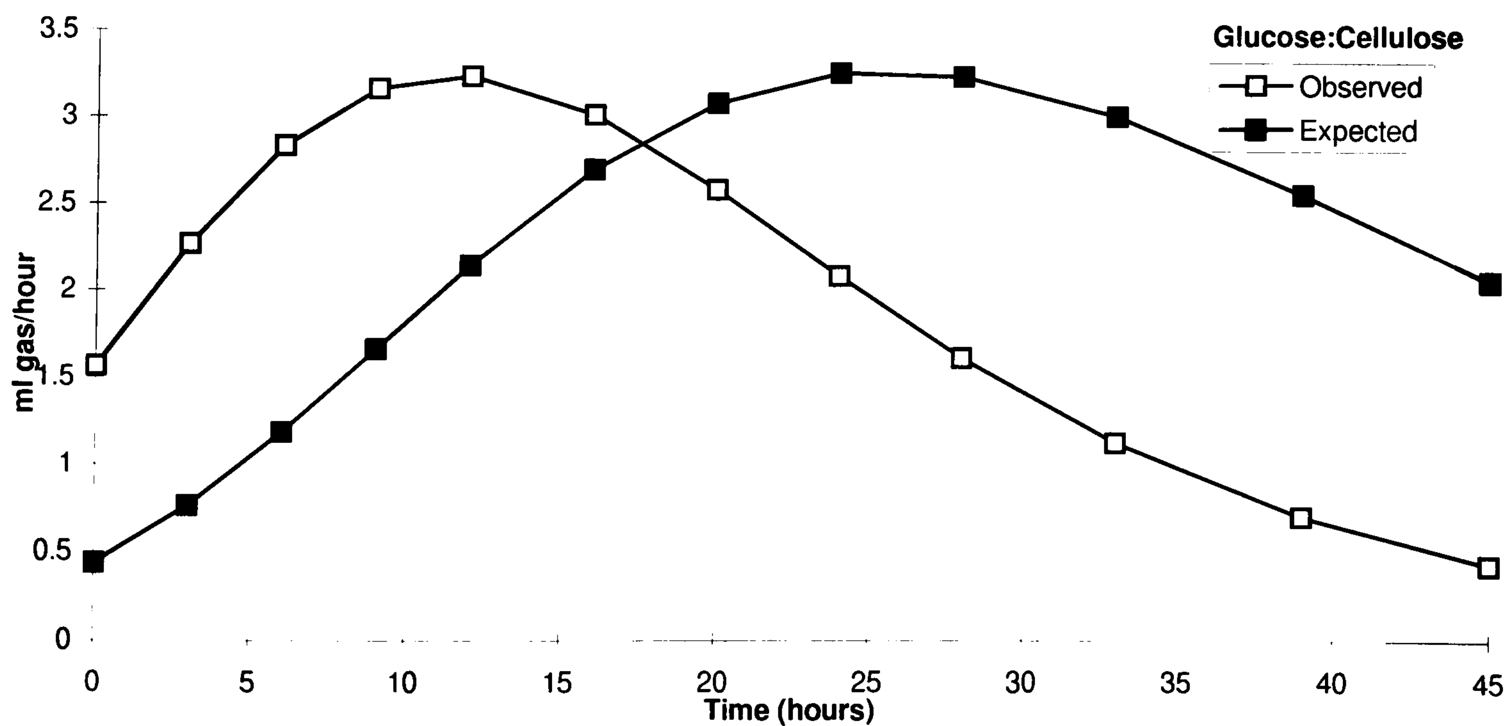
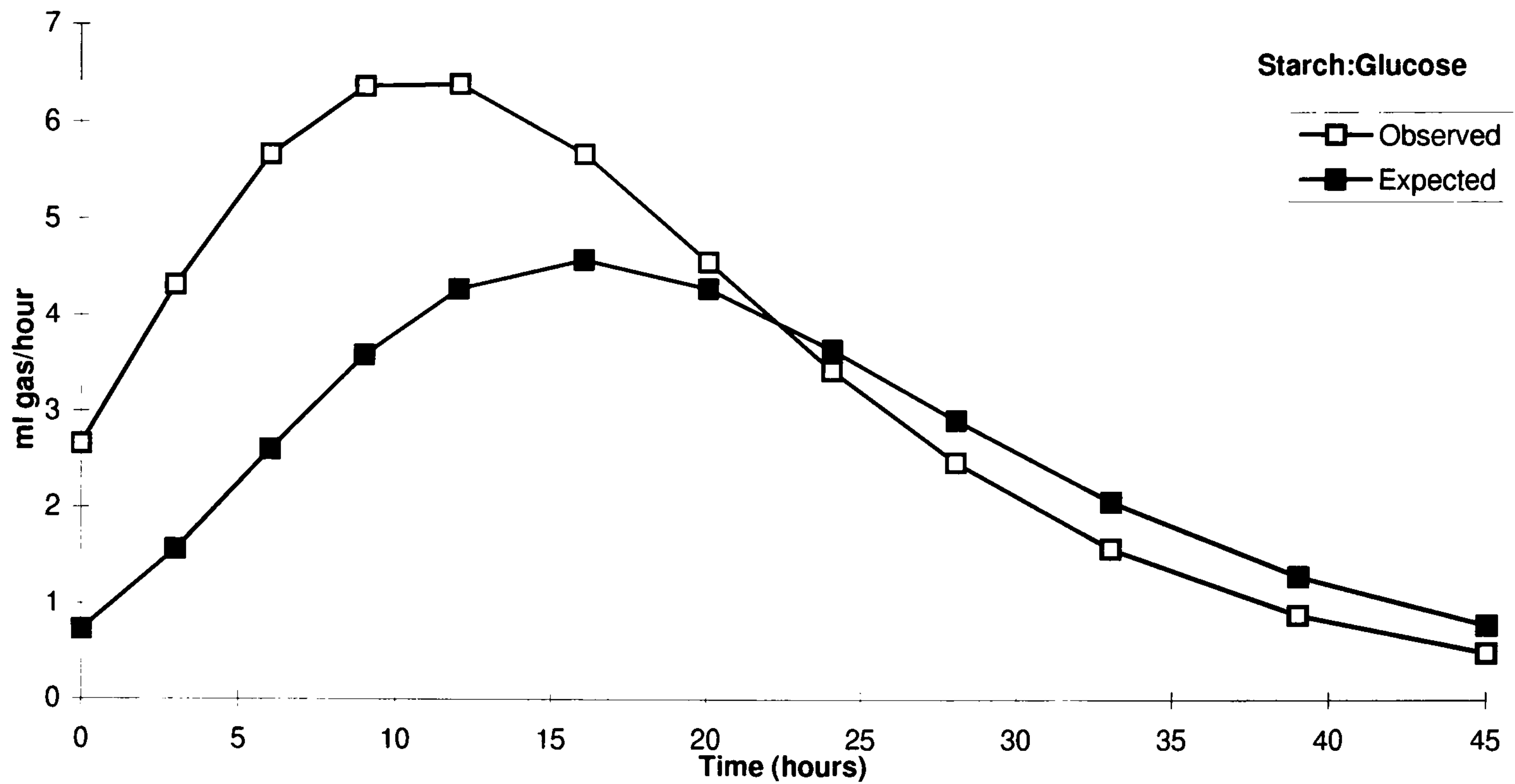
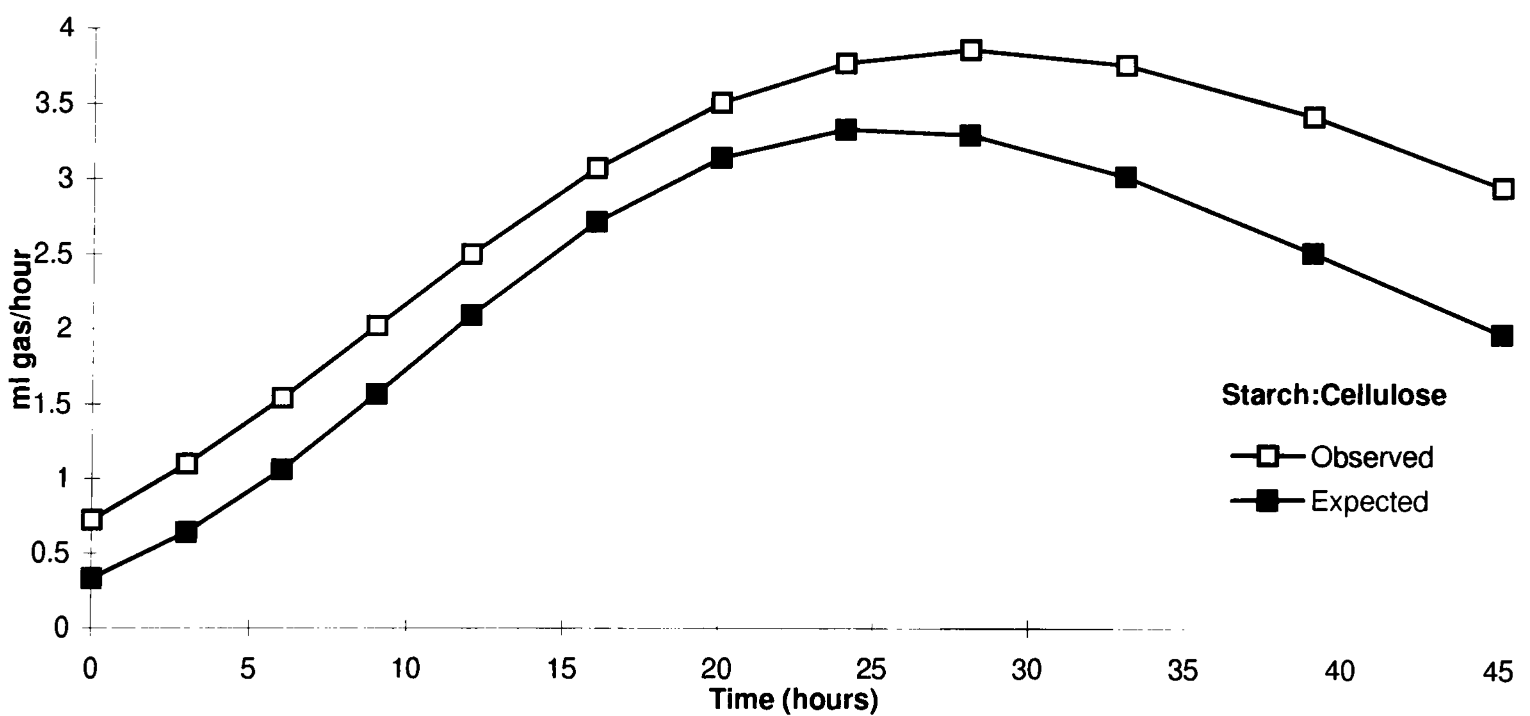


Figure 5.15: *Gas production rates (ml/hour) for associative effects of mixtures of carbohydrates at a level of 80mgN/l from BSA. Figures are not to the same scale.*





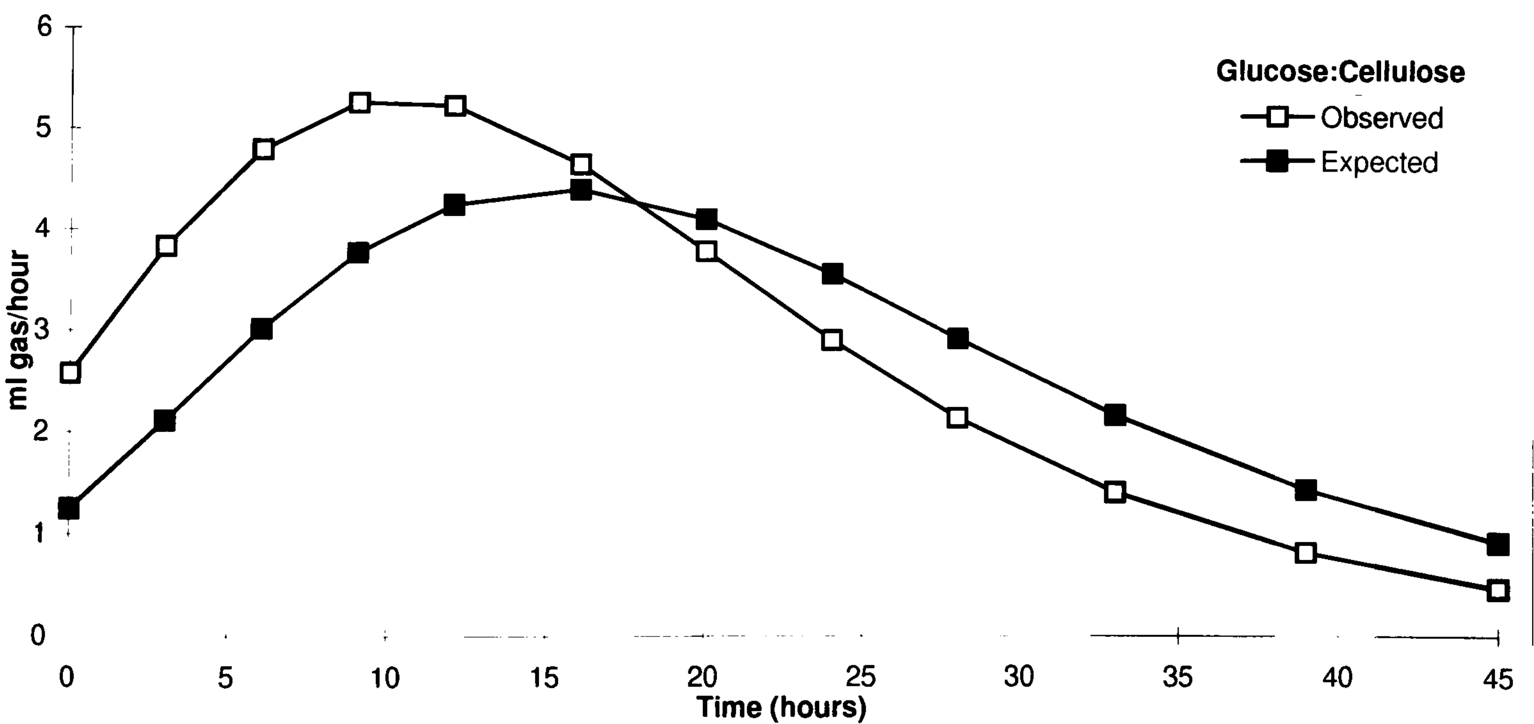


Figure 5.16: *Gas production rates (ml/hour) for associative effects of mixtures of carbohydrates at a level of 80mgN/l from casein. Figures are not to the same scale.*

The response to the protein in the medium varied widely according to the type of carbohydrate and the level of nitrogen. Minimum levels of nitrogen for future experiments were established as 20mgN/l for glucose and 30 mgN/l for starch and cellulose. The concept that fermentability depends on the solubility of a protein is challenged. Results showed that BSA is more soluble than casein, but it appears to release nitrogen more slowly. However, the released nitrogen appeared to be more fermentable than that from casein. It was determined that BSA was a better protein to ferment starch. This carbohydrate was fermented at a faster rate and produced a greater gas pool size when fermented with BSA. Casein was identified as a better protein to ferment glucose and cellulose. Glucose fermented faster, with a shorter lag phase and a greater gas pool size with casein than with BSA. Cellulose was fermented with a shorter lag phase and to a greater extent when fermented with casein.

The response of the mixtures of carbohydrates to the two proteins was complex. Mixtures fermented in the presence of casein fermented faster and with a shorter lag phase. Mixtures of starch:cellulose were fermented to a greater extent independently of the source of nitrogen. Mixtures of rapidly fermentable material produced more gas in the early stages whereas mixtures of slowly fermentable material were more important towards the end of the fermentation.

Associative effects were shown to exist for mixtures of chemical entities. Associative effects showed a change in magnitude with time. Two main responses were identified. A sigmoidal response (positive at the beginning and negative at the end) was characteristic when BSA was the source of nitrogen. The negative associative effects are characteristic of the "carbohydrate effect" reported in the literature. The second response was exponential (positive associative effect) and characteristic when mixtures were fermented with casein. This effect was probably due to the more rapid availability of casein. Positive associative effects are characteristic of the "protein effect" reported in the literature.

The greatest associative effects were found when the mixture had components of the relative same fermentability. This was observed for both '*slow*' (starch:cellulose:BSA) and '*fast*' (starch:glucose:casein) combinations. This suggested a synchronisation in the availability of nutrients due to the fermentability of the substrates. The point at which the rate is maximum was proposed as the time of maximum availability of nutrients for the fermentation. This point of synchronisation was shown to occur earlier for the mixtures with casein.

According to these results, utilisation of nitrogen by the rumen microbes (in the presence of the right carbohydrate) is a function of its availability and fermentability and not of its solubility. Positive effects can be achieved by using protein with a rapid release of nitrogen. On the other hand, negative effects can be obtained with a protein that releases nitrogen slowly. Condensed tannins may slow down the fermentability or availability of the protein and, therefore, they may contribute to the interactions. This will be studied in Chapter 7. The results showed that it is possible to manipulate the rate of fermentation by the strategic use of materials of different fermentabilities and obtain different associative effects. It is important to have in mind that leaves are a far more complex substrate than the mixtures tested here, but modelling the fermentation kinetics with mixtures of chemical compounds will help later to explain associative effects of fodder tree leaves.

Chapter 6

FERMENTATION KINETICS OF MIXTURES OF CHEMICAL ENTITIES AND FODDER TREE LEAVES

6.1 INTRODUCTION

Results from the previous chapter have shown that associative effects of a mixture depended on the synchronisation of the release of nutrients from the different components. This was modelled by combining carbohydrates and proteins of different fermentabilities. In a nitrogen-free medium, the fermentability of the carbohydrates in a mixture will depend largely on the fermentability of the protein. As tree leaves are the sources of protein in the system, the characterisation of the effects of carbohydrates on the fermentation of fodder tree leaves is important in order to understand the associative effects of mixtures of tree leaves. This characterisation was carried about by fermenting the tree leaves in the presence of the chemical entities evaluated in the previous chapter.

OBJECTIVES

The objectives of this chapter are to:

- study the dynamics of the fermentation of fodder tree leaves as complements for structural and non-structural carbohydrates.

- study the associative effects of mixtures of fodder tree leaves and carbohydrates.

6.2 MATERIALS AND METHODS

To assess the effects of carbohydrates on the fermentation of leaves from fodder trees, the plant species selected in Chapter 4 were fermented in the presence of carbohydrates: glucose, starch and cellulose. The plant species were: *Trichanthera gigantea*, *Gliricidia sepium*, *Inga* sp., *Leucaena leucocephala* and *Erythrina edulis*. All possible combinations of single carbohydrate:single plant species were assessed. Controls of carbohydrates and plant species fermented individually were also carried out. The total amount of substrate was 1g for the combination (0.5g of each component), and 0.5g for single controls. All determinations were carried out in duplicate for 70 hours. In this experiment, no protein

was added to the Menke's medium. Leaves were therefore the only source of nitrogen in each combination. The effects of the carbohydrates were assessed by comparing the gas produced by the plant material with and without the carbohydrates.

Associative effects were studied by comparing the observed response curve of the mixtures (carbohydrate:plant species) against the expected curve calculated from the response profile from each component of the mixture fermented individually. The expected profile was calculated as the sum of half of the gas produced by each single component (carbohydrate and plant species) at a given time.

STATISTICAL ANALYSIS

The lag phase, rate and extent of the fermentation of the mixtures and single substrates were determined by fitting the Gompertz model. This model was fitted to each replicate separately. Significant differences between the estimated parameters due to the effect of the carbohydrates, plant species and their interactions were estimated by the General Linear Model (GLM). Differences in cumulative gas production at 12, 24, 45 and 70 hours were also tested by GLM.

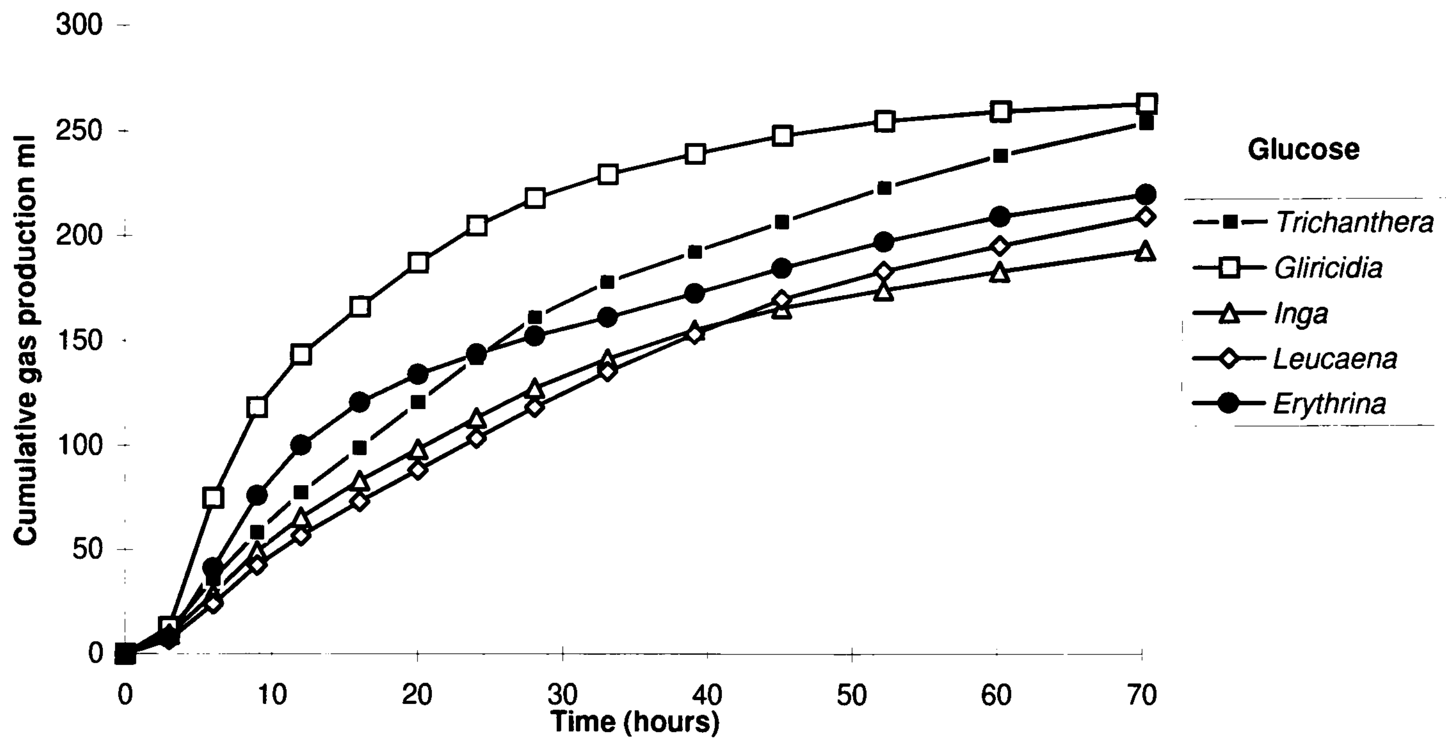
Associative effects between the components of the mixtures were tested at 12, 24, 45 and 70 hours by means of Student *t* tests as described in Chapter 3.

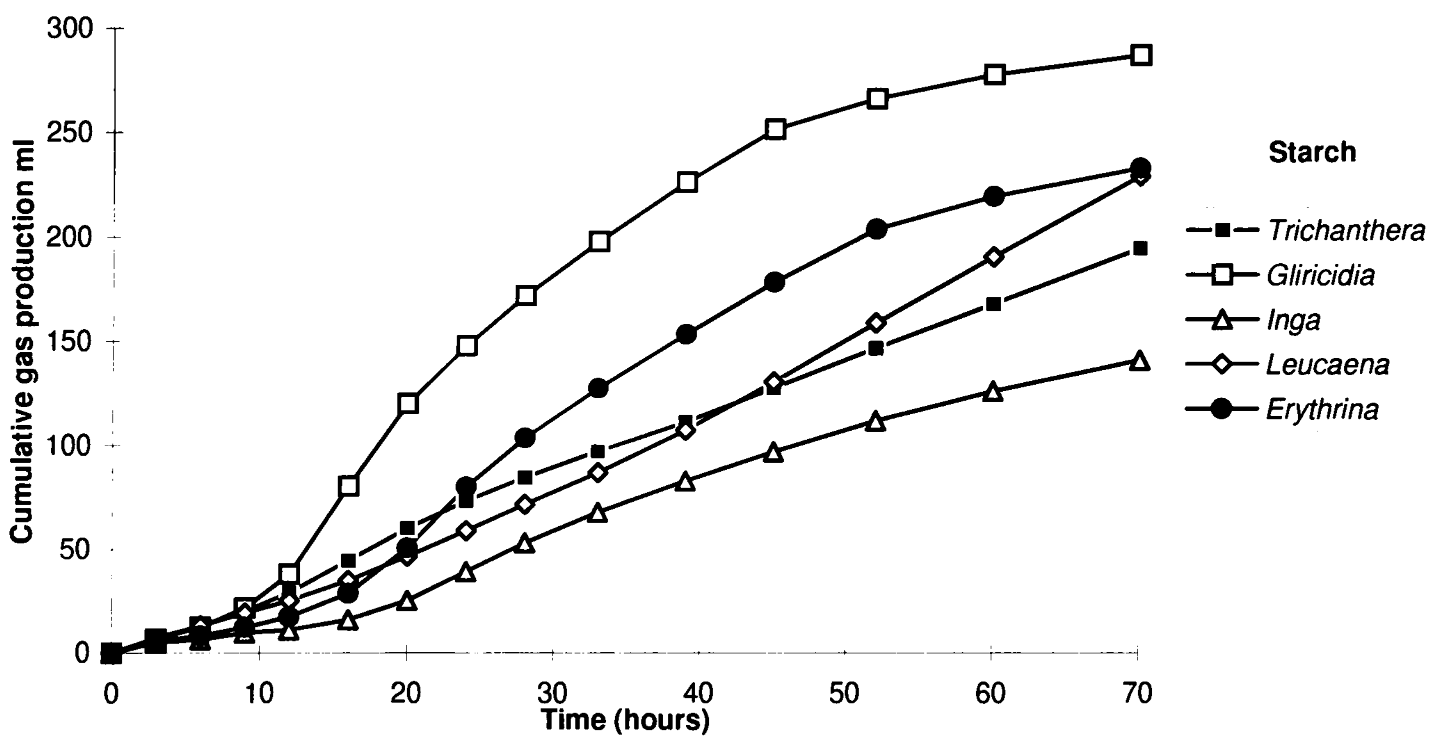
6.3 RESULTS AND DISCUSSION

EFFECT OF THE CARBOHYDRATES

All curves fitted well (R^2>99%). Figure 6.1 shows the cumulative gas production for the mixtures of fodder tree leaves and carbohydrates. In the case of glucose, the highest profile was obtained with *Gliricidia sepium*. *Trichanthera gigantea* had almost the same gas production at 70 hours as *Gliricidia sepium* but there was a major difference during the first 60 hours of incubation. The mixture *Erythrina edulis*:glucose produced more gas between 0 and 24 hours than the combination *Trichanthera gigantea*:glucose.

In the mixtures with starch, the highest profile was obtained with *Gliricidia sepium*. Both *Erythrina edulis* and *Leucaena leucocephala* reached the same cumulative gas production at 70 hours but there was an important difference in the profiles, especially between 24 and 60 hours. *Trichanthera gigantea* was not as good a complement to starch as it was to glucose, compared with the other species.





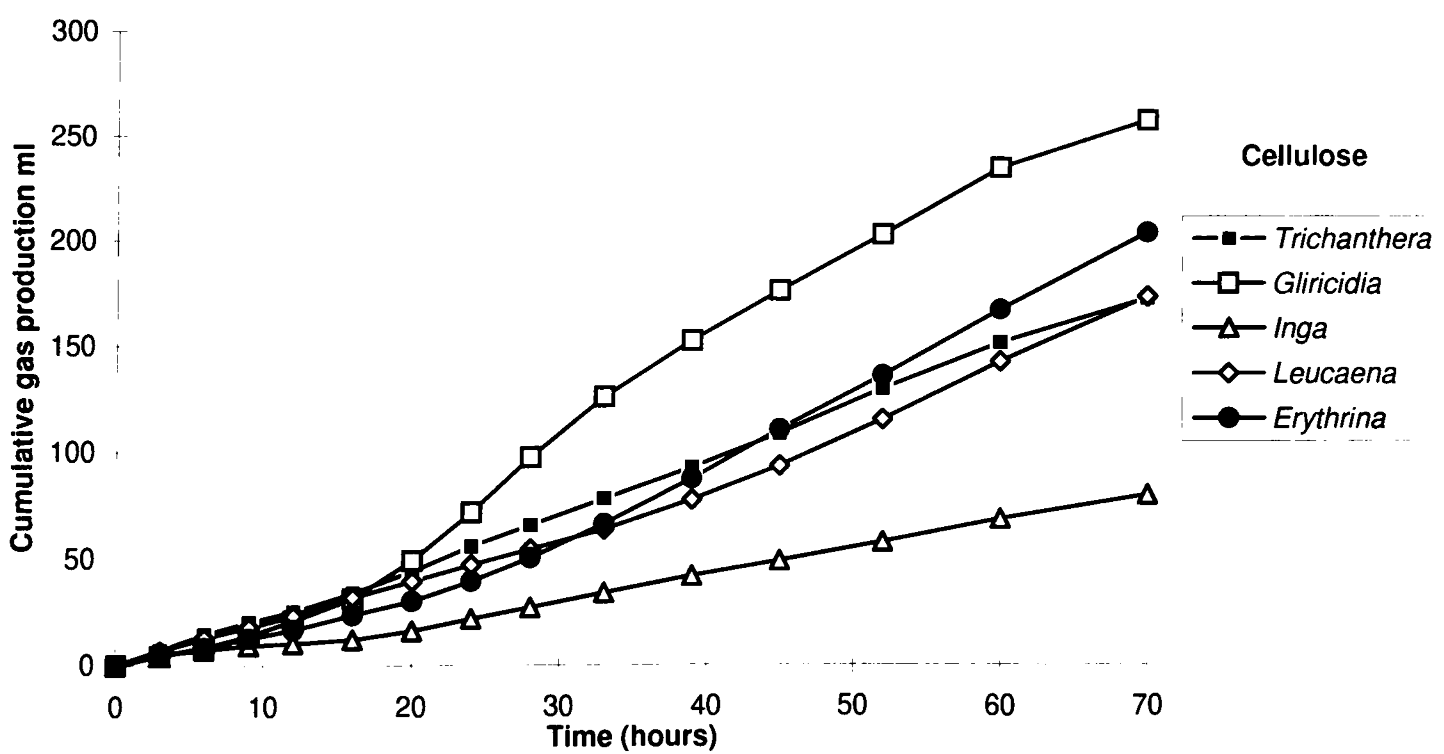


Figure 6.1: *Effect of carbohydrates on the fermentation of fodder tree leaves.*

In the case of the mixtures with cellulose, *Gliricidia sepium* showed the highest gas profile. *Leucaena leucocephala, Trichanthera gigantea* and *Erythrina edulis* showed mixed responses to cellulose, i.e. they ranked differently at different times. The response of *Inga* sp. to all carbohydrates was the lowest.

The effect of the carbohydrates on the fermentation rates of the mixtures can be seen in Table 6.1. There were significant effects of the source of carbohydrates, fodder species and their interactions ($p<0.001$).

Table 6.1: *Effect of different carbohydrates on the rate of gas production (hour^{-1}) from fermentation of fodder tree leaves.*

	Control	Glucose	Starch	Cellulose
Trichanthera gigantea	0.064	0.063	0.039	0.032
Gliricidia sepium	0.088	0.112	0.080	0.051
Inga sp.	0.012	0.068	0.051	0.030
Leucaena leucocephala	0.054	0.055	0.028	0.021
Erythrina edulis	0.050	0.080	0.064	0.029
Means	0.053	0.076	0.052	0.033

S.E.D(20 df_{error})=0.0025

The effect of glucose was an increase in the rate constant. The fastest fermentation rate was found with *Gliricidia sepium* but the greatest increase compared with the control was with *Inga* sp. Glucose had no effect on the rate of gas production of *Leucaena leucocephala.*

The effect of starch was unclear. It decreased the rate of gas production with *Trichanthera gigantea* and *Leucaena leucocephala* but increased the rate with *Inga* sp. and *Erythrina edulis*. The fastest rate was found for *Gliricidia sepium* and the slowest for *Leucaena leucocephala.*

Cellulose decreased the rate constant of the fodder tree leaves except with *Inga* sp. The fastest fermentation rate was found for *Gliricidia sepium.*

With regard to the lag phase, there were significant effects of the carbohydrates, fodder species and their interactions ($p<0.001$). Results are shown in Table 6.2.

Table 6.2: *Effect of different carbohydrates on the lag phase (hours) in fermentation of fodder tree leaves.*

	Control	Glucose	Starch	Cellulose
Trichanthera gigantea	20.7	15.7	29.6	37.4
Gliricidia sepium	14.5	8.2	19.6	30.6
Inga sp.	55.3	14.4	30.7	42.5
Leucaena leucocephala	17.5	18.7	47.2	63.5
Erythrina edulis	21.6	10.6	26.6	51.7
Means	25.9	13.5	30.7	45.1

S.E.D(20 df_{error})= 2.07

The effect of the carbohydrates on the lag phase of fodder tree leaves was a decrease in the presence of glucose and an increase with starch and cellulose. The case of *Inga* sp. was an exception as there was a decrease in the lag phase with all carbohydrates. *Inga* sp. is the forage with the lowest fermentability in Menke's medium. When this forage was fermented on its own, the result was an extremely long lag phase, so it is not surprising that this situation improved with the addition of carbohydrates. The longest lag phase was found with the mixtures of *Leucaena leucocephala* and all carbohydrates and the shortest was found with mixtures of carbohydrates and *Gliricidia sepium*.

Table 6.3 shows the effects of the different carbohydrates on the gas pool size. There were significant effects of the sources of carbohydrates, tree leaves and their interactions ($p<0.001$).

Table 6.3: *Effect of different carbohydrates on the gas pool size(ml) from fermentation of fodder tree leaves.*

	Control	Glucose	Starch	Cellulose
Trichanthera gigantea	85.3	252.2	230.7	244.0
Gliricidia sepium	87.7	253.2	287.8	291.3
Inga sp.	40.9	191.4	159.6	124.1
Leucaena leucocephala	86.8	217.5	384.4	415.1
Erythrina edulis	71.5	205.7	246.9	368.2
Means	74.4	224.0	261.9	288.5

S.E.D (20 df_{error})= 11.6

The effect of the carbohydrates was a significant increase in the gas pool size resulting from the fermentation of the fodder tree leaves. The highest gas pool size with glucose was found when it was combined with *Trichanthera gigantea* and *Gliricidia* sepium. With regard to starch and cellulose, the highest gas pool sizes were found with *Leucaena leucocephala*. The lowest gas pool was found for *Inga* sp. in all cases.

Table 6.4 shows the cumulative gas production at 12, 24, 45 and 70 hours of the fodder tree leaves when fermented with the different carbohydrates. According to the analysis of variance there were significant differences (p<0.001) due to the carbohydrates, plant species and their interactions.

Table 6.4: *Effect of carbohydrates on the cumulative gas production (ml) at 12, 24, 45 and 70 hours of fodder tree leaves.*

	Control	Glucose	Starch	Cellulose
12 hours				
Trichanthera gigantea	15.5	77.5	29.0	25.4
Gliricidia sepium	24.8	143.8	38.0	21.1
Inga sp.	8.3	65.7	11.2	10.5
Leucaena leucocephala	23.2	56.8	25.4	23.4
Erythrina edulis	14.5	100.2	17.4	16.9
Means	17.2	88.8	24.2	19.4
S.E.D(20 df_{error})	2.21			
24 hours				
Trichanthera gigantea	38.0	142.2	73.4	55.8
Gliricidia sepium	58.5	205.4	148.0	71.7
Inga sp.	10.1	113.5	39.2	21.9
Leucaena leucocephala	43.5	103.6	59.0	47.1
Erythrina edulis	30.0	144.0	80.2	39.4
Means	36.0	141.7	80.0	47.2
S.E.D (20 df_{error})	4.19			
45 hours				
Trichanthera gigantea	69.3	207.3	127.7	108.7
Gliricidia sepium	79.9	248.2	251.9	176.0
Inga sp.	12.2	166.2	97.2	48.9
Leucaena leucocephala	68.2	170.3	130.5	93.3
Erythrina edulis	52.2	185.2	178.5	110.5
Means	56.4	195.4	157.2	107.5
S.E.D (20 df_{error})	4.49			
70 hours				
Trichanthera gigantea	81.0	254.7	194.8	172.7
Gliricidia sepium	89.4	263.7	287.5	257.7
Inga sp.	17.4	194.0	140.9	80.0
Leucaena leucocephala	82.0	210.1	229.2	173.5
Erythrina edulis	65.5	220.6	233.0	204.0
Means	67.0	228.6	217.1	177.6
S.E.D (20 df_{error})	4.79			

From Table 6.4 two main observations can be made. The first is the effect of the different carbohydrates on the leaves as compared with the control (no carbohydrate) and the second is the best combination (carbohydrate:plant species) in terms of the highest gas production at a given time.

Glucose had the biggest effect in increasing the fermentation of *Inga* sp. at all times. This was followed by *Erythrina edulis* and the lowest increment was found for *Leucaena leucocephala*. This pattern was the same throughout the incubation.

The effect of starch was greatest on *Trichanthera gigantea* and *Gliricidia sepium* at 12 hours. After that period of time, the biggest effect of starch was again on *Inga* sp, followed by *Erythrina edulis* and with *Leucaena leucocephala* the lowest.

Cellulose showed the greatest increment in gas production with *Trichanthera gigantea* and *Inga* sp. during the first 24 hours and with *Inga* sp. and *Erythrina edulis* from 45 hours onwards.

In general, there was a positive effect of the carbohydrates on the fermentation of fodder tree leaves. This effect was greatest on the plant material with the lowest fermentability. The effect also depended on the fermentability of the carbohydrate, being greatest with glucose followed by starch and cellulose. Müller *et al.* (1989) fermented 46 different African fodder trees and shrubs in the presence of glucose, pectin, starch and cellulose with the Menke's gas production method (Menke and Steingass, 1988). Fermentation was carried out with 300mg of plant material and 100mg of carbohydrate for 48 hours. The authors found a positive effect of the addition of carbohydrates with 39 of the 46 species. This is in accordance with the positive effects of carbohydrates in cumulative gas production found in this thesis. No information was provided on the effects of carbohydrates on the fermentation kinetics of the fodder trees.

The other important observation relates to how the two components of the mixture complement each other. The best combination was *Gliricidia sepium* with starch. This plant material showed the highest cumulative gas production with all carbohydrates at all times. It may be explained by the fact that *Gliricidia sepium* had the highest amount of crude and soluble protein (see Table 4.1). The protein appeared to be a good complement to the added carbohydrates. It is also possible that protein from *Gliricidia sepium* have different fractions of different fermentability that are appropriate for each carbohydrate. *Gliricidia sepium* had among the lowest amounts of starch, total sugars and NDF (see Tables 4.2 and 4.3). Therefore is not surprising the response to the added carbohydrates given its high protein content (relation 'crude protein:(starch+total sugars)' = 1.5).

Trichanthera gigantea combined well with starch and cellulose during the first 24 hours and with glucose from 45 to 70 hours. This plant material is highly fermentable and high in total sugars and water soluble carbohydrates (see Table 4.2). These components could boost the fermentation in the first few hours. The effect of added glucose is less in the initial stages and the fermentation of starch and cellulose are favoured by that initial boost.

Erythrina edulis reacted in the opposite way to *Trichanthera gigantea*. It combined well with glucose during the first 24 hours and with starch and cellulose from 45 hours onwards. *Erythrina edulis* is high in starch but low in total and soluble sugars.

Leucaena leucocephala combined well with starch and cellulose in the first 24 hours. In general, this species did not yield high gas values when combined with the carbohydrates. The same was true of *Inga* sp. Although these two species have high crude protein contents, they are also high in condensed tannins and protein precipitation activity. It is possible that the protein from these species is bound to tannins and therefore unavailable for the fermentation of the added carbohydrates. *Leucaena leucocephala* is well balanced in terms of the relation 'crude protein:(starch+total sugars)' of 1.1, this may be another reason for the lack of response to carbohydrates.

The fermentation rates can help to illustrate the fermentability of the different components of the mixture. As glucose is rapidly and completely fermentable, the kinetics of its mixture with fodder tree leaves (in a nitrogen free medium) may indicate, at least partially, the availability and/or fermentability of the nitrogen from the leaves. (It is important to bear in mind that other rapidly fermentable material from the leaves can benefit from the initial boost in the fermentation by having the combination of glucose and rapidly available and fermentable nitrogen.) This can be supported by the work of Raab *et al.* (1983) who devised a method for the *in vitro* determination of protein degradation in rumen fluid using the Menke gas production method (Menke and Steingass, 1988). This was based on measurements of ammonia concentration and gas production when the feedstuff was incubated with increasing amounts of starch in a nitrogen-free medium. The method has been used to determine the protein degradation of fodder tree leaves (Müller *et al.*, 1989). In this study, the fermentation of the leaves with carbohydrates can give an approximation of the fermentability of the protein from the leaves. It is important to have in mind the carbohydrate contents of the leaves and their balance in terms of the protein:energy ratio.

Figure 6.2 shows the effects of carbohydrates on the fermentation rates of the plant species.

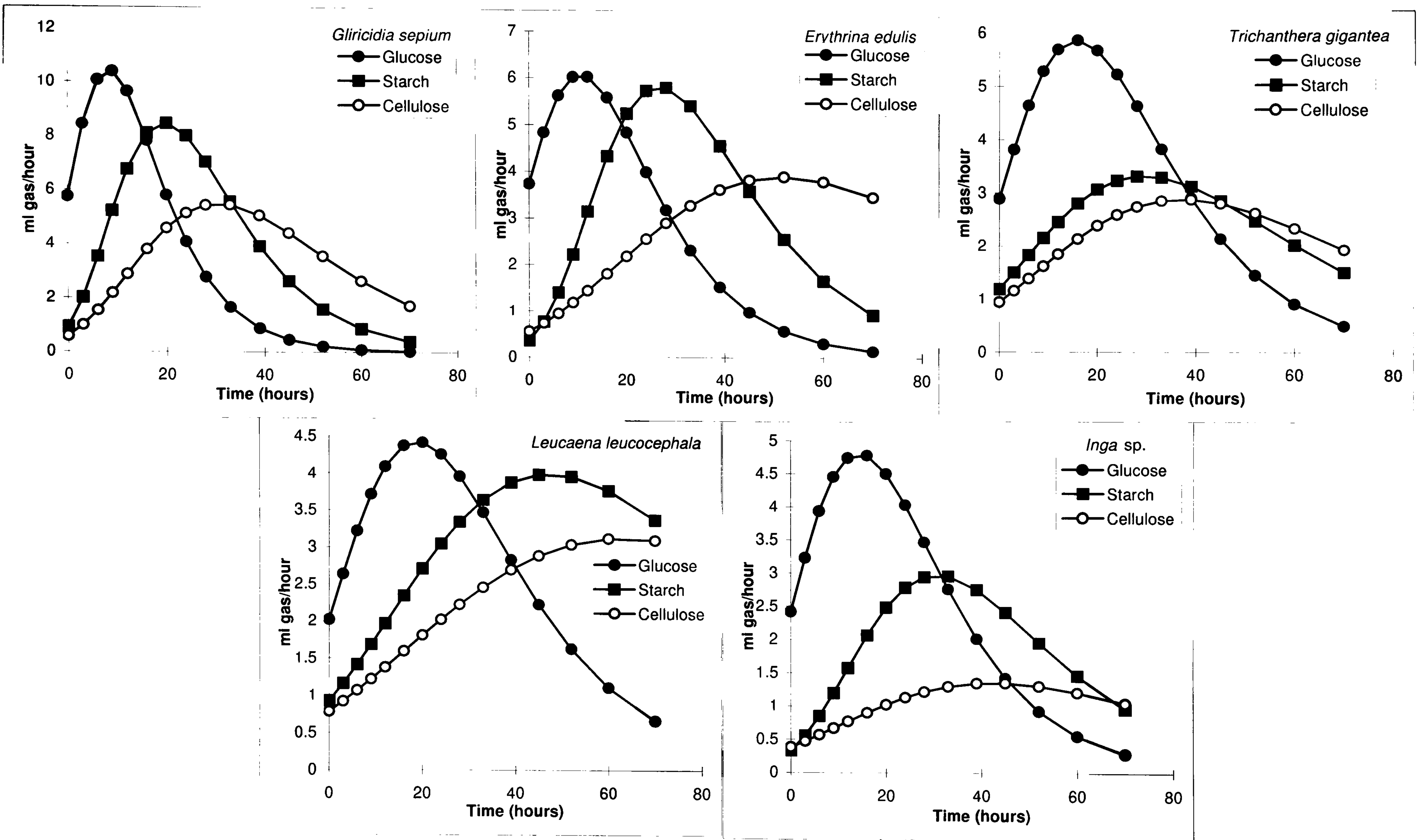


Figure 6.2: *Gas production rates (ml/h) of mixtures of carbohydrates and fodder tree leaves. Figures are not to the same scale.*

In the case of *Gliricidia sepium*, the profiles of their mixture with glucose show an early peak at 9 hours and a rapid decrease to reach zero at 70 hours. Starch produces a later second peak (20 hours) and cellulose peaks even later (33 hours) as is expected for these types of substrates. In the case of glucose, the fermentation of the mixture was carried out completely. This indicates that *Gliricidia sepium* may have a rapidly fermentable nitrogen fraction. The rates with starch and cellulose were faster than when these carbohydrates were combined with other leaves (see Table 6.1). The rates also seemed to peak earlier with *Gliricidia sepium*. This plant material may have different nitrogen fractions of different fermentabilities which may be appropriate for the different carbohydrates. *Gliricidia sepium* was the plant material with both the highest soluble and insoluble protein but their carbohydrate contents were among the lowest. This hypothesis is supported by the fact that *Gliricidia sepium* clearly showed the three distinct peaks associated with each carbohydrate.

Erythrina edulis was very similar to *Gliricidia sepium* except that the peaks of the mixtures with starch and cellulose were at later stages (28 and 52 hours). They were, after *Gliricidia sepium,* the highest peaks of all mixtures. This shows that *Erythrina edulis* is a good complement to starch and cellulose and suggests that it may have nitrogen fractions of different fermentabilities that combine well with each carbohydrate. This apparent late availability of nitrogen may be due to the nature of the protein itself (like in the case of BSA) as this plant has no tannins. *Erythrina edulis* was high in starch but had the lowest contents of total sugars.

Trichanthera gigantea profiles peaked at 16, 28 and 39 hours with glucose, starch and cellulose respectively. The fermentation rate with glucose, was very similar to that of the mixture with *Inga* sp. whereas the rate of fermentation of starch was similar to that with *Erythrina edulis*. The fermentation profile of its mixture with cellulose peaked earlier than all plant material except *Gliricidia* sepium. This suggests that its nitrogen may be more fermentable towards the middle of the fermentation (between 16 and 39 hours).

The rate profiles of *Leucaena leucocephala* were late compared with the other plant materials. Mixtures of this species with glucose peaked at 20 hours, the mixture with starch at 45 and that with cellulose at 60 hours. This suggests that the protein from *Leucaena leucocephala* may not be highly fermentable or that it is made available at a later stage compared with the other species. This species is high in phenols, condensed tannins and protein precipitation activity. It is also well balanced in the protein:energy ratio. The hypothesis that its protein is not highly fermentable is supported in the literature. Aii and Stobbs (1980) suggested that a considerable quantity of N in *Leucaena leucocephala* may be resistant to ruminal degradation. Flores *et al.* (1979) found that supplementation with

Leucaena leucocephala increased milk production to a similar degree as that for casein protected with formaldehyde. Goodchild and McMeniman (1994) found that *Leucaena leucocephala* increased rumen ammonia concentration at the same low levels as slowly fermentable proteins like cowpea straw and formaldehyde-treated casein. *Inga* sp. is also very high in phenolic compounds and had the greatest effect on protein precipitation. However, the fermentation rates of mixtures of carbohydrates with this species did not peak as late as in the case of *Leucaena leucocephala*.

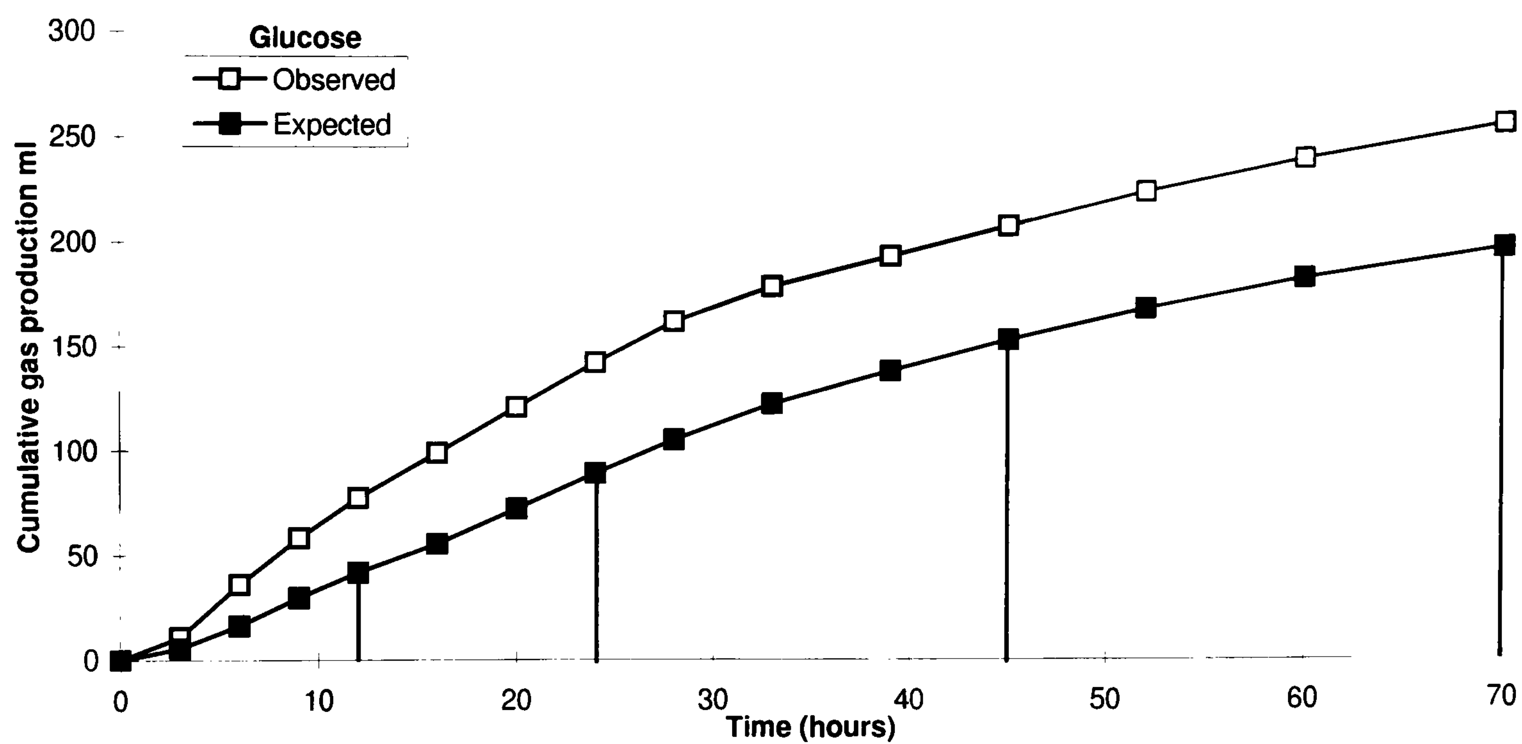
The results showed that *Gliricidia sepium* would be appropriate for the fermentation of the different types of carbohydrates. *Erythrina edulis* is a good match for the fermentation of starch whereas *Trichanthera gigantea* combines well with glucose. Plant species high in tannins and protein precipitation activity like *Leucaena leucocephala* and *Inga* sp. combine well with starch and cellulose. This is important to understand the associative effects of mixtures of tree leaves (see Chapter 8).

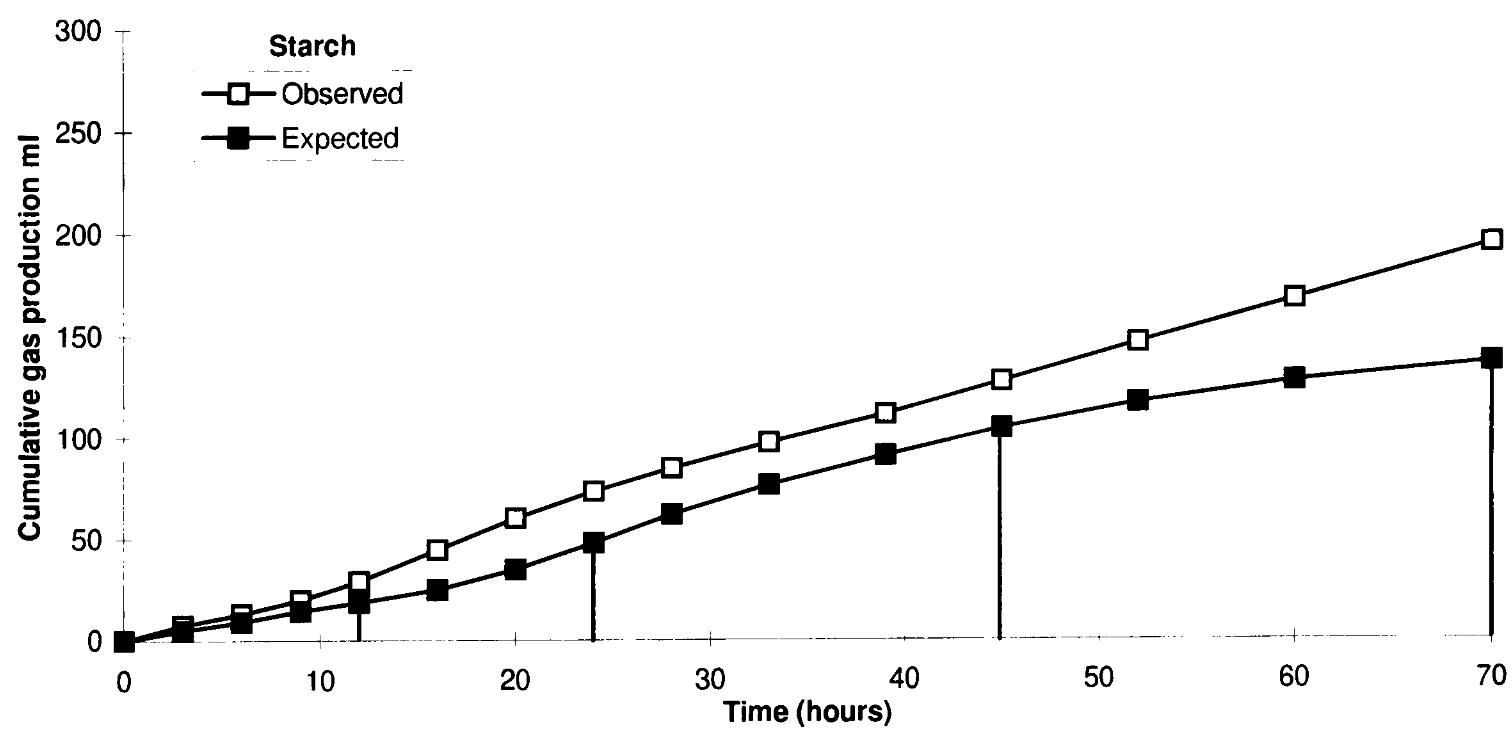
ASSOCIATIVE EFFECTS

There were significant differences between the sum of the gas produced by single components and that of most mixtures at the different times tested. In these cases, the null hypothesis that the gas produced by the mixture was equal to the sum of gas production by individual components was rejected. This means that the fermentation of carbohydrates and fodder tree leaves is not additive. Figures 6.3 to 6.7 show the associative effects of the mixtures.

According to the figures, there were two types of responses. The first type was exponential and it was shown in all mixtures with glucose. This effect was generally large except in the case of the mixture *Leucaena leucocephala*:glucose. This mixture showed associative effects only at 45 hours. As previously shown, this particular mixture had the lowest gas production of all.

The second type was characteristic of the mixtures with starch and cellulose in which there was an initial period of no effect or a negative effect, followed by a rapid increase in the response to finally show a positive associative effect. This is a sigmoidal response but is sometimes nearly linear. The only exception to this was the mixture *Gliricidia sepium*:starch which was exponential. This is not surprising as it was shown that this is the ideal combination of substrates to maximise gas production under these conditions.





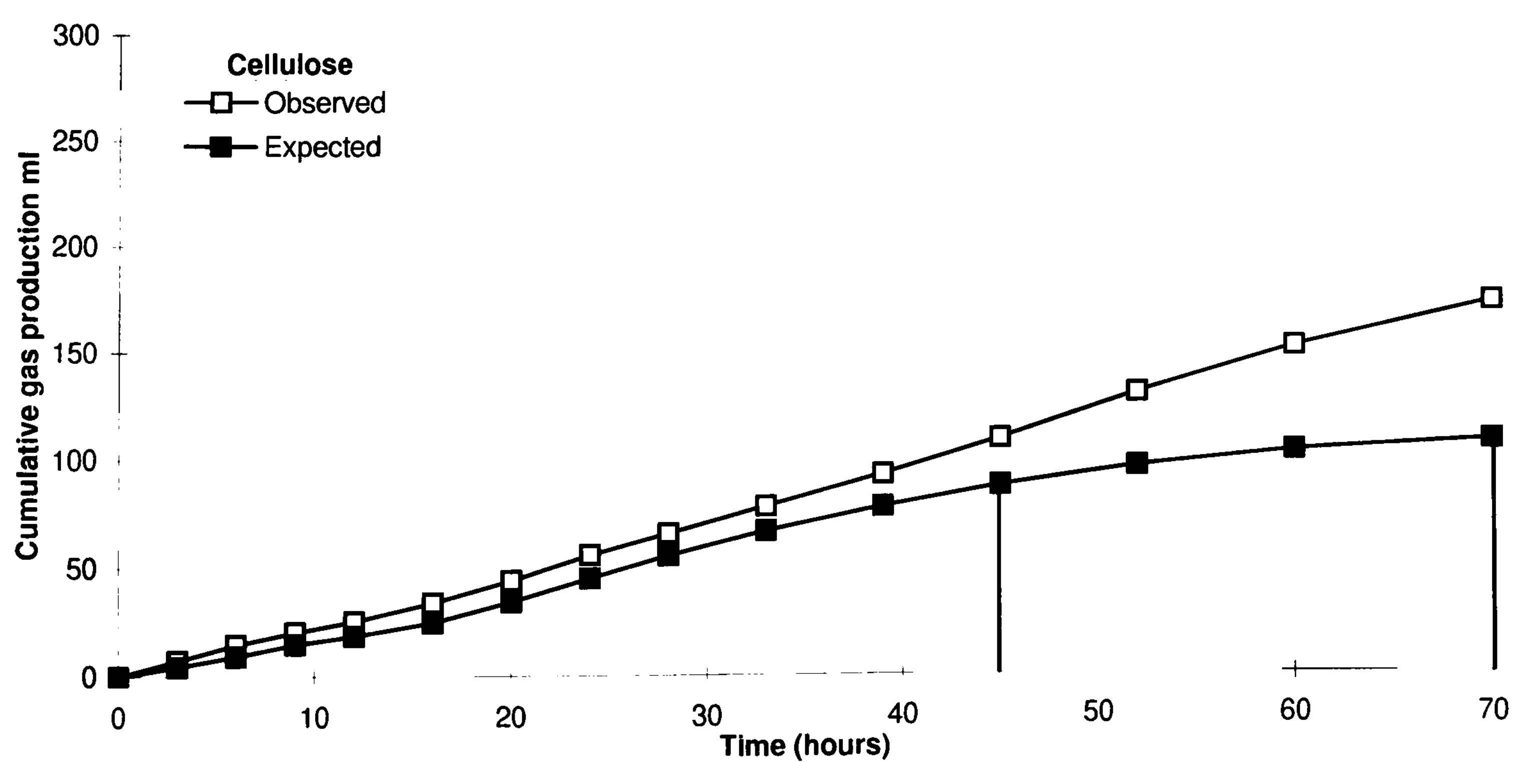


Figure 6.3: *Associative effects of a mixture of Trichanthera gigantea and carbohydrates.* Associative effects were tested at 12, 24, 45 and 70 hours. *Vertical lines show the times at which significant differences were found.*

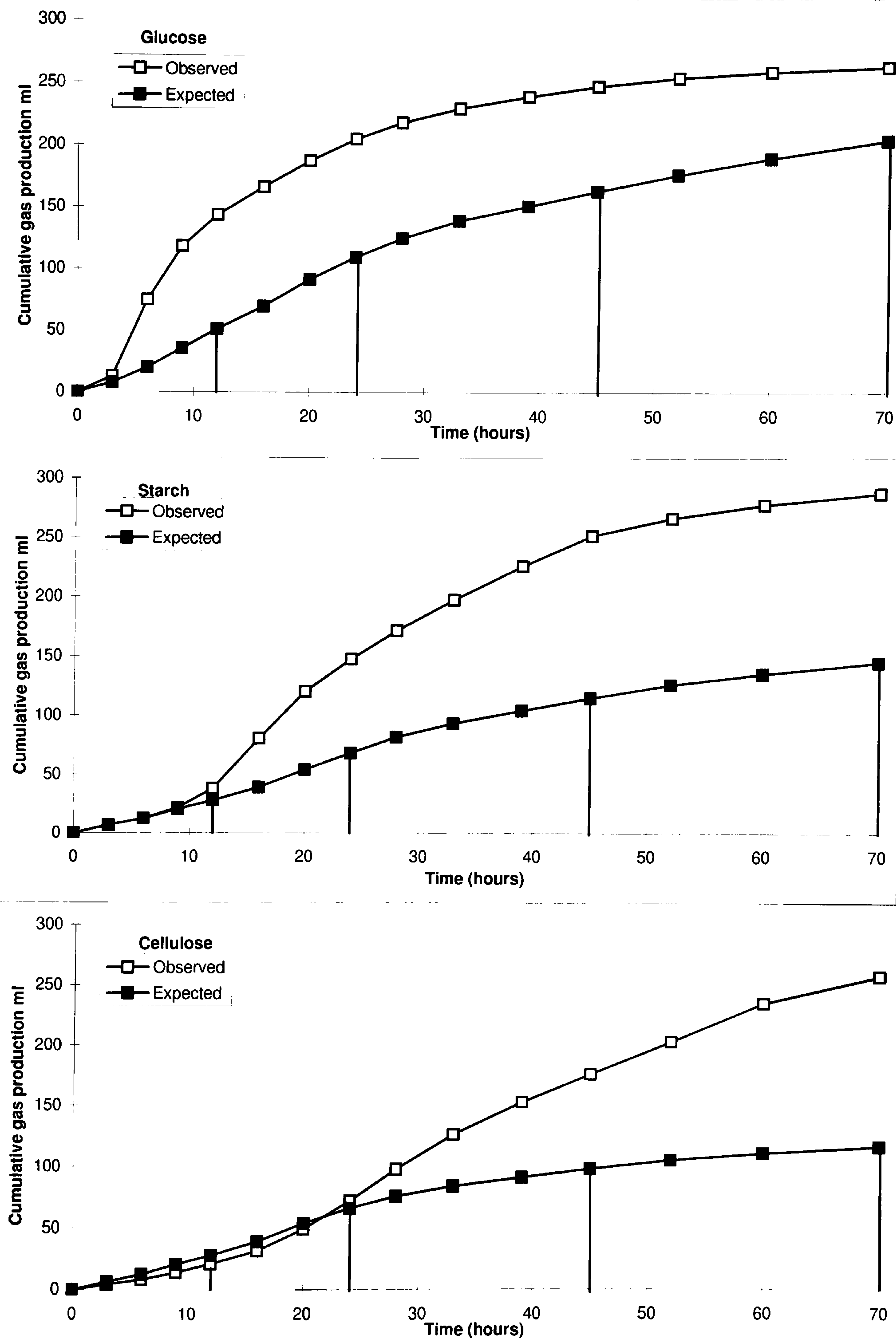


Figure 6.4: *Associative effects of a mixture of Gliricidia sepium and carbohydrates.* Associative effects were tested at 12, 24, 45 and 70 hours. *Vertical lines show the times at which significant differences were found.*

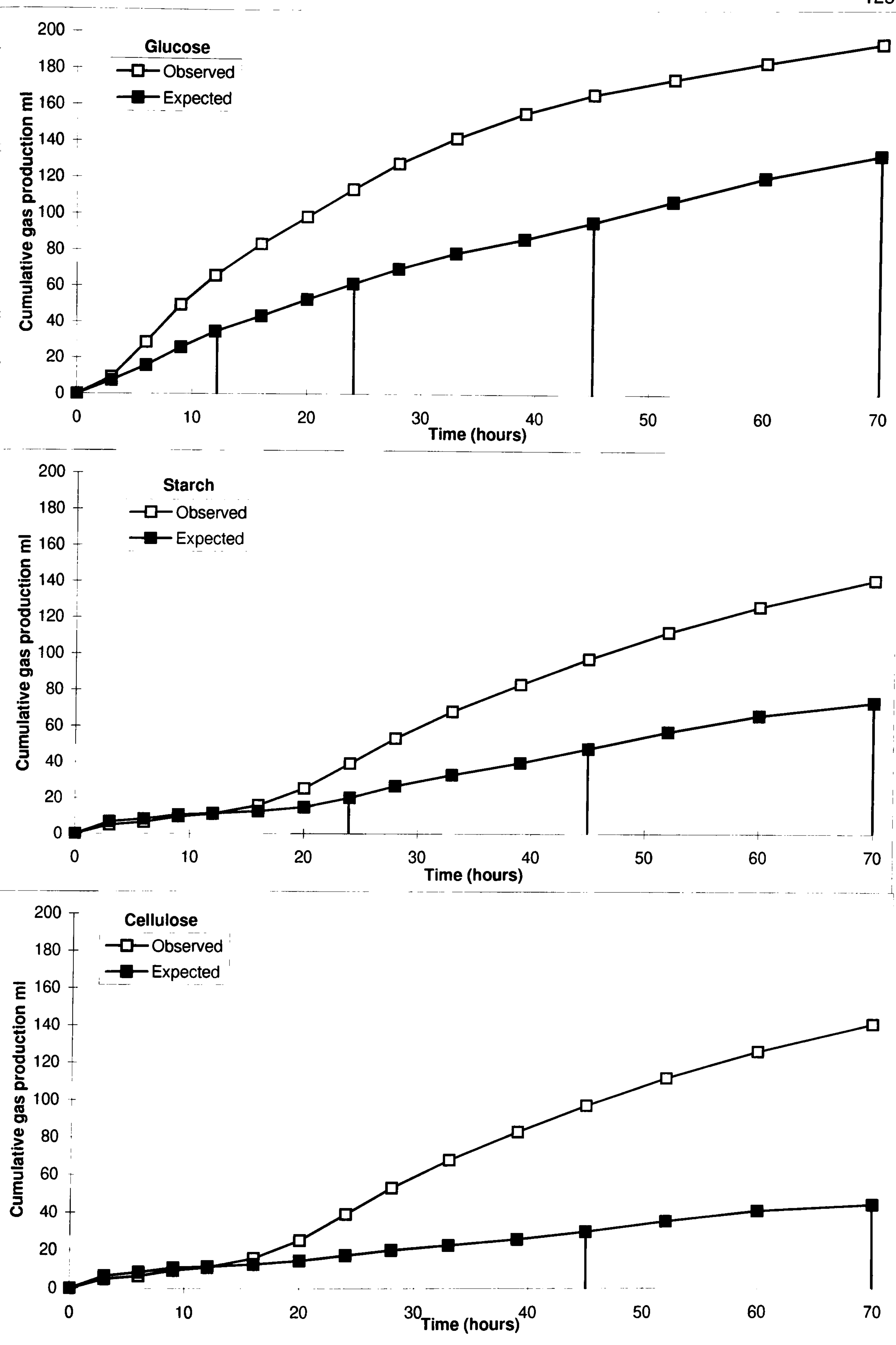
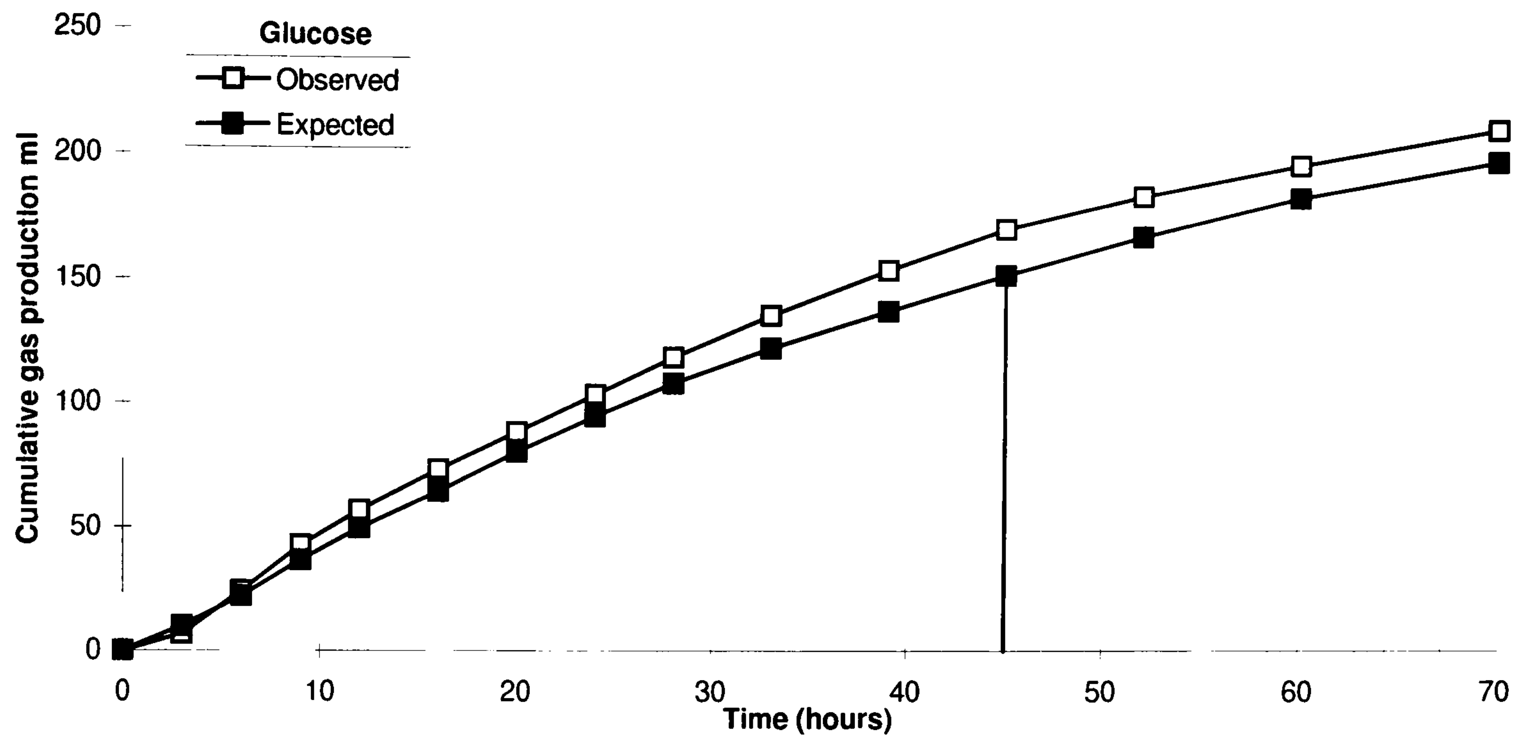
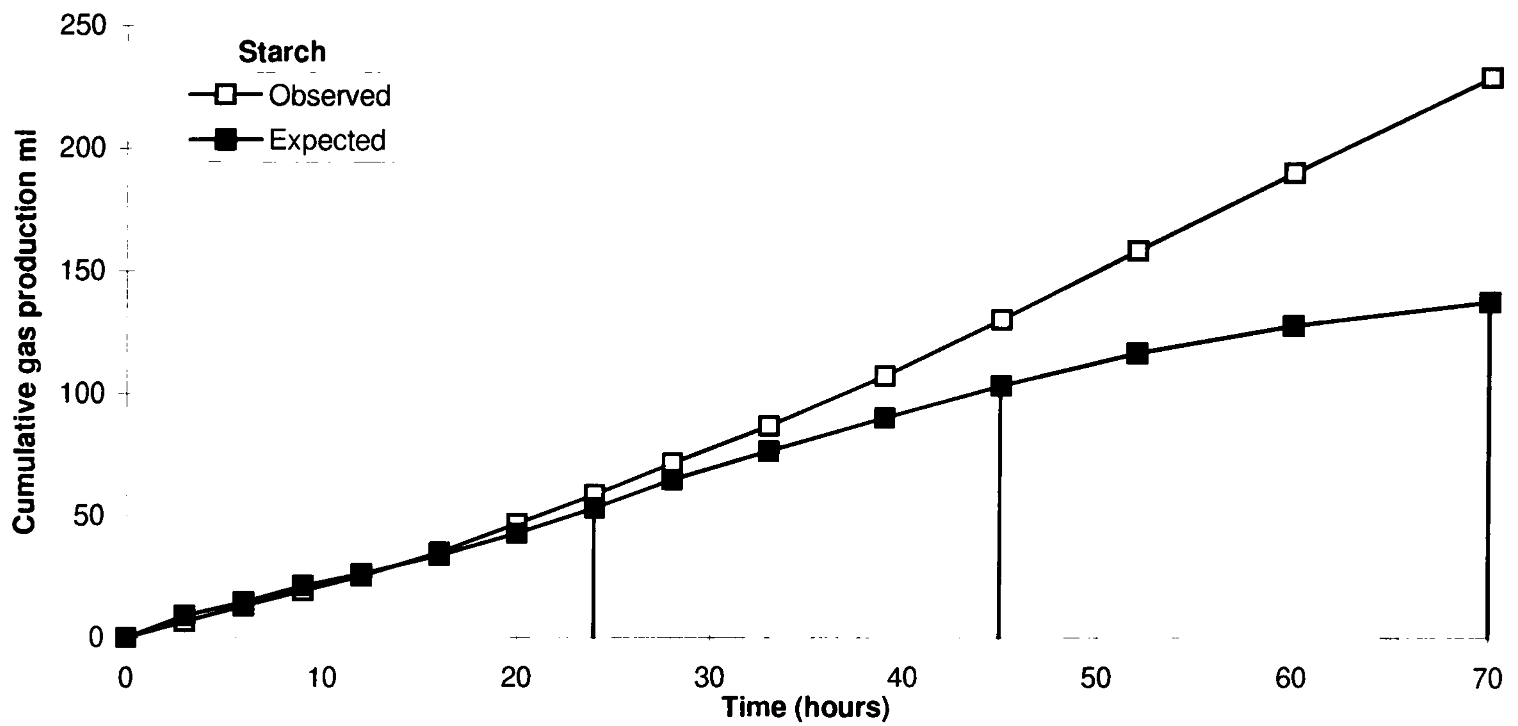


Figure 6.5: *Associative effects of a mixture of Inga sp. and carbohydrates.* Associative effects were tested at 12, 24, 45 and 70 hours. *Vertical lines show the times at which significant differences were found.*





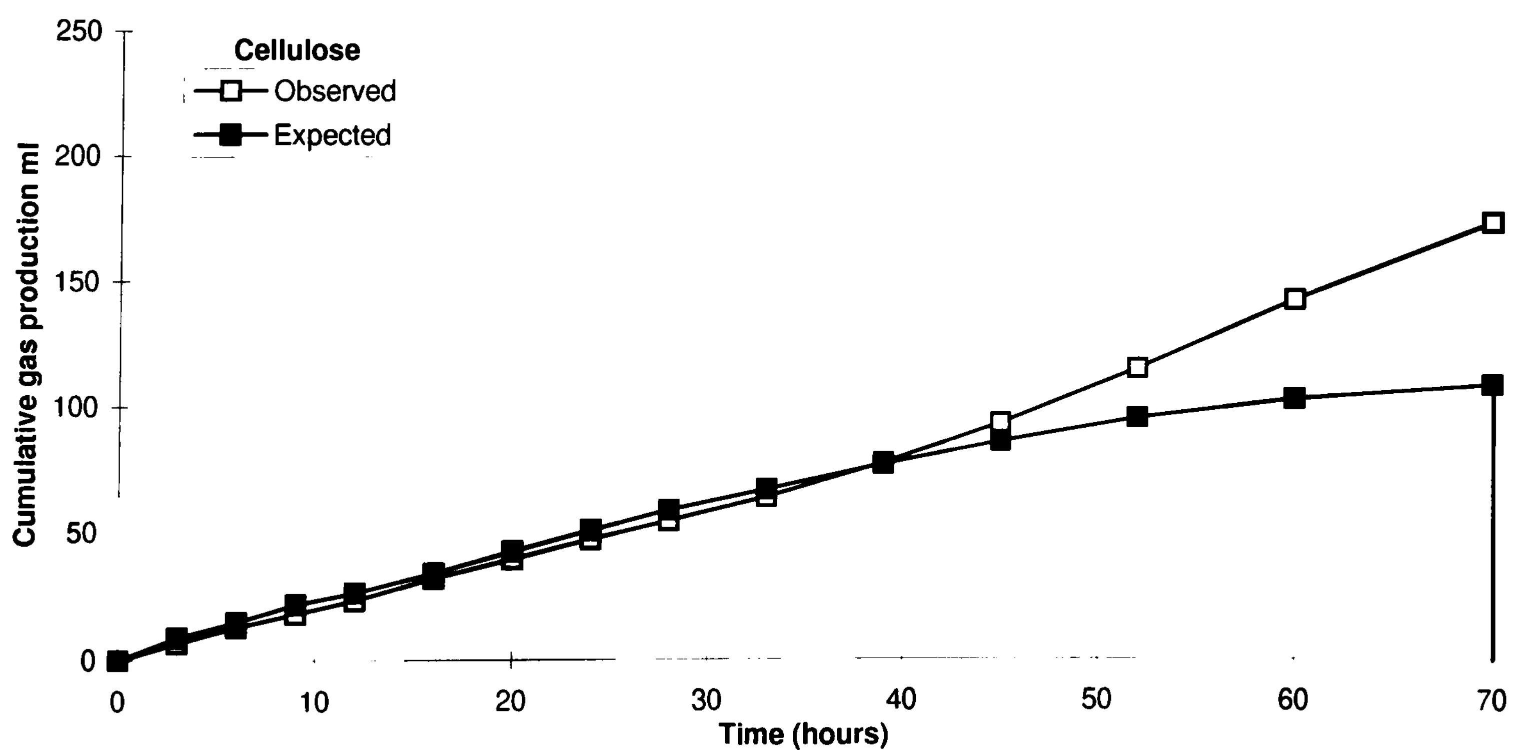
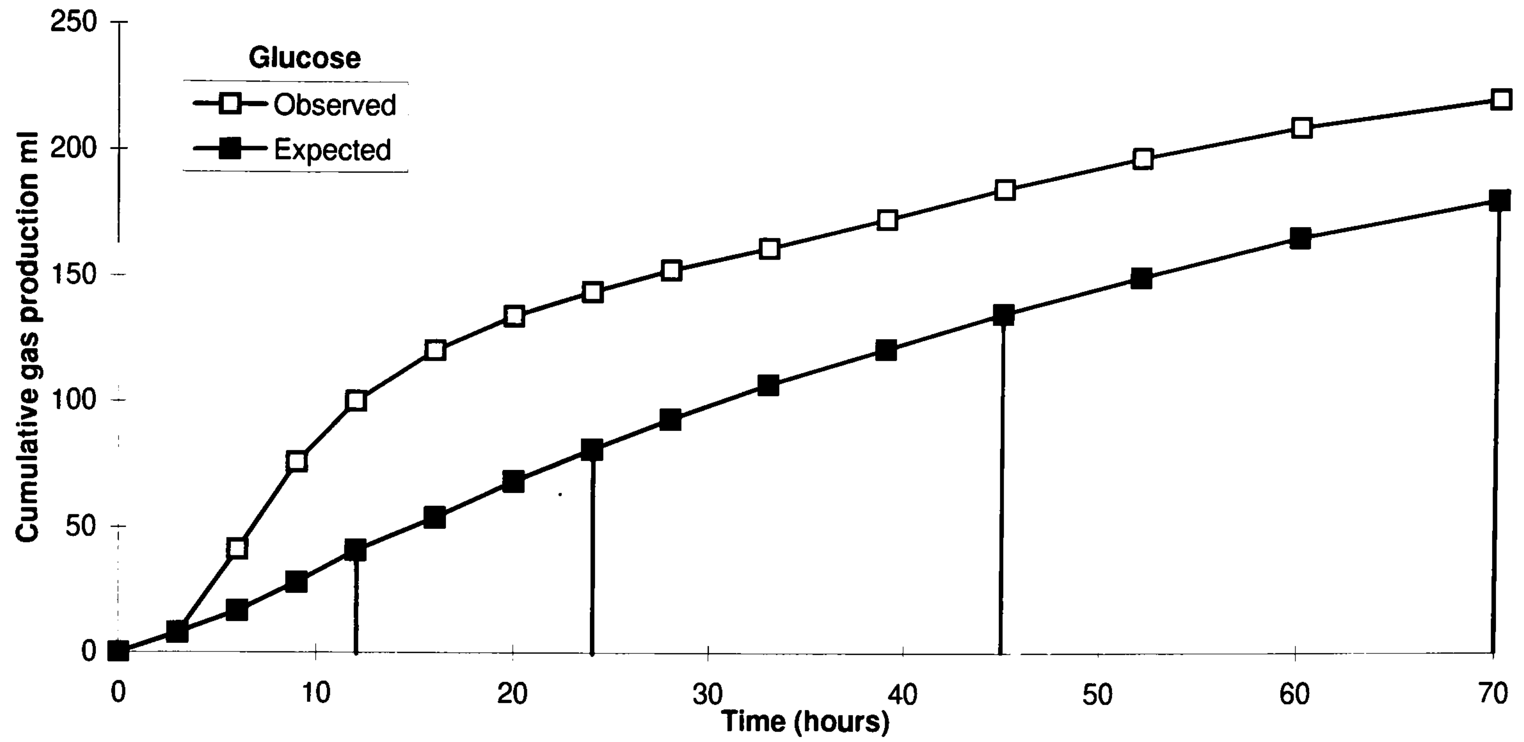
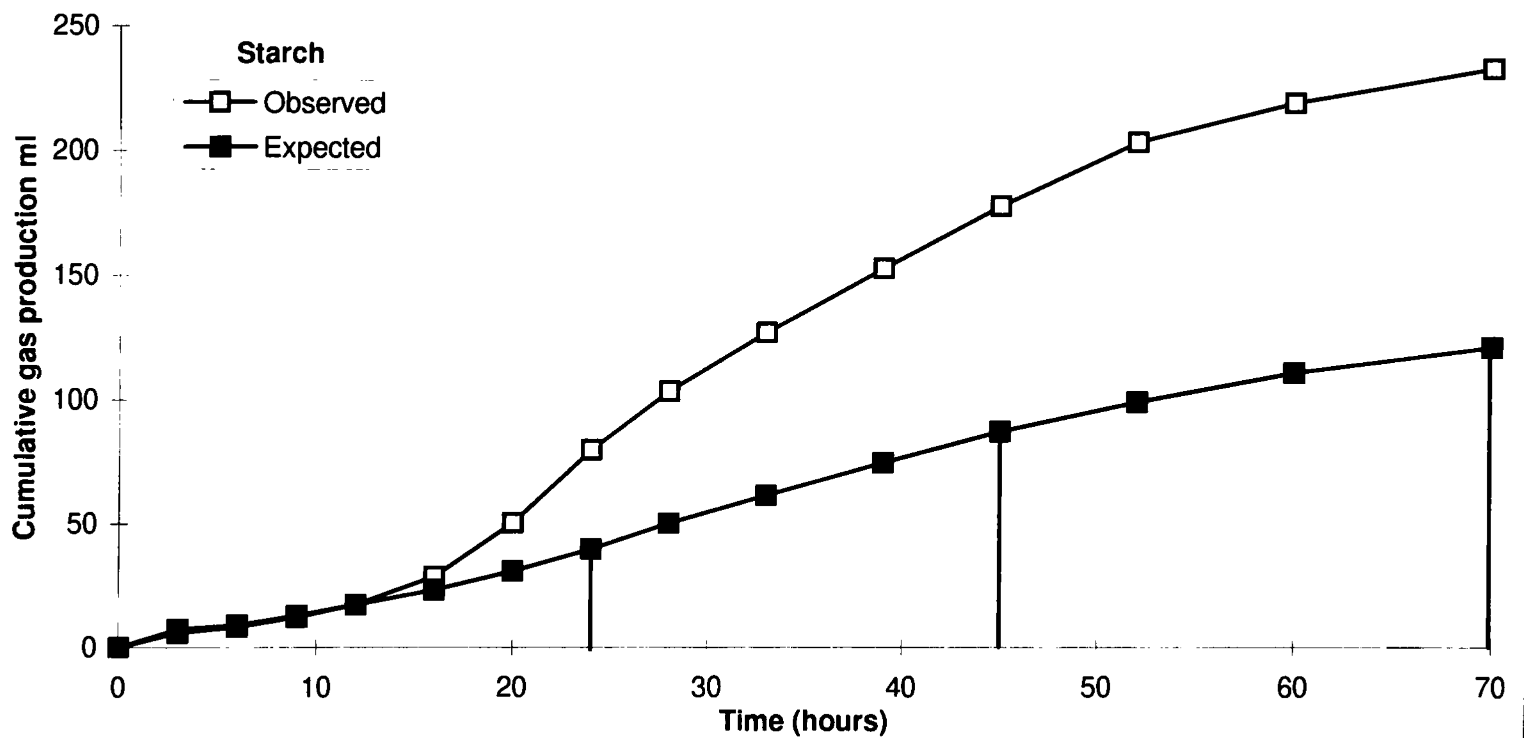


Figure 6.6: *Associative effects of a mixture of Leucaena leucocephala and carbohydrates.* Associative effects were tested at 12, 24, 45 and 70 hours. *Vertical lines show the times at which significant differences were found.*





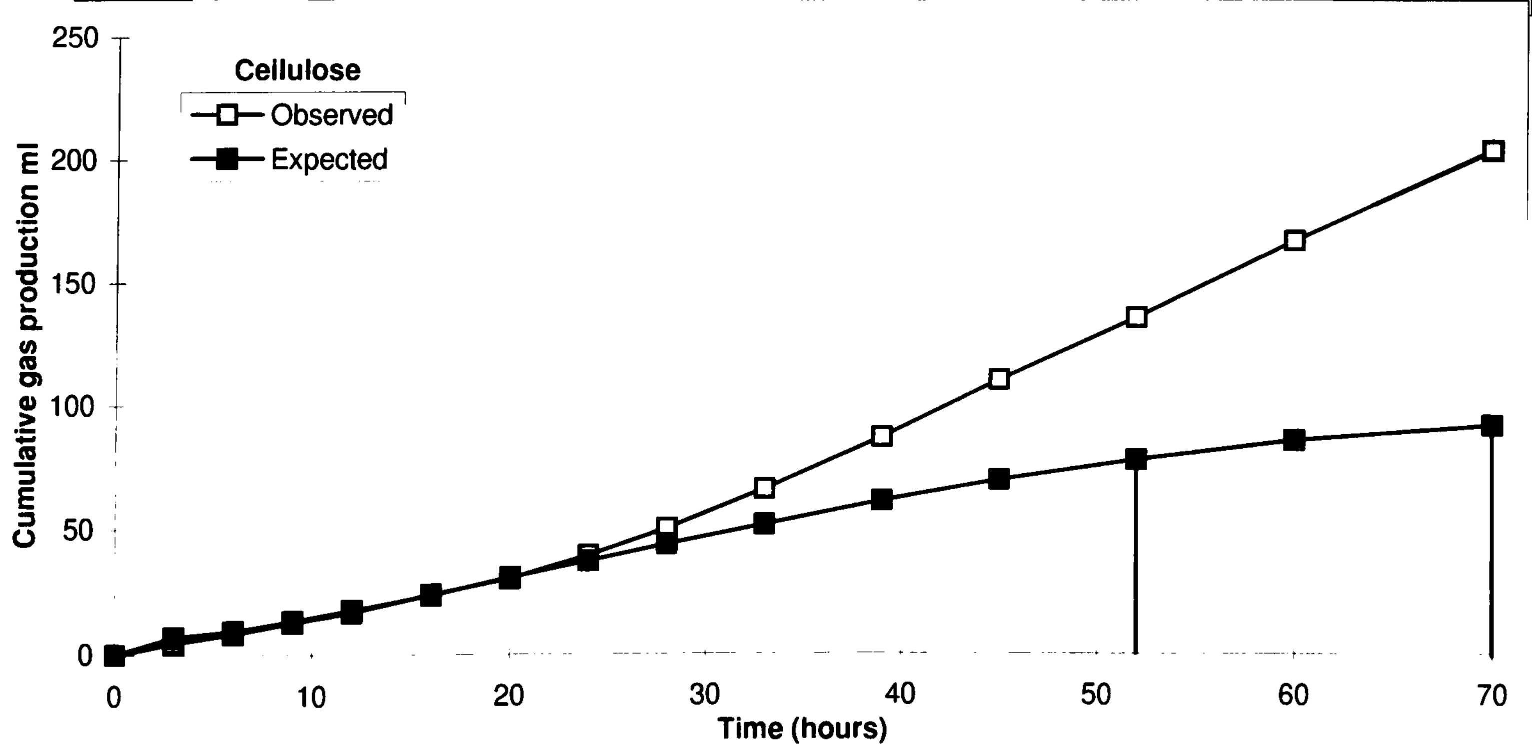


Figure 6.7: *Associative effects of a mixture of Erythrina edulis and carbohydrates.* Associative effects were tested at 12, 24, 45 and 70 hours. *Vertical lines show the times at which significant differences were found.*

In Chapter 5 it was shown that an exponential response was characteristic of mixtures in which there is an early synchronisation of the release of nutrients. This was the case of mixtures of glucose:starch and glucose:cellulose in the presence of casein. The sigmoidal response was characteristic of mixtures with BSA in which the synchronisation occurs at a later stage of the fermentation. In the case of the fodder tree leaves both types of responses were observed for each species suggesting that synchronisation in the release of nutrients from tree leaves may occur both early or late. This also supports the hypothesis that different fractions of nitrogen from the leaves are being used with the different carbohydrates. Associative effects of the mixtures of the plant species and carbohydrates can be seen in Table 6.5

Most of the associative effects were positive. Tree leaves are sources of nitrogen and are therefore more likely to show positive associative effects resulting from the protein supplementation effect. There were however some cases of negative associative effects in the early stages of fermentation. This effect was greatest with the mixture *Gliricidia sepium*:cellulose in the early stages but later it changed to a positive effect.

This illustrates that associative effects with fodder tree leaves can be of a composite nature. Initial negative effects are presumably, due to the depression in fibre digestibility, as the available nitrogen is fermented with rapidly fermentable carbohydrates. Later positive effects are observed presumably when more nitrogen is made available to provide amino acids and peptides that stimulate cellulolytic bacterial growth. The late availability of nitrogen may be related to the presence of phenolic compounds or to the nature of the protein itself.

The associative effects tended to be greater in mixtures with starch and cellulose especially towards the end of the incubation period (except in the case of *Trichanthera gigantea*). This is explained by the fact that the effect of these two carbohydrates on the fermentation was a decrease in the fermentation rate and an increase in the lag phase. These conditions may favour late synchronisation in the release of nutrients as tree leaves also have long lag phases by themselves. This was shown in Chapter 4 as the effect of Menke's medium on the fermentation of the tree leaves was a reduction in the rate constant and an increase in the lag phase.

Associative effects with glucose were higher at the beginning and decreased towards the end of the fermentation as would be expected with a rapidly fermentable substrate. The effect of glucose on the fermentation kinetics was an increase in the rate constant and a reduction in the lag phase. The presence of glucose as rapidly fermentable carbohydrate may mean that the available nitrogen is used

rapidly, boosting the fermentation in the first stage of the incubation period. Brown and Pitman (1991) studied the associative effects of mixtures of grasses and legumes. They observed positive associative effects with *in vitro* NDF digestion and concluded that the likely mode of action was a reduction of the lag phase due to the contribution of soluble and/or degradable nitrogen from the legumes.

Table 6.5: *Associative effects (%) on cumulative gas production of mixtures of carbohydrates and fodder tree leaves.†*

	Time (hours)			
	12	**24**	**45**	**70**
Trichanthera gigantea:glucose	14.2**	7.3**	4.2**	3.4**
Trichanthera gigantea:starch	22.4**	8.7**	5.0**	8.3***
Trichanthera gigantea:cellulose	29.8**	5.5n.s	4.1*	7.2**
Gliricidia sepium:glucose	23.9***	9.9***	6.4***	3.5**
Gliricidia sepium:starch	17.4**	26.2***	26.3***	28.1***
Gliricidia sepium:cellulose	-51.3***	4.7*	16.0***	18.9***
Inga sp.:glucose	16.7**	10.7**	7.6**	4.4**
Inga sp.:starch	-1.9n.s	34.2**	54.6***	72.1***
Inga sp.:cellulose	-7.8n.s	14.3n.s	15.4**	16.4**
Leucaena leucocephala:glucose	5.1n.s	2.6n.s	2.3*	1.0n.s
Leucaena leucocephala:starch	-4.5n.s	9.1*	14.8***	34.3***
Leucaena leucocephala:cellulose	-12.2n.s	-4.0n.s	2.1n.s	10.0***
Erythrina edulis:glucose	21.8***	8.7**	4.0**	2.3**
Erythrina edulis:starch	-0.3n.s	8.3**	10.1***	8.5***
Erythrina edulis:cellulose	-2.1n.s	1.7n.s	11.3**	13.4***

†Calculated as: (observed-expected/observed)x100.
n.s Not significant.
* Significant at 5% level.
** Significant at 1% level.
*** Significant at 0.1% level.

6.4 GENERAL CONCLUSIONS

The effect of carbohydrates on the fermentation of fodder tree leaves (compared to the control without carbohydrate) was an increase in the rate constant with glucose and a decrease with starch and cellulose. The effect on the lag phase was reversed. Glucose decreased the lag phase and starch and cellulose increased it. All carbohydrates increased the gas pool size.

In terms of cumulative gas production of the mixtures, there was a positive effect of the carbohydrates on the fermentation of fodder tree leaves. This effect was greater on the plant material with the lowest fermentability. The effect also depended on the fermentability of the carbohydrate, being greatest with glucose followed by starch and cellulose. The fermentability of the plant material also played an important role. The results showed that *Gliricidia sepium* was a good complement to all the different types of carbohydrates. *Erythrina edulis* was a good match for the fermentation of starch whereas *Trichanthera gigantea* combined well with glucose. The plant species high in tannins and protein precipitation activity, *Leucaena leucocephala* and *Inga* sp., combined well with starch and cellulose. The results suggested the presence of different fractions of protein of different fermentability in the tree leaves that may explain the differences in the time at which the maximum fermentation rate was achieved. In the case of fodder tree leaves, a late availability of nitrogen may be due to the nature of the protein itself or to the presence of phenolic compounds.

Associative effects between fodder tree leaves and carbohydrates were shown to occur. Two types of responses were identified; the first was exponential and characteristic of the mixtures with glucose. The second was sigmoidal (it resembled a linear response in some mixtures) and it was characteristic of mixtures with starch and cellulose. These two responses in associative effects were similar to those obtained with mixtures of carbohydrates, casein or BSA where the synchronisation in the release of nutrients occurred at early and late stages respectively.

Associative effects with fodder tree leaves may be of a composite nature as they can be both negative and positive.

The associative effects tended to be greater in mixtures with starch and cellulose, especially towards the end of the incubation period. The mode of action was a decrease in the fermentation rate and an increase in the lag phase of the mixtures. The associative effects with glucose were higher at the beginning and decreased towards the end of the fermentation. The mode of action was an increase in the rate constant and a reduction in the lag phase.

Chapter 7

EFFECTS OF TANNINS FROM TROPICAL FODDER TREE LEAVES ON THE FERMENTATION KINETICS OF MIXED SUBSTRATES

7.1 INTRODUCTION

Tannins are widely distributed in nature and are the fourth most abundant group of plant constituents after cellulose, hemicellulose and lignin. They do not seem to have a purely physiological function but have been shown to influence growth, development and reproduction of higher plants. It has been suggested that tannins constitute a unique quantitative defence against predators, as a repellent and by decreasing the feeding value of some forages (Clausen *et al.*, 1992). However, tannins in some species actually enhance nutrition in ruminants. Much protein from highly digestible feed is lost by microbial degradation and subsequent absorption as ammonia in the rumen. *In vitro* and *in vivo* studies have shown that some plant tannins, if not in excess, reduce the rate of ammonia produced and make the tanned protein available for enzymatic digestion beyond the rumen, improving the efficiency of protein utilization (Barry, 1988). This is only possible if a stable tannin-protein complex is formed that is not susceptible to metabolism by microorganisms yet does not interfere with subsequent enzymatic digestion of protein in the lower gut. The role of tannins (and other phenolic compounds) in the fermentation is less clear. Ingested tannins can be metabolized by, or can be toxic to, rumen microbes. The effect of several plants species containing tannins on the fermentation has been studied *in vitro*. Reports show that tannins can reduce the degradation of cellulose, starch, pectin and glucose, and protein from several sources (Mueller-Harvey and McAllan, 1992). Because of the chemical diversity, the biological response to tannins depends on their nature and varies between plant species.

Information about the effects of tannins on the main components of plant material, and their interaction, is required to understand fully the fermentation kinetics of the selected plant species and the possible role of tannins and phenolic compounds in mixtures of fodder tree leaves.

OBJECTIVES

The objective of this chapter is to analyse the role of condensed tannins and phenolic compounds

during *in vitro* fermentation and how this affects the kinetics of gas production by the main components of the feeds, structural and non-structural carbohydrates and different proteins.

The general strategy was to use a protective agent to bind the tannins in the plant material; thus the inhibition of the fermentation by tannins could be estimated from the difference in gas production between the presence and absence of the tannin-binding agent.

7.2 EFFECT OF THE CONCENTRATION OF A TANNIN BINDING AGENT, POLYETHYLENE GLYCOL 4000 (PEG) ON THE FERMENTATION

There are a large number of compounds that can be used to mitigate or remove adverse effects of tannin rich plants. These tannin-complexing agents include polyethylene glycol (PEG), polyvinylpirrolidone (PVP), and polyvinylpolypirrolidone (PVPP) (Clausen *et al.*, 1992). The ability of these inert agents to react with condensed tannins enables them to be used to study the condensed tannins-protein-animal interactions (Kumar, 1992b; Kumar and Vaithiyanathan, 1990; Mangan, 1989). A series of preliminary experiments to test the protective agents PVP and PVPP in the radial diffusion and gas production techniques was carried out with forages with different amounts and kinds of phenolic compounds (data not shown). Different levels of PVP and PVPP were tested and compared with PEG in the gas production technique for short- and long-term fermentations (12, 45, 166 hours) with Theodourou's medium. All the agents used significantly ($p<0.05$) increased gas production, but results were inconsistent as the response of the different species was different according to the agent used. PVP appeared to increase the fermentation to a greater extent than PVPP, but both also increased the fermentation of forages with no assayable phenols or tannins. The effect of PEG was more consistent. It did not increase gas production of tannin-free forages. The maximum increment in gas production due to the chemicals was found at very different times in each forage (between 9 and 20 hours for PEG and PVP, and between 20 and 45 for PVPP). Khazaal and Ørskov, 1994, also found inconsistencies in the results when using PVP in Menke's gas production technique with different plant material. The increase in gas production was not found to be significantly ($p>0.05$) correlated with any phenolic assay used. Based on these preliminary results, it was decided to use PEG as the binding agent to determine the effects of tannins on the fermentation.

OBJECTIVES

The objective of this section was to determine an optimum level of PEG to overcome the effects of tannins on the fermentation; this was then used in the later experiments.

DETERMINATION OF THE OPTIMUM LEVEL OF PEG TO OVERCOME THE EFFECTS OF TANNINS BY THE RADIAL DIFFUSION TECHNIQUE

Materials and methods

As no reference was found concerning the optimum level of PEG to overcome the effect of tannins on gas production, a wide range of levels was tested. An initial range was selected by the radial diffusion technique. The principle being to maximise the binding of tannins to PEG thus reducing their binding to protein (protein precipitation activity).

Two experiments were designed to establish the optimum range by using polyethylene glycol 4000 (BDH 44273) as the binding agent and tannic acid (SIGMA 1401-55-4) as a source of tannins.

In the first experiment, six levels of PEG (0, 20, 40, 60, 80 and 100mg/ml) were tested. Two solutions, one of 200mg of PEG/ml and the other one of 20mg of tannic acid/ml in distilled water were made up to volume (10ml) with acetone to give the six solutions for the different levels. 15μl of each of the final solutions were taken in duplicate and placed on two haemoglobin - agarose plates according to the radial diffusion method (see Chapter 3). Results are expressed on a fresh matter basis. There were 4 replicates for each level.

The second experiment was designed according to the results of the first experiment and, in this case, six different levels of PEG (0, 8, 16, 24, 32 and 40 mg/ml) were tested. In this experiment, the solution of PEG was 8mg/ml and the tannic acid remained unchanged. The experimental conditions were the same as with the previous experiment.

Statistical analysis

Results of the two experiments (protein precipitated cm^2/g) were analysed by one-way analysis of variance. The total number of observations for each experiment was 24.

Results and discussion

According to the analysis of variance, there were significant differences ($p<0.001$) between the levels of PEG in both experiments. Figure 7.1 shows the response in protein precipitation activity to the different levels of PEG. In the first experiment, there was a sharp decrease ($p<0.001$) in the

precipitation of protein from 0.97 to 0.20cm^2/g, corresponding to 0 and 20mg/ml of PEG. There was no further significant decrease from 20mg/ml onwards. It was therefore decide to test a narrower range (from 0 to 40mg/ml).

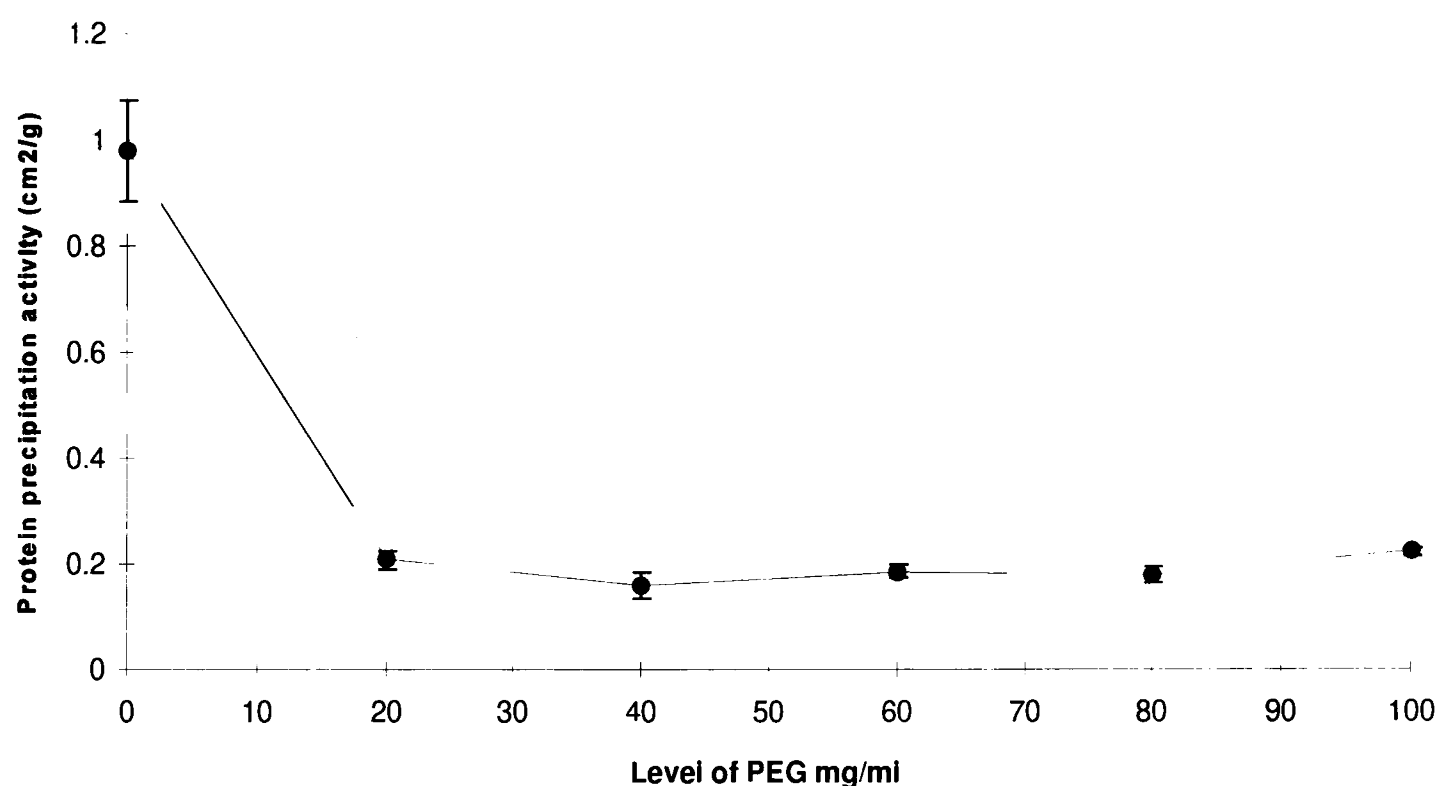


Figure 7.1: *Effect of different levels of polyethylene glycol (PEG) on the protein precipitation activity of tannic acid.*

The results of the second experiment, covering that range, are illustrated in Figure 7.2. The response to the different levels of PEG was again a reduction in the protein precipitated by tannic acid as the levels of PEG increased. There was a significant decrease ($p<0.001$) in the protein precipitated between the levels 0 and 8 and between 8 and 16mg/ml of PEG. There was no further significant effect of adding more PEG. The amount of PEG required to reduce the activity of tannic acid was calculated to be around 10 mg/ml (between the levels 8 to 16 mg/ml). These experiments also show that under controlled conditions, PEG did not completely overcome the effect of tannic acid as the amount of precipitated protein never reached zero (lowest point 0.1814cm^2/g). This suggests that PEG may not be able to react completely with tannins to stop them reacting with the protein. During fermentation with plant material, the result may be different. It is important to bear in mind that tannic acid, although the standard for tannin assays, is a purified hydrolysable tannin, and may not represent the effect of all tannins.

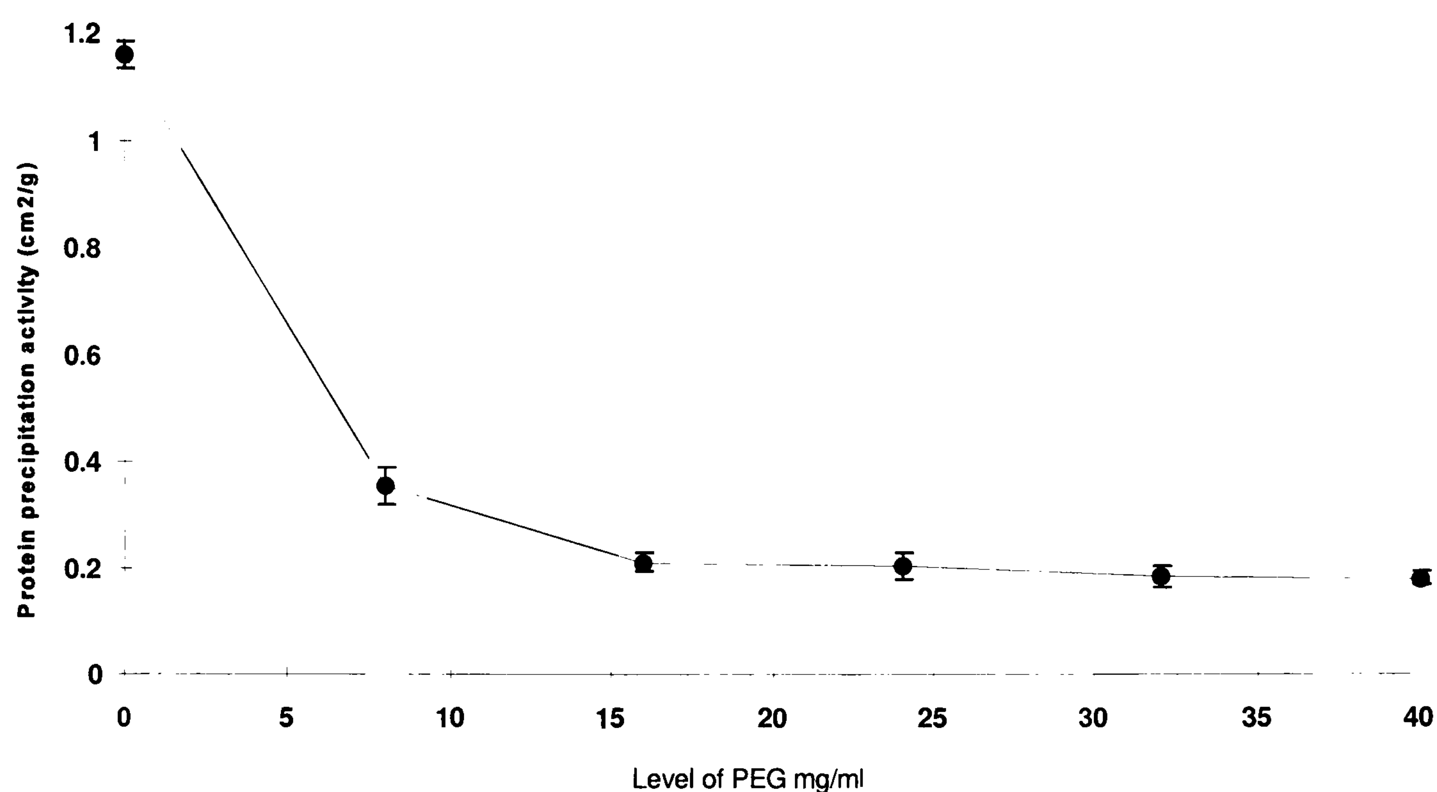


Figure 7.2: *Effect of the different levels of polyethylene glycol (PEG) on the protein precipitation capacity of tannic acid.*

Conclusions

The main conclusions derived from these experiments were:

- The level of PEG to overcome the protein precipitation capacity of tannic acid was between 8 and 16mg/ml (10mg/ml from the chart);
- PEG did not overcome completely the effect of tannic acid, and
- the range of levels to test in the gas production from plant material was selected as between 0 and 40mg/ml.

EFFECT OF THE LEVEL OF PEG TO OVERCOME THE EFFECTS OF TANNINS ON THE FERMENTATION

Objectives

The optimum level of PEG may vary according to the plant species and the effect on gas production may be due not simply to tannin-protein complexes. The next experiment was therefore designed to establish an optimum level of PEG in which to study the effects of tannins on the fermentation of plant material in different mixtures.

Materials and methods

Two species containing condensed tannins, *Inga* sp., and *Leucaena leucocephala*, were selected to test the response of the plant material to different levels of PEG with Menke's medium. They were fermented with nine different levels of PEG (0, 0.1, 0.5, 1, 2.5, 5, 10, 20, 40 mg/ml in the final mixture).

The fermentation was carried out in duplicate for 45 hours and the weight of the sample was 1g. The buffer was prepared to a final concentration of 70ml/bottle and PEG was dissolved in water and dispensed in the remaining 20 ml. A stock solution of 200g/l of PEG was prepared and the volumes of PEG needed for the final volume were dispensed accordingly. Small volumes were dispensed with the help of a Hamilton syringe.

Statistical analysis

The Gompertz model was used to fit the response curves. Each replicate was fitted separately (11 data points per replicate). The rate constant, lag phase and gas pool size were estimated, but they are not discussed here as the main objective was to establish the appropriate levels of PEG and not to assess its effects on the fermentation kinetics, which are discussed more thoroughly later in this chapter (see 7.3). The cumulative gas production at 45 hours was analysed for significant differences by means of one-way analysis of variance.

Results and discussion

The model fitted well for all treatments (R^2>99%). The effect of increasing levels of PEG on the cumulative gas production at 45 hours, with *Inga* sp. and *Leucaena leucocephala*, can be seen in Figure 7.3. There were highly significant differences ($p<0.001$) between levels of PEG for *Inga* sp. The response increased and peaked at a level of 2.5mg/ml, and then remained constant (differences between contiguous levels after this point were not significant); but differences from the control (0 PEG), were statistically different ($p<0.001$) from 0.1mgPEG/ml onwards. In the case of *Leucaena leucocephala* (Figure 7.4) differences between levels were highly significant ($p<0.001$). The response to the different levels of PEG increased and reached a maximum at the level of 20mg/ml. The minimum level of PEG to produce a significant increment in gas production in this species, with respect to control (0 PEG) was 5mg/ml.

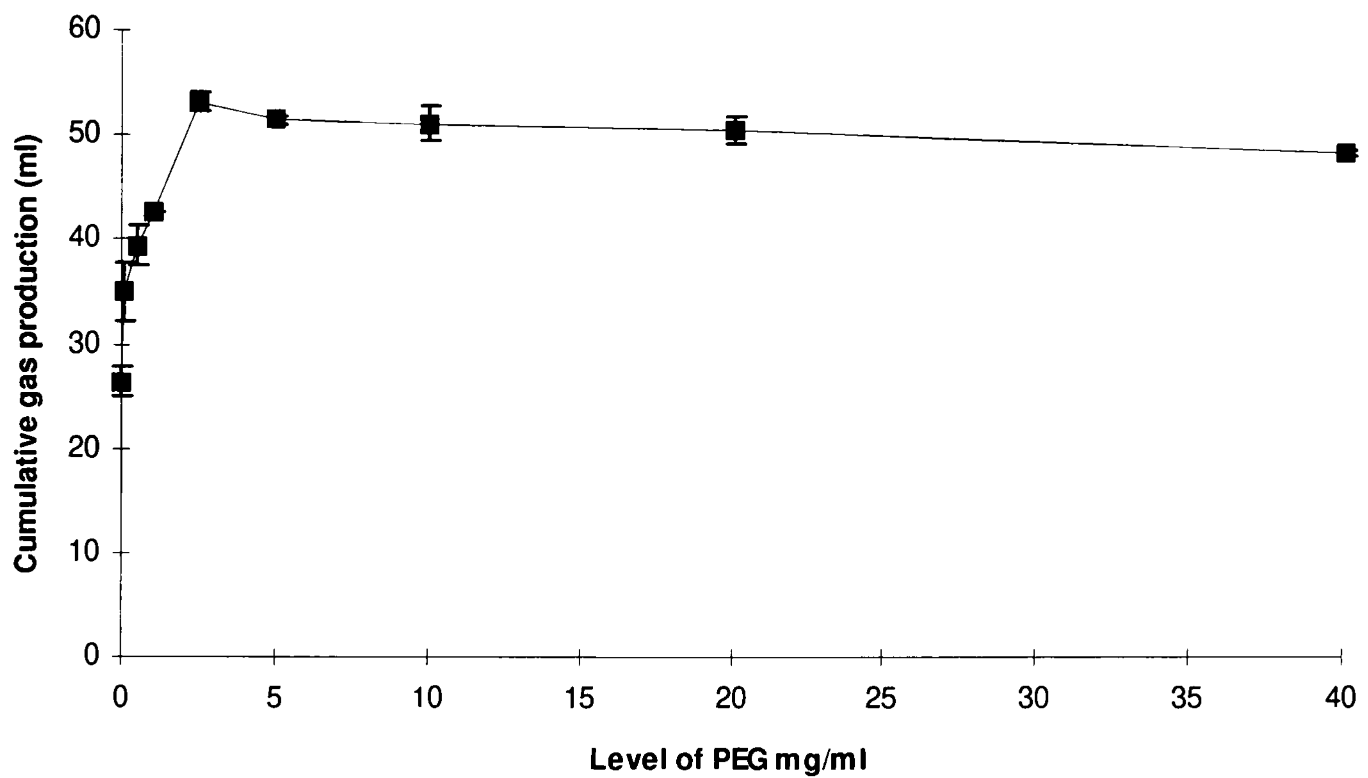


Figure 7.3: *Effect of different levels of polyethylene glycol (PEG) on the fermentation of Inga sp. at 45 hours.*

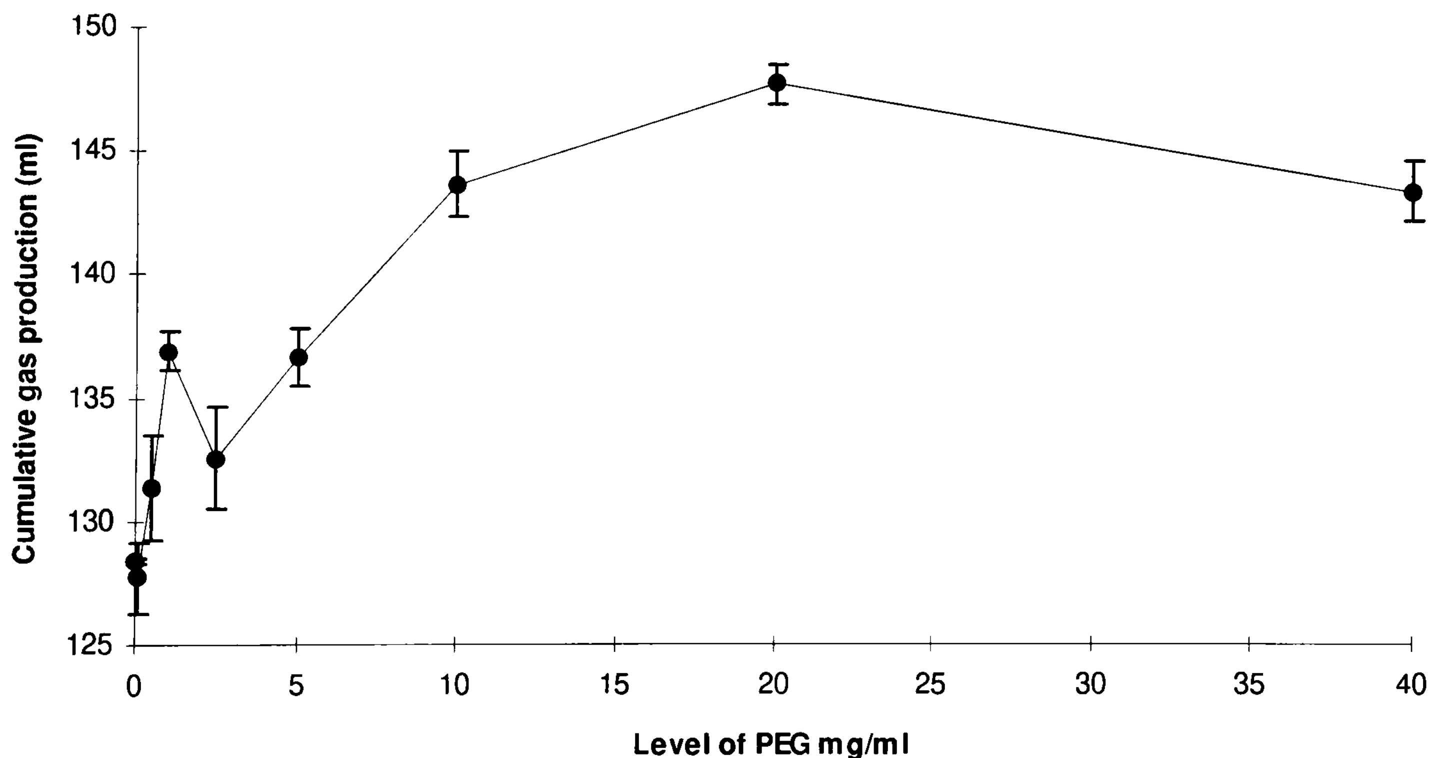


Figure 7.4: *Effect of different levels of polyethylene glycol (PEG) on the fermentation of Leucaena leucocephala at 45 hours.*

These results show that the maximum response to PEG depends on the plant material. This seems logical as different species have different amounts and types of tannins and phenolic compounds. In the case of *Inga* sp., a maximum difference of 98% more gas produced than the control was reached at the level of 2.5mg/ml of PEG, whereas for *Leucaena leucocephala*, the maximum difference was 15% at 20mg/ml of PEG. As *Inga* sp. had more tannins, the response to PEG is greater and at lower

levels than with *Leucaena leucocephala*. This last species needed a greater amount of PEG to produce a maximum response. This suggests that the response to PEG does not only depend on the amount of tannins but also on the chemical characteristics of the tannin itself (hydrolysable or condensed) or other components of the plant material (i.e., some plant material may prove more difficult to react with PEG than others because their tannins are more firmly attached to fibre for example).

Conclusions

As the response to PEG changes with the plant material, ideally, the level of PEG should be specific for each plant material. However, this would require a new series of experiments leading to establish optimum levels for each species. The disadvantages of having specific levels of PEG would be that this may be difficult to handle in large experiments and that the different levels of PEG could be a factor affecting results, making it difficult to assess the effects of the treatments when mixtures of 2 or 3 substrates are fermented. As *Inga* sp. and *Leucaena leucocephala* have the highest contents of tannins and phenolic compounds among the selected species, and as the response did not increase at the highest level, it was decided to keep PEG in excess, at 10 and 40mg/ml for all plant material.

7.3 MODELLING THE EFFECTS OF TANNINS ON THE FERMENTATION OF MIXED SUBSTRATES

OBJECTIVES

The objective of this section is to analyse the effects of tannins on the fermentation of the main components of the feeds. This is achieved by fermenting mixtures of different chemical entities (structural and non-structural carbohydrates, proteins and phenolic compounds). To study their individual roles in the presence of tannins the mixtures were also fermented with and without PEG.

DETERMINATION OF THE TANNIN:PROTEIN RATIO

The effect of tannins was modelled by fermenting different mixtures of carbohydrates and proteins in the presence of quebracho tannin. This tannin was selected as it may represent the effects of a mixture of tannins better than tannic acid (section 7.2). Quebracho tannin is a commercial extract from a South American tree, *Schinopsis balansae* Engl., *S. lorentzii* Engl. It contains both condensed and hydrolysable tannins. An analysis of quebracho tannin yielded resorcinol, gallic acid, seven phenolic

methyl-esters and lignin among several other phenolic compounds. It also showed traces of residual sugars (Streit and Fengel, 1994). It has been shown that quebracho does not ferment in the rumen (Parrinder *et al.*, 1991).

To model the effects of tannins on the fermentation it was necessary to determine appropriate levels of substrates with which to study these effects. Minimum levels of two proteins, BSA and casein, were selected for three different carbohydrates (glucose, starch and cellulose) in Chapter 5. These minimum levels are ideal for this study because, in a nitrogen free medium, any effect of tannins on reducing the available protein will show dramatically. These minimum levels are in the linear part of the response to nitrogen for each particular mixture of carbohydrate-protein. It was decided that the level of PEG of 40mg/ml, determined in previous experiments for different phenolic compounds from fodder tree leaves, was more than enough to model the effect of PEG on quebracho tannin.

The appropriate level of quebracho for each protein under these conditions was unknown. Therefore it was necessary to determine a suitable ratio of tannin:protein for the fermentation.

Objectives

- To determine a suitable ratio of tannin:protein on which to base the next experiments, and
- to assess the capacity of PEG to react with tannins in the fermentation in its ability to stop the formation of the tannin-protein complex and to dissolve the complex once formed.

Materials and methods

As there was no reference to an appropriate ratio of tannin:protein for the fermentation, it was decided to start with a wide range of tannin:protein ratios using BSA and glucose as a model. However, there was no reference for a starting point either. A starting point ratio was determined by using the radial diffusion technique as follows.

In the original paper describing the radial diffusion technique (Hagerman, 1987), 0.6mg of sorghum tannin per well delivered in 15μl, was used to precipitate BSA. This is a concentration of tannin of 40mg/ml. To replicate these conditions, two solutions were prepared by using 0.4g of quebracho tannin in 10ml of 70% acetone (as opposed to the normal extraction of 0.5g of substrate in 5ml of 70% acetone) and 15μl of the solutions were planted in duplicate on two agarose plates according to the normal procedure. In this case, BSA was used instead of haemoglobin. Plates were incubated for 5

days. After this period of time, the diameters of the precipitated rings were measured and the amount of protein precipitated by quebracho was calculated to be 0.202mg of BSA/band. As this protein was precipitated by 0.6mg of quebracho tannin, the tannin:protein ratio was calculated to be 2.96. A starting point for the fermentation was estimated to be a tannin:protein ratio of 3:1.

To establish an appropriate ratio in which to study the effects of tannins on the fermentation, it was decided to test six different tannin:protein ratios, using quebracho tannin and BSA in the presence of glucose. These ratios were: 0, 0.1, 1, 3, 6 and 10. PEG was added at a concentration of 40mg/ml in two treatments, one to stop and another to dissolve the tannin-protein complex.

As there was no evidence that the tannin-protein complex could form in the fermentation buffer (formation of the complex is pH dependent), it was decided to use the radial diffusion buffer as a medium for this complex to occur. Several attempts to establish the ratio by measuring (with a spectrophotometer) the disappearance of the protein in the Menke's buffer in the presence of quebracho at 280nm, failed. It was realized later that the pH of the Menke's buffer was about 8 when it is in a non-reduced state. When reduced, the pH goes down to about 6.8. The radial diffusion buffer has a pH of 5, this pH is the optimum for the reaction with BSA (see Hagerman, 1987).The pH of the quebracho solution was measured as 4.9 and PEG solution was 4.8. The pH of the final solution in the radial diffusion buffer, including glucose and BSA, was 4.9.

A solution of BSA-glucose was prepared in radial diffusion buffer. The minimum level of BSA for 0.5g of glucose was 20mg nitrogen/l, as determined in Chapter 5. Five ml of this BSA-glucose solution were transferred into the bottles and increasing amounts of the quebracho solution were placed in the bottles to get the desired ratios. PEG was added before and after the quebracho addition according to the treatments. The addition of the different solutions was made under a constant stream of CO_2 and the bottles were sealed with the butyl rubber stoppers to keep the anaerobic conditions but they were not crimped. The time span between addition of substrates and the further addition of PEG was 1 hour. Menke's medium was added 1 hour later. The experimental plan is shown in Table 7.1.

Statistical analysis

The Gompertz model was fitted to the data of cumulative gas production, for each replicate separately (14 data points per replicate) and the three parameters were estimated. The cumulative gas production at 70 hours was analysed for the effects of the tannin:protein ratio, presence of PEG and their interaction, by GLM.

Table 7.1: *Experimental plan for determining the level of quebracho tannin to model the effects of tannins and PEG in the fermentation*

Fermentation using Menke's medium and carried out in duplicate with a nominal sample weight of 0.5g. Fermentation lasted 70 hours.

PEG (40mg/ml) was added to each tannin:protein ratio (0, 0.1, 1, 3, 6 and 10) in three treatments. In the first one, PEG was added before quebracho to stop the formation of the tannin-protein complex. In the second treatment PEG was added after the tannin-protein complex was formed. The third treatment was the control (0 PEG).

The medium was prepared to be dispensed in 60ml, the volume needed to complete the bottles (90ml) was dispensed as follows: 20ml of PEG solution, 5ml of a solution Glucose-BSA (in radial diffusion buffer, pH 5), and 5ml of a solution of Quebracho, as follows:

Treatments	Ratio T/P	Glucose-BSA Solution ml	Quebracho Solution ml	PEG Solution ml	Water ml
With PEG	0	5	0	20	5
	0.1	5	0.05	20	4.95
	1	5	0.5	20	4.5
	3	5	1.5	20	3.5
	6	5	3	20	2
	10	5	5	20	0
Without PEG	0	5	0		25
	0.1	5	0.05		24.95
	1	5	0.5		24.5
	3	5	1.5		23.5
	6	5	3		22
	10	5	5		20

Solutions for other treatments were dispensed accordingly.

Results and discussion

The model fitted the data very well (R^2 >99%). The parameters are not discussed here as they are discussed in more detail later in this chapter and because the aim of this experiment was to set up an appropriate ratio rather than to study the fermentation kinetics. There were significant differences ($p<0.001$) in gas production at 70 hours for the tannin:protein ratio, the addition of PEG and for their interactions. Results are shown in Table 7.2.

The response to PEG increased as the tannin:protein ratio increased, indicating a significant effect of the tannin in decreasing the gas production. This suggests that quebracho tannin is binding BSA, protecting it from microbial degradation, in the presence of glucose. This effect was significantly greater at the higher levels, which means that perhaps neither the previously selected ratio 3 nor the top level of 10, were high enough to inhibit completely the fermentation of the substrate. Therefore, it was decided to test higher levels for the next experiment.

Table 7.2: *Response of a mixture of glucose-BSA to different levels of quebracho tannin and the recovery due to the presence of PEG added before and after the formation of the tannin-protein complex. Results are the average of cumulative gas production at 70 hours.*

Ratio Tannin:Protein	Addition of PEG			
	Before	No PEG	After	Means
0	186.4	190.9	185.9	187.7
0.1	185.4	189.4	183.4	186.0
1	193.4	184.1	181.6	186.3
3	192.1	175.9	182.6	183.5
6	203.6	164.4	188.4	185.4
10	198.1	120.9	194.6	171.2
Means	193.1	170.9	186.0	

S.E.D (18 df_{error})=3.29

PEG significantly recovered the gas production from the depletion caused by quebracho. This means that PEG is capable of both stopping the formation of the tannin-protein complex and releasing the nitrogen from the complex in the fermentation (this is assuming that the complex was formed one hour after mixing quebracho and BSA in the radial diffusion buffer in the presence of glucose). Jones and Mangan (1977) found that ageing a complex of Fraction 1 leaf protein and sainfoin tannins for more than 10 minutes before the addition of PEG was enough for the complex to form. They also found that the reaction between PEG and the complex was complete within 15 minutes of the addition of PEG at a pH of 6.4. The time span given in this experiment (1 hour between the addition of the substrates) is more than enough given the great affinity of quebracho for BSA (Asquith and Butler, 1986).

The effect of PEG was significantly greater when PEG was added before quebracho. The average gas production across the treatments when quebracho was added before was 193.1ml and when it was added after was 186ml. It should have had an average of approximately 191ml (the gas production when quebracho was not present). This means that PEG is more efficient at stopping the formation of the tannin-protein complex rather than freeing the protein from the complex. Figure 7.5 illustrates, at the maximum level, how the effect of quebracho tannin and PEG were constant along the fermentation.

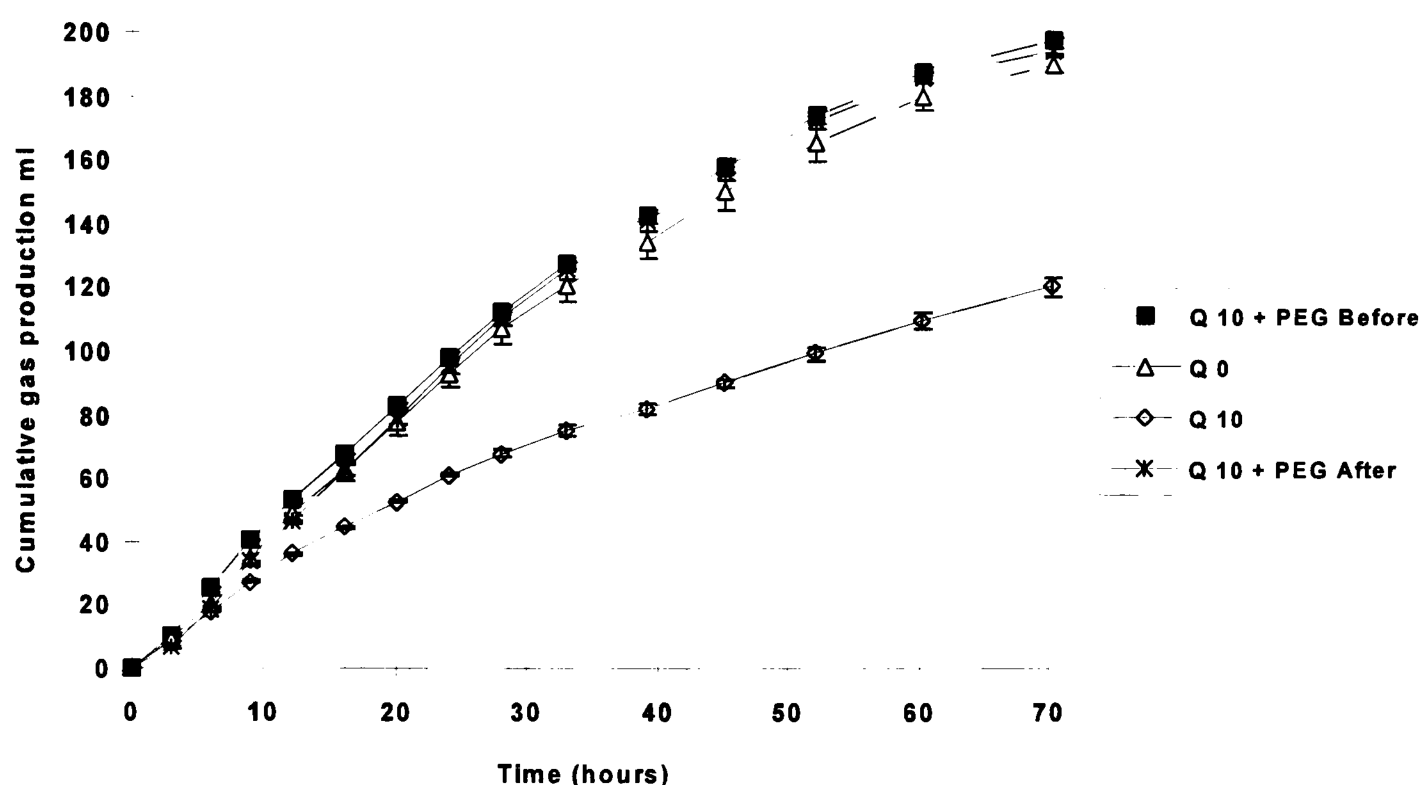


Figure 7.5: *Effect of ratio quebracho tannin:protein of 10:1 on the fermentation of a mixture of glucose-BSA and how this effect is alleviated by the addition of PEG, both before and after the formation of the complex tannin-protein.*

Conclusions

The conclusions for this part of the work were:

- The gas production system is suitable for studying the effects of tannins on the fermentation.
- Quebracho tannin reduces the fermentation of glucose in the presence of BSA, and appears to be a good model for further research. However, greater levels of this tannin would be needed for a greater inhibition of the fermentation of the substrate.
- PEG can mitigate the effects of tannins in the system as it restores the gas production from the depletion caused by the tannin and this can be used to study the effect of tannins in the fermentation. However, PEG may not completely restore the protein from the complex in more diverse substrates like fodder tree leaves but is very efficient at stopping the formation of the tannin-protein complex under these conditions.

EFFECTS OF TANNINS ON THE FERMENTATION OF CHEMICAL ENTITIES

Objectives

To study the effects of tannins on the fermentation kinetics and gas production of mixtures of carbohydrates and proteins. The maximum effect is sought by using increasing levels of the tannin.

Materials and methods

Quebracho tannin, as a model for condensed tannins, was fermented with mixtures of carbohydrates and proteins, according to the results obtained in previous experiments. Three carbohydrates: glucose, starch and cellulose, were fermented with 2 proteins: BSA and casein with five quebracho tannin:protein ratios: 0, 5, 10, 20 and 50. In chapter 5, minimum levels of nitrogen to ferment carbohydrates were found to be 20mgN/l for glucose and 30mgN/l for cellulose and starch, in the final mixture. These two levels of nitrogen for each carbohydrate were selected for this experiment.

BSA was delivered in solution as opposed to casein which was delivered as a solid. The objective was to see how the system quebracho-protein-PEG reacted to a less soluble protein. As in the previous experiment, to ensure that the reaction tannin-protein occurred the radial diffusion buffer was used as a vehicle for the protein.

According to the previous experiment there was a significant response to the tannin:protein ratio 10 for a BSA-glucose mixture, but it appeared that the system did not reach a maximum effect. Also it may be possible that the response at the same ratio is not the same for the different carbohydrate-protein mixtures. A wider range was proposed here to study the effect of tannins at high levels. The experimental plan is shown in Table 7.3.

Table 7.3: *Experimental plan to determine the effects of tannins on mixtures of carbohydrates and proteins.*

Fermentation using Menke's medium and carried out in duplicate with a nominal weight sample of 0.5g. Fermentation lasted 70 hours.

Experimental arrangement:
3 Carbohydrates - 2 proteins - 5 ratios tannin:protein.

A medium was prepared for a concentration of 60mls/bottle. The final volume (90ml) was completed as follows:

Ratio Tannin:protein	Protein Solution ml*	Quebracho Solution ml**	Water ml
0	5	0	25
5	5	0.5	24.95
10	5	1	24
20	5	2	23
50	5	5	20

Solutions for the treatments were dispensed accordingly.

*BSA was dispensed in 5 ml of radial diffusion buffer in two solutions: 2.48mg/ml for the level 20mgN/l and 3.74mg/ml for the level 30mgN/l. Casein was weight out and dispensed as a solid into bottles containing 5 ml of radial diffusion buffer.

** Two solutions of quebracho in distilled water were used: 124mg/ml for the level 20mgN/l and 187mg/ml for the level 30mgN/l.

Statistical analysis

The effects of the treatments on the fermentation kinetics were assessed by fitting the Gompertz model to the data and the resultant curve parameters (gas pool size, lag phase and rate constant), were analysed by analysis of variance according to the General Linear Model (GLM) procedure. The effects of sources of carbohydrate, protein, tannin:protein ratio and their interactions were tested. The number of observations for all tests was a total of sixty (n=60). The effects on the cumulative gas production were determined at 12, 24, 45 and 70 hours by an analysis of variance (GLM) of the fitted values at those times, calculated from the equations, at the highest level of tannin.

Results and discussion

In general there was a very good fit (R^2>99%). The effects of the treatments on the gas pool size are presented in Table 7.4. There was a significant effect ($p<0.001$) of the tannin:protein ratio and the source of carbohydrate ($p<0.001$). The interactions, carbohydrate x protein ($p<0.01$), carbohydrate x ratio ($p<0.001$), protein x ratio ($p<0.001$) and carbohydrate x protein x ratio ($p<0.001$) were significant. There was no significant effect of the source of protein ($p=0.554$).

A significant effect of the source of carbohydrate is to be expected as different carbohydrates ferment differently, and will therefore have different gas pool sizes. The same can be said for the significant interaction between sources of carbohydrate and protein. The lack of significance of the source of protein is understandable as, in a medium lacking nitrogen, the source of N may not be as important as the presence itself. However, as the two proteins were supplied to the microorganisms in two different forms as liquid (BSA) and as solid (casein), this may indicate that quebracho tannin is reacting in the same way with both proteins independently of their solubility. In other words, tannins from quebracho are reacting with soluble and insoluble protein in the gas production method in the presence of radial diffusion buffer. These results also give more confidence that the selected minimum levels of nitrogen from BSA and casein were adequate as they had no influence in the results.

There was a significant effect ($p<0.001$) of the increasing tannin:protein ratio in decreasing the gas pool size. This reduction was highly significant from the lowest up to the highest levels, which means that a tannin:protein ratio as low as 5:1 can affect the gas pool size of the mixture but a ratio of 50:1 is not yet the maximum.

The interaction carbohydrate x ratio in terms of the gas pool size of the treatments with starch was affected significantly ($p<0.05$) from a ratio of 5:1 onwards. However the maximum differences ($p<0.001$) were found at the ratios 20 and 50:1. At these levels, the system was still responding to tannins. Glucose was affected significantly ($p<0.001$) from level 5:1. Highly significant differences were also found at higher levels, giving evidence that the system is still responding to the high levels of quebracho. On the other hand, highly significant differences were found for cellulose ($p<0.001$) from the ratios 0 to 10:1. There were no significant differences from that level onwards, which means that the limit was reached for this particular carbohydrate. In general, carbohydrates of low fermentability may be affected to a greater extent at low ratios than more rapidly fermentable carbohydrates. The general effect of quebracho tannin was a significant reduction in the gas pool size from the ratio 5:1 onwards for all treatments. Whereas, the more fermentable mixtures responded significantly to the higher levels, mixtures of cellulose with BSA and casein reached a limit at the ratio 20 (see Table 7.4).

Table 7.4: *Effect of quebracho tannin on the Gas Pool Size(ml) of different mixtures of carbohydrates-proteins.*

	Ratio Tannin:Protein				
	0	**5**	**10**	**20**	**50**
Starch:BSA	170.8	157.3	123.2	52.9	32.3
Starch:Casein	142.0	125.5	126.4	110.0	35.1
Means	156.4	141.4	124.8	81.4	33.7
Glucose:BSA	170.4	137.6	101.1	86.0	65.2
Glucose:Casein	142.6	130.0	98.9	93.1	77.2
Means	156.5	133.8	100.0	89.5	71.2
Cellulose:BSA	100.3	36.3	20.2	22.2	23.2
Cellulose:Casein	87.5	71.9	20.2	24.3	25.5
Means	93.9	54.1	20.2	23.3	24.4
Means Ratio	135.6	109.7	81.6	64.7	43.0

S.E.D (30 df_{error})= 4.78

Table 7.5, shows the effects of quebracho tannin on the lag phase of the mixtures. There was a significant effect ($p<0.001$) of the tannin:protein ratio and the source of carbohydrate ($p<0.001$). The interactions, carbohydrate x protein ($p<0.001$), carbohydrate x ratio ($p<0.001$), protein x ratio ($p<0.05$) and carbohydrate x protein x ratio ($p<0.001$) were also significant. There was no significant effect of the source of protein ($p<0.249$). This was a similar case as the gas pool size.

Table 7.5: *Effect of quebracho tannin on the lag phase (hours) of different mixtures of carbohydrates-proteins.*

	Ratio Tannin:Protein				
	0	**5**	**10**	**20**	**50**
Starch:BSA	31.9	36.3	32.6	35.6	20.0
Starch:Casein	34.0	34.0	41.2	37.1	22.2
Means	33.0	35.2	36.9	36.3	21.1
Glucose:BSA	24.0	26.0	24.2	21.3	21.1
Glucose:Casein	17.9	20.8	21.0	20.0	17.9
Means	21.0	23.4	22.6	20.6	19.5
Cellulose:BSA	31.1	25.0	6.7	12.0	15.4
Cellulose:Casein	28.4	49.9	7.6	12.2	12.7
Means	29.7	37.4	7.1	12.1	14.0
Means Ratio	27.9	32.0	22.3	23.1	18.2

S.E.D (30 df_{error}) = 3.03

In general, there was a significant effect in reducing the lag phase as the tannin:protein ratio increased, and there appears to be a limit at the ratio 10:1 (there was no significant difference from this ratio onwards). The effect of the ratio of quebracho tannin:protein on the different carbohydrates was: a significant decrease in the lag phase at the highest level of quebracho with starch ($p<0.001$); no significant effect on the lag phase with glucose; and a highly significant decrease ($p<0.001$) from the ratio of 10:1 upwards with cellulose. Looking at specific treatments, the mixture starch-BSA significantly decreased the lag phase ($p<0.001$) only at the ratio of 50:1, whereas when fermented with casein, starch showed a significant decrease in the lag phase ($p<0.05$) from ratio 10:1 and it decreased even more at ratio 50:1 ($p<0.001$). This is not surprising as it was shown in previous chapters that BSA is a better protein for fermenting starch and that, when starch is fermented at a high level of nitrogen, the characteristic "S" shape of its response is more evident. As at the highest ratio there is less protein available (as a result of the interaction with quebracho tannin), the lag phase is shorter compared with those treatments with low ratios of quebracho tannin, which would have more protein available, and therefore the characteristic shape (with a longer lag phase) can manifest itself. The lag phase of glucose was not affected when fermented with BSA or casein, suggesting that lag phases of highly fermentable materials may not be affected by high levels of tannins. In contrast, the lag phase of cellulose was affected from the ratio 10:1 ($p<0.001$) when fermented with BSA and from ratio 5:1 ($p<0.001$) when casein was the source of nitrogen.

The effects of the tannin:protein ratio on the rate constant can be seen in Table 7.6. There were highly significant effects ($p<0.001$) for source of carbohydrate, ratio, and the interactions carbohydrate x protein, carbohydrate x ratio and carbohydrate x protein x ratio. There was no effect of the source of

protein ($p<0.394$) nor of the interaction protein x ratio on the rate constant ($p<0.135$). In general, although significant, the effect of the different concentrations of tannin on the rate constant was unclear. There were alternate increases and decreases in the rate constant as the tannin:protein ratio increased. There was no effect on the rate of the mixture glucose-BSA, but there was a significant effect ($p<0.05$) for glucose-casein from the ratio 5:1 onwards. The tannin:protein ratio affected the rate constant of all remaining mixtures from the levels 5 and 10 onwards, but the effect was not clear within the different mixtures.

Table 7.6: *Effect of quebracho tannin on the rate constants of gas production (hour^{-1}) of different mixtures of carbohydrates-proteins.*

	Ratio Tannin:Protein				
	0	**5**	**10**	**20**	**50**
Starch:BSA	0.063	0.057	0.047	0.031	0.055
Starch:Casein	0.052	0.053	0.038	0.044	0.045
Means	0.057	0.055	0.043	0.038	0.050
Glucose:BSA	0.055	0.047	0.049	0.057	0.060
Glucose:Casein	0.070	0.054	0.057	0.058	0.063
Means	0.062	0.051	0.053	0.057	0.061
Cellulose:BSA	0.040	0.034	0.102	0.077	0.076
Cellulose:Casein	0.044	0.023	0.119	0.071	0.075
Means	0.042	0.028	0.111	0.074	0.075
Means Ratio	0.054	0.045	0.069	0.056	0.062

S.E.D (30 df_{error}) = 0.00436

The effects of quebracho tannin on the cumulative gas production of the different mixtures were studied at the highest level of the tannin. The analysis of variance (GLM) showed significant differences in the gas production for the values of the mixtures at the selected times of 12, 24, 45 and 70 hours. However, there were no differences at 12 hours due to the interaction of carbohydrate x protein x ratio ($p=0.319$) which means that, at that time, the effects of the different sources of carbohydrates and proteins were significantly ($p<0.001$) more important for the fermentation than their interaction with quebracho and the differences due to treatments could not be detected.

The reduction due to the addition of quebracho in the presence of the different mixtures was calculated from the difference between mixtures with and without tannin, at the highest level, and expressed as a percentage in Table 7.7. Effects at 12 hours are not presented as they were not significant ($p>0.05$).

Table 7.7: *Percentage reduction in the predicted cumulative gas production at different times of mixtures of carbohydrates and proteins, due to high levels of quebracho tannin. Results were calculated as the difference of the mixtures with and without quebracho at the highest tannin-protein ratio (50). All the differences were highly significant ($p<0.001$).*

Mixture	24 hours	45 hours	70 hours
Starch:BSA	56.0	77.2	80.5
Starch:Casein	46.7	69.6	74.4
Glucose:BSA	55.0	58.7	60.7
Glucose:Casein	47.2	47.4	46.4
Cellulose:BSA	48.3	63.3	72.0
Cellulose:Casein	36.1	56.7	66.2

The main conclusion from this table is that there is an important reduction in the cumulative gas production due to the presence of quebracho tannin across time for all mixtures. This is not surprising since it has been shown that tannins can reduce the degradation of cellulose, starch, pectin and glucose and protein from several sources (Mueller-Harvey and McAllan, 1992). There was a greater reduction in the mixtures with starch and cellulose at 45 and 70 hours. The mixtures of BSA with glucose tended to have a more linear response with the time. Also, there was a smaller reduction in the mixtures with this carbohydrate. This suggests that more fermentable carbohydrates may be less affected by tannin than less fermentable materials such as starch and cellulose. This is supported by the fact that the most fermentable combination glucose:casein was the least affected by quebracho. Tannins have been implicated in the reduction of the degradability of structural carbohydrates. Jung (1985) found that a significant depression of *in vitro* dry matter disappearance (IVDMD) of cellulose with increasing concentrations of benzoic acid, cinnamic acid, caffeic acid and ferulic acid. The response with hemicellulose was very different. None of the components depressed IVDMD significantly and *p*-coumaric acid actually increased it. This illustrates that the affinity of tannins for structural carbohydrates is specific. Phenolic compounds are also involved in the inhibition of IVDMD of cellulose and starch (Jung and Fahey, 1983). They found that the phenolic monomers commonly found in forages decreased IVDMD of cellulose and starch and that this effect increased with phenolic concentration. The cause of the reduction of IVDMD of cellulose for vanillin appears to be a reduction in the bacterial attachment to structural carbohydrates in the presence of the phenol rather than a toxic effect (Varel and Jung, 1986).

Mixtures with glucose did not show a reduction close to 100%, as expected when no nitrogen was available and as the level of quebracho increased, suggesting that more quebracho is needed to completely bind the protein. However, this tannin:protein ratio of 50:1 was adequate for the purposes of this modelling and another experiment was not considered necessary.

The reduction with BSA and each carbohydrate was also greater than the reduction with casein. This implies that quebracho is binding more BSA than casein. This is supported by the literature, as it is well know that tannins have a great affinity for BSA, to such an extent that BSA can be used instead of PEG to protect dietary protein (Clausen *et al.*, 1992). In addition BSA was already dissolved when added to the fermentation bottles, as opposed to casein which was delivered as a solid. Apart from showing that quebracho tannin can react with soluble and less soluble protein, this also suggests that quebracho tannin reacted more with the more soluble protein. Although these two factors (affinity and solubility) cannot be separated by this experiment, it seems logical that tannins may react more easily with a protein that is already in solution. According to Mueller-Harvey and McAllan, (1992) solubility is not a prerequisite for interaction, as insoluble (non-extractable) polyphenols can also absorb proteins from solution and *vice versa*. On the other hand, quebracho and BSA have shown to have high affinity for each other. Asquith and Butler (1986) and Asquith *et al.* (1987) found that the relative affinity of quebracho and BSA was among the highest compared to a other combinations phenol:protein. No reference was found relating to the affinity of quebracho and casein, but according to Luck *et al.* (1994) all caseins are strongly hydrophobic. This property permits strong mutual association and facilitates strong complexation with polyphenolic substrates. According to this study, the affinity of quebracho for casein seems to be lower than that of quebracho for BSA.

Conclusions

The conclusions drawn from this section are:

In general, there was a clear reduction due to quebracho tannin in the cumulative gas production of all carbohydrate-protein mixtures. This effect was repeated for the variable gas pool size, which decreased as more tannin was put in the mixtures. However, even small amounts of tannin (tannin:protein ratio 5:1) produced a significant response.

Carbohydrates or mixtures of low fermentability were affected to a greater extent than more fermentable mixtures or carbohydrates. Also, these latter mixtures needed greater levels of tannins (beyond the maximum tested in this experiment) to reach a maximum reduction in fermentation.

The effects of quebracho tannin started to have significant effect in the fermentation after 12 hours.

Quebracho tannin produced a greater reduction of the fermentation of the mixtures with BSA than with casein. Results showed that this tannin reacted with both soluble and insoluble protein and with

solubilized and not solubilized protein in the presence of carbohydrates. Although quebracho represented a "free" tannin, the results suggest that tannins from tree leaves could react with proteins (independently of their solubility). Taking the appropriate precautions with respect to pH, gas production can be used to study the effects of tannins from fodder tree leaves on dietary protein.

7.4 EFFECT OF THE TANNINS IN TROPICAL FODDER TREE LEAVES ON GAS PRODUCTION

OBJECTIVES

To study the effects of tannins on the fermentation kinetics of fodder tree leaves at two levels of PEG and to assess the magnitude of these effects on the gas production.

MATERIALS AND METHODS

As PEG reacts with tannins, the difference between a plant material fermented with and without PEG should illustrate the effect of tannins on the fermentation. To measure the effect of the tannins from the selected fodder tree leaves on the fermentation, the plant material was fermented with and without PEG. A sample of *Urera baccifera* was also fermented to illustrate the effect of adding PEG to a substrate with no detected phenolic compounds. The phenolic components of these species were reported in Chapter 4. To ensure the reaction with the maximum amount of tannins, PEG was provided at two levels, 10 and 40mg/ml. The experiment was a factorial design of 6 plant species and 3 levels of PEG. Other experimental conditions were the same as in the previous experiment.

Statistical analysis

The Gompertz equation was fitted to all the curves for each replicate separately (11 data points per replicate) and the three parameters (rate constant, lag phase and gas pool size) were estimated. Significant differences between the estimated parameters due to the treatments and treatment interactions were detected using the General Linear Model (GLM) procedure. Differences in cumulative gas production at 45 hours were also tested by GLM.

RESULTS AND DISCUSSION

There was a very good fit for all the curves (R^2>98%).There were significant differences for all treatments for the variables rate, lag phase and gas pool size and for all interactions ($p<0.001$). Results are summarized in Table 7.8.

Table 7.8: *Gompertz curve parameters for the fermentation profiles of six plant materials under three treatments with polyethylene glycol.*

	Level of PEG mg/ml		
Plant material	**0**	**10**	**40**
Rate Constant (hours^{-1})			
Trichanthera gigantea	0.059	0.063	0.064
Gliricidia sepium	0.097	0.092	0.102
Inga sp.	0.054	0.071	0.055
Leucaena leucocephala	0.073	0.078	0.074
Erythrina edulis	0.084	0.096	0.090
Urera baccifera	0.093	0.095	0.094
Mean PEG	0.077	0.082	0.080
S.E.D (18 df_{error}) =0.00417			
Lag phase (hours)			
Trichanthera gigantea	19.2	18.4	16.5
Gliricidia sepium	17.0	16.5	13.7
Inga sp.	16.9	16.4	17.8
L.eucaena leucocephala	15.0	16.4	16.4
Erythrina edulis	18.5	15.2	15.0
Urera baccifera	13.5	13.3	13.4
Mean PEG	16.7	16.0	15.5
S.E.D (18 df_{error}) =0.801			
Gas Pool size (ml)			
Trichanthera gigantea	181.5	176.9	190.1
Gliricidia sepium	126.1	136.4	172.1
Inga sp.	32.7	58.4	60.8
Leucaena leucocephala	143.4	160.2	162.3
Erythrina edulis	127.5	136.3	147.1
Urera baccifera	152.0	152.5	151.9
Mean PEG	127.2	136.8	147.4
S.E.D (18 df_{error}) =2.509			

There was no significant effect of the level of PEG on the rates, except for *Inga* sp, at the level of 10mg/ml. There was also no effect of PEG on the lag phase in most of the plant material. However, a high level of PEG had the effect of decreasing the lag phase in *Trichanthera gigantea*, *Erythrina edulis* and *Gliricidia sepium*. These plants have no tannins but they do have total phenols.

In general, there was a significant effect of adding PEG in increasing the gas pool size of the tree leaves, except for the lowest level of PEG in *Trichanthera gigantea* and both levels in *Urera baccifera*. PEG had a more important effect on the gas pool size than on other parameters of the curves (PEG did not increase or decrease the speed at which the substrates were fermented and its effect on the lag time was to decrease it in forages without tannins). Provided there is a good fit, gas pool size is an estimate of the total gas production at the end point (45 hours). Therefore, it is not surprising that the differences in the gas pool size and on the cumulative gas production at 45 hours are very similar. Differences in gas production at 45 hours were tested by GLM. There was a highly significant ($p<0.001$) effect of plant material, level of PEG and their interactions. Data from gas production at 45 hours at 10 and 40mg/ml, were subtracted from the control (0 PEG) and the results are presented on Table 7.9.

Table 7.9: *Effect of tannins and phenolic compounds on the fermentation of fodder tree leaves measured in gas produced over 45 hours, as the increment due to the addition of PEG.*

	Increment due to PEG (ml)		**Effect of tannins (%)**	
	Level 10mg/ml	**Level 40mg/ml**	**Level 10**	**Level 40**
			%	**%**
Trichanthera gigantea	-0.3n.s	14.78***		10.0
Gliricidia sepium	11.55***	48.17***	9.6	40.2
Inga sp.	24.49***	22.23***	90.7	82.3
Leucaena leucocephala	14.62***	15.05***	11.2	11.5
Erythrina edulis	14.23***	24.01***	12.2	20.6
Urera baccifera	0.58n.s	-0.67n.s		
Mean PEG	10.86***	20.6***		

S.E.D (18 df_{error})=1.974
n.s Not significant
*** Significant at 0.1% level

There was an increment in gas production due to the addition of PEG at two levels for all forages except for *Trichanthera gigantea* at level 10 mg/ml PEG and for *Urera baccifera* at both levels. The effect of PEG on forages without tannins suggests that PEG is also reacting with other phenolic compounds. Longland *et al.* (1994) found that PEG reacted with plant material containing both soluble

and bound condensed tannins in the gas production with Theodorou's medium. No tannins or phenols were detected in *Urera baccifera,* which confirms that PEG does not decrease the fermentation of forages without phenolic compounds (there was no effect of PEG on the rate, lag phase or gas pool size for this species either).

Phenolic compounds from the species studied thus appear to suppress the gas production from the plant material over a wide range from 9.6 to 90%. This suppression, however, does not seem to be related simply to the amount of phenolic compounds (see Table 4. 5 for phenolic contents). The greater response from *Inga* sp. could be attributed to the high amount of phenolic compounds that this plant material contains, but the same cannot be said of *Gliricidia sepium*, for example. The suppression in gas production may also be related to the nature of the phenolic compounds present in the plant material. This is illustrated by the cases of *Gliricidia sepium* and *Erythrina edulis.* Both species are very similar in their phenolic contents but their response to PEG is very different. Phenolic compounds from *Inga* sp., *Gliricidia sepium* and *Erythrina edulis* seem to be more powerful suppressors of fermentation than those from *Leucaena leucocephala* and *Trichanthera gigantea.*

However, it is important to bear in mind that the response to PEG will depend on three factors:

- the capacity of PEG to inactivate all phenolic compounds from the plant material (or the resistance of phenolic compounds to be inactivated);
- the presence of enough carbohydrates (or the right type) in the plant material or in the medium to ferment the protein released by PEG;
- The complexation of the freed tannin with carbohydrates; and
- the susceptibility of the released protein itself to fermentation by micro-organisms.

The effects of tannins and phenolic compounds were general suppression of the fermentation. This suppression may have important consequences in associative effects of mixtures of tree leaves. The fact that PEG did not increase the fermentation (or any curve parameters) of *Urera baccifera*, suggests that it did not have any effect on other components of the plant material.

CONCLUSIONS

The conclusions drawn from this work are:

PEG reacts with phenolic components besides tannins but does not have an effect on other components of the plant material.

Except in one case, phenolic components of the plant material did not appear to increase the fermentation rate. PEG decreased the lag phase in forages without tannins, which suggests that phenolic compounds may increase the lag phase (fermentation starts later), but the effects of tannins may not be to prolong the lag phase, but rather to decrease the gas pool size.

7.5 EFFECT OF TANNINS FROM TROPICAL FODDER TREE LEAVES ON THE FERMENTATION OF CARBOHYDRATES

OBJECTIVES

The main objective of this experiment was to determine the effects of tannins from fodder tree leaves on the fermentation of carbohydrates. This effect was measured by using PEG to release protein from the tannin. This protein thus became available for fermentation with carbohydrates in the medium and thus increasing gas production. The effect of the tannin was calculated as the difference due to the presence of PEG.

MATERIALS AND METHODS

To determine the effects of the tannins from the selected fodder tree leaves on carbohydrates, the leaves were fermented in the presence of glucose, starch and cellulose, with and without PEG. Controls were carried out for the tree species without carbohydrates and with and without PEG, but results are not discussed here, as this has been already discussed before in the previous section. The experimental plan is described in Table 7.10.

Table 7.10: *Experimental plan to determine the effects of tannins on the fermentation of carbohydrates.*

Fermentation using Menke's medium and carried out in duplicates with a nominal sample weight of 0.5g. The extent of the fermentation was 70 hours.

Experimental arrangement:
5 Fodder tree species - 3 Carbohydrates - 2 Levels of PEG (0 and 40mg/ml)

Controls were carried out for tree species, carbohydrates, and control no substrate, all in the presence and absence of PEG.

The medium was prepared to be dispensed in 60ml, the volume needed to complete the bottles (90ml) was dispensed as follows: 20ml of PEG solution and 10ml of water for the PEG treatments and 30ml of water for treatments without PEG.

The effective level of PEG for this experiment was in fact double that of 7.3. In that experiment the concentration of PEG was 40mg/ml in the final mixture and the amount of substrate was 1g. In this experiment, the concentration of PEG was kept the same, but the weight of the sample was 0.5g; so the concentration of PEG per gram of substrate was practically doubled. It was shown in 7.2 that high concentrations of PEG (beyond 20mg/ml) did not significantly increase gas production.

The effects of the phenolic compounds from fodder tree leaves on the cumulative gas production of the different mixtures were calculated as a percentage of the difference on the mixtures with and without PEG.

Statistical analysis

As for the previous experiments, the main effects and interactions of the treatments in the fermentation kinetics were assessed by analysis of variance (GLM) of the curve parameters as produced by the Gompertz model. The effects on the cumulative gas production at different times were assessed by GLM of fitted values at 12, 24, 45 and 70 hours. The number of observations for all tests was a total of sixty (n=60).

RESULTS AND DISCUSSION

There was a good fit (R^2>99%). In all cases one particular mixture, *Erythrina edulis*:cellulose (both components exhibit a long lag phase) showed a lag phase that was so pronounced that to accommodate it, the model yielded values for lag phase of 111.6 hours and a gas pool size of 1041.5ml. The fit was extremely good (R^2=99.6%). This illustrates that in extreme cases the model can produce parameters that may not have a biological significance (a lag phase of 111 hours when the fermentation was

carried out for 70) but still produces a good fit. However, because these extremes values can have a large influence in the results, curve parameters of *Erythrina edulis* and their mixtures were excluded from the statistical analysis. They were not excluded from the analysis of the cumulative gas production because this analysis is carried out with the real values obtained from the fermentation and therefore not influenced by the mathematical model. Other models were not tested.

According to the analysis of variance there were significant effects ($p<0.001$) of fodder tree species, source of carbohydrate, presence of PEG and their interactions for the variable gas pool size (ml). Differences between tree species are expected as they have different fermentation kinetics. The same can be said for the differences between the sources of carbohydrates and for the interactions tree leaves x carbohydrates.

There was, in general, a significant effect of PEG ($p<0.001$) on the gas pool size (see Table 7.11) When comparing the effect of PEG for each particular combination of tree leaves and carbohydrates, the *t* test showed that the gas pool sizes of most mixtures were not affected by PEG. The only significant effects were a reduction in the gas pool size of the mixture *Gliricidia sepium*:glucose ($p<0.05$) and an increase in the gas pool size of *Inga* sp.:cellulose in response to PEG. The effect of PEG on the mixture *Gliricidia sepium*:glucose is not clear. It may have been influenced by the fact that, in the absence of PEG, this mixture had a very high gas production during the first 12 hours (143ml, more than 50% of the gas being produced during this period) (kinetics of this mixture were studied in Chapter 6). There is a clear effect of PEG on the mixture *Inga* sp.:cellulose, because of the presence of tannins, but that does not explain the lack of effect with other carbohydrates. In general, PEG did not affect the gas pool size of the mixtures. A very similar situation was found for the lag phase (see Table 7.12).

Table 7.11: *Effect of PEG on the gas pool size (ml) of mixtures of fodder tree leaves and carbohydrates.*

	Glucose		Starch		Cellulose	
	No PEG	PEG	No PEG	PEG	No PEG	PEG
Trichanthera gigantea	252.2	239.2	230.7	207.4	244	247.5
Gliricidia sepium	253.2	165.8	287.8	283.7	291.3	296.7
Inga sp.	191.4	212.4	159.6	198.7	124.1	219.7
Leucaena leucocephala	217.5	276.7	384.3	363.5	415.0	472.3
Means	228.5	223.5	265.6	263.3	268.6	309.0

S.E.D (24 df_{error})= 10.44

Table 7.12: *Effect of PEG on the lag phase of mixtures of fodder tree leaves and carbohydrates.*

	Glucose		Starch		Cellulose	
	No PEG	PEG	No PEG	PEG	No PEG	PEG
Trichanthera gigantea	15.7	15.3	29.6	24.9	37.4	41.0
Gliricidia sepium	8.3	12.8	19.6	18.1	30.6	29.0
Inga sp.	14.4	12.2	30.7	30.6	42.5	40.4
Leucaena leucocephala	18.7	15.8	47.2	32.7	63.5	60.4
Means	14.2	14.0	31.7	26.5	43.5	42.7

S.E.D (24 df_{error})= 1.92

There were significant effects of the source of carbohydrates ($p<0.001$), plant material ($p<0.001$) and the presence of PEG ($p<0.05$) on the lag phases of the different mixtures, according to the GLM. Their interactions were also significant. However, when comparing the effect of PEG for each particular mixture, there were no significant differences except for the mixture *Leucaena leucocephala*:starch. PEG decreased the lag phase which means that the mixture started the fermentation earlier. It may be favoured by an early release of protein due to PEG. The effect on the rate constant, however, was more consistent (see Table 7.13).

There were significant effects of plant material, carbohydrate and PEG and their interactions on the rate constants of the mixtures (Table 7.13). PEG had no effect on the mixtures *Trichanthera gigantea*:glucose and cellulose and *Leucaena leucocephala*:cellulose, but it had a significant effect in reducing ($p<0.001$) the rate constant of the *Gliricidia sepium*:glucose.

Table 7.13: *Effect of PEG on the rate constant (hours^{-1}) of mixtures of fodder tree leaves and carbohydrates.*

	Glucose		Starch		Cellulose	
	No PEG	PEG	No PEG	PEG	No PEG	PEG
Trichanthera gigantea	0.063	0.062	0.039	0.049	0.032	0.033
Gliricidia sepium	0.112	0.093	0.080	0.099	0.051	0.052
Inga sp.	0.068	0.074	0.051	0.057	0.030	0.036
Leucaena leucocephala	0.055	0.062	0.028	0.039	0.021	0.023
Means	0.074	0.072	0.049	0.061	0.033	0.036

S.E.D (24 df_{error})= 0.00161

The general effect of PEG was a significant increase in the rate constant. This suggests that, in general, phenolic compounds from these species decreased the potential speed at which their mixtures with carbohydrates should ferment. This may prove important for predicting the associative effects of mixtures as it has been suggested that they rely on a synchronisation of the release of nutrients, and here phenolic compounds are shown as potential regulators of that synchrony.

The effects of phenolic compounds over time are shown in Table 7.14. According to the analysis of variance (GLM), there were significant differences in the cumulative gas production of the mixtures at the selected times. There were no differences at 12 hours due to the presence of PEG ($p=0.501$). As in the previous experiments, the sources of carbohydrates, the plant material and their interactions affected the fermentation to a greater extent ($p<0.001$), so differences due to the treatments could not be detected. Therefore, the effects at 12 hours are not presented in the table.

Table 7.14: *Effect of phenolic compounds (%) on the cumulative gas production from fodder tree leaves on the fermentation of carbohydrates.†*

Plant species	Glucose	Starch	Cellulose
24 hours			
Trichanthera gigantea	-4.4n.s	9.7n.s	-17.9*
Gliricidia sepium	-45.3***	13.7***	14.1*
Inga sp.	22.6***	19.5n.s	62.5**
Leucaena leucocephala	47.2***	58.6***	3.6*
Erythrina. edulis	12.0***	36.6***	-28.5*
45 hours			
Trichanthera gigantea	-5.3*	6.6n.s	-7.7n.s
Gliricidia sepium	-36.7***	4.7*	6.4*
Inga sp.	14.8***	29.9***	92.2***
Leucaena leucocephala	36.5***	48.2***	17.2**
Erythrina edulis	3.1n.s	3.3n.s	-32.9***
70 hours			
Trichanthera gigantea	-5.3*	-1.2n.s	-2.2n.s
Gliricidia sepium	-34.8***	-0.3n.s	3.3n.s
Inga sp.	11.8***	28.1***	95.9***
Leucaena leucocephala	30.3***	27.2***	23.2***
Erythrina edulis	0.1n.s	-12.9***	-19.5***

†Calculated as: (gas production PEG-gas production No PEG/Gas production PEG)x100.

n.s Not significant.

* Significant at 5% level.

** Significant at 1% level.

*** Significant at 0.1% level.

There was a wide variety of responses between the mixtures to the addition of PEG. The responses were greater in those forages with condensed tannins. In the case of *Inga* sp., the response increased with time in the mixture with starch and there was an even greater response in the mixture with cellulose. The response with glucose was also important, although it decreased with time. In the case, of *Leucaena leucocephala*, the greater responses were found for the mixtures with starch and with glucose, both decreasing with time. The response of the mixture with cellulose increased with time, making the responses of these two species completely opposite. In the case of forages with no condensed tannins, but with phenolic compounds, the responses were less consistent. There were

positive and negative effects. A positive effect like those with condensed tannins suggests that PEG is reacting with tannins and releasing protein which is fermented with the available carbohydrates. This protein may be released early or it may be rapidly available, favouring mixtures with medium to rapidly fermentable carbohydrates (starch - glucose) as in the case of *Leucaena leucocephala*; or the protein may be released or available at a later stage, favouring mixtures with starch and cellulose, as in the case of *Inga* sp.

Negative effects suggest that when the protein is released or is made available, it does not ferment well with the present carbohydrate or simply there is not enough carbohydrate or the right type to boost the fermentation at the time of release. In other words, it suggests an asynchrony. This can be sustained by studying the cases of *Gliricidia sepium* and *Erythrina edulis*. In the case of *Gliricidia sepium* the release of protein may be a late one, that is how it exhibits positive effects with starch and cellulose. Because of the high response of this species to glucose, especially in the first 12 hours (as was discussed previously in this chapter and in Chapter 6), by the time of the release of the protein by PEG, the glucose would have been fermented, leaving the nitrogen with no available carbohydrate and therefore showing a negative effect. The case of *Erythrina edulis* is the opposite but is also a case of asynchrony. With this species, the release of protein may be early, so it is used to ferment rapidly fermentable carbohydrates, giving positive effects especially in the early hours. Because of this early release, this protein is fermented with the available carbohydrates leaving no nitrogen to make use of the extra cellulose, therefore exhibiting negative effects with this carbohydrate in the mixture.

CONCLUSIONS

The general effect of PEG was an increase in the rate constant, which implies that tannins can reduce the rate constant of the substrates. Fodder tree leaves with condensed tannins have a greater effect on the fermentation, in the presence of carbohydrates. Forages with phenolic compounds showed positive and negative effects. The explanation proposed for these effects was synchrony and asynchrony of the release of protein.

7.6 GENERAL CONCLUSIONS

The results showed that PEG reacted not just with condensed tannins but also with other phenols and its effect was specific to the plant material. PEG was not able to overcome completely the effects of tannins in the radial diffusion technique, but it was able to do so in the fermentation. It can stop the

formation of the tannin-protein complex and release the protein from the complex. However, it may not be able to completely recover the protein from the complex in diverse substrates like fodder tree leaves. It appeared not to have an effect on other components in the plant material.

Quebracho tannin, as a model for a mixture of tannins, was capable of reacting with protein of different solubilities in the presence of structural and non-structural carbohydrates. The effect of quebracho tannin on mixtures of carbohydrates and proteins was a reduction in the gas pool size and in the lag phase. The lag phase of glucose, when fermented with BSA or casein, was not affected by the tannin, suggesting that the lag phases of highly fermentable material may not be affected by high levels of tannins. In contrast, the lag phase of cellulose in the presence of both proteins was significantly reduced. There was a clear reduction in gas production due to the presence of quebracho. This tannin started to show an effect from 12 hours onwards. The greatest reduction was with starch and cellulose at 45 and 70 hours. This confirms that more fermentable carbohydrates may be less affected by tannins than less fermentable material such as starch and cellulose. Quebracho reacted with both soluble and insoluble protein but it reacted more with the more soluble protein.

The effect of PEG on the fermentation of forages with tannins was an increase in the rate constant, which implies that tannins have the potential to reduce the rate constant of the substrates. On the other hand, there was an effect of PEG in decreasing the lag phase of forages without tannins, which suggests that phenolic compounds may increase the lag phase. The effects of tannins were not shown in the lag phase, but in decreasing the gas pool size. Some differences between the findings with quebracho and phenols from the tree leaves are expected as quebracho is a free tannin.

When fermented in the presence of carbohydrates, tannins freed from PEG started to have a significant effect on the fermentation after 12 hours which confirms the findings with quebracho. Equally, mixtures of low fermentability were affected to a greater extent than more fermentable mixtures. Highly fermentable mixtures need greater levels of tannins to reach a maximum depletion of the fermentation. The effect of phenolics in mixtures with carbohydrates was an increased gas pool size and increased fermentation rate. This can affect the associative effects of mixtures of plants high in tannins and high in carbohydrates

Fodder tree leaves with condensed tannins showed a marked effect in reducing the fermentation, in the presence of carbohydrates. Forages with phenolic compounds showed positive and negative effects. These effects were explained as a synchrony or asynchrony in the release of protein.

Chapter 8

ASSOCIATIVE EFFECTS OF MIXTURES OF FODDER TREE LEAVES

8.1 INTRODUCTION

In previous chapters, it has been shown how important is the synchrony in the release and/or fermentability of nutrients, in producing associative effects on the gas production of a mixture of substrates. The synchrony of rapidly fermentable/available proteins and carbohydrates produced greater associative effects than when non-synchronized nutrients were fermented together (i.e. proteins that ferment fast with very slowly fermentable carbohydrates). A late synchrony occurred when slowly fermentable proteins and carbohydrates were mixed and associative effects were more evident towards the end of the fermentation.

Mixtures of fodder tree leaves with carbohydrates were fermented to characterise their response, in terms of the associative effects. The synchronization of fermentation rates of fodder tree leaves was studied. Results showed that associative effects tended to be greater in mixtures with starch and cellulose, especially towards the end of the fermentation. The mode of action was a decrease in the fermentation rate and an increase in the lag phase of the mixtures. Associative effects with glucose were higher at the beginning and decreased towards the end of the fermentation. The mode of action was an increase in the rate constant and a reduction of the lag phase.

The effects of phenolic compounds on the fermentation was studied and their role in affecting the synchronization was discussed. These results are brought together in this chapter to explain the associative effects of mixtures of fodder tree leaves and to give the basis for their prediction by analysing the response curves.

OBJECTIVES

The objectives of this chapter are to:

- test the existence of associative effects in mixtures of fodder tree leaves; and
- explain these effects according to the knowledge gained from previous chapters.

This will meet the general objective of this thesis of developing the scientific knowledge of the mechanisms that govern the associative effects of combining different tree leaves on gas production during *in vitro* incubation.

8.2 MATERIALS AND METHODS

Mixtures of the five tree species: *Trichanthera gigantea, Gliricidia sepium, Inga* sp., *Leucaena leucocephala* and *Erythrina* edulis, were tested for associative effects at two levels of nitrogen in the medium: 0 and 60mgN/l. Urea was the source of nitrogen. All possible combinations of two species were mixed on a 50:50 basis and the total amount of substrate was 0.5g. A control containing 0.5g of each individual species was fermented to calculate the expected response curve as explained in section 3.4. Mixtures of trees were fermented in triplicate but six replicates of the single species controls were fermented in order to increase the precision of the predicted value. The experimental plan is shown in Table 8.1.

Table 8.1: *Experimental plan to determine associative effects of mixtures of fodder tree leaves.*

Fermentation using Menke's medium and carried out for 70 hours. Three replicates were used for the mixtures and six replicates for control single substrates. Nominal weight 0.5g (0.25g for each component in the mixtures).

Mixtures tested were (on a 50:50 basis):

Trichanthera gigantea:Gliricidia sepium
Trichanthera gigantea:Inga sp.
Trichanthera gigantea:Leucaena leucocephala
Trichanthera gigantea:Erythrina edulis
Gliricidia sepium:Inga sp.
Gliricidia sepium:Leucaena leucocephala
Gliricidia sepium:Erythrina edulis
Inga sp.:*Leucaena leucocephala*
Inga sp.:*Erythrina edulis*
Leucaena leucocephala:Erythrina edulis

Single species control; control no substrate; 0 Nitrogen and control no substrate 60mgNitrogen/l were also carried out.

The medium was prepared with a concentration of 85ml/bottle. A solution of urea (46%N) of 2.6mg/ml was prepared and dispensed in the remaining 5ml for treatments at 60mgN/l. Treatments with no nitrogen were completed with distilled water.

Experiments reported in this thesis up to now have been carried out with nitrogen-free media, using substrates of tree leaves and proteins of different fermentabilities as the sources of nitrogen. For this experiment, it was decided to use a level of nitrogen that resembled an *in vivo* situation. In recent trials in the Cauca Valley (Colombia) (data not published), it was determined that the consumption of urea-

molasses block containing 20% of urea by growing cattle, was 13% of the total intake. Assuming the total weight of substrate in the bottle (0.5g) as total intake, the amount of urea to be put into the bottles to replicate that *in vivo* conditions was 0.013g. This amount of urea in 100 ml/bottle would give a level of nitrogen of 60mgN/l, hence the choice of this level of experiment. Of course, this level of nitrogen does not take into account of any endogenous nitrogen through recycling of urea to the animal.

STATISTICAL ANALYSIS

Student's *t* tests were used to test for associative effects on the cumulative gas production of the different mixtures at 12, 24, 45 and 70 hours. The null hypothesis was that gas production of a mixture was equal to the sum of the gas production of its individual components. These tests were carried out as described in section 3.4.

The effect of urea at the level of 60mgN/l was studied by comparing the fermentation of the substrates with and without urea. The Generalized Linear Model procedure was used to test differences in gas production at 12, 24, 45 and 70 hours from fodder tree leaves in the presence and absence of urea. The effects of urea on the fermentation kinetics of single substrates was studied by fitting the Gompertz equation (constant=0) to the data and the resultant parameters were statistically tested by GLM. The interaction between urea and fodder tree leaves was also tested.

8.3 RESULTS AND DISCUSSION

The model fitted the data very well (R^2>99%).

EFFECT OF UREA

The effect of urea at 60mgN/l can be studied by comparing the fermentation of the substrates with and without urea. According to the analysis of variance there was a significant effect of the presence of urea ($p<0.001$) on gas production at 45 and 70 hours and a significant effect of the plant species ($p<0.001$) at all incubation times. There was no significant interaction ($p>0.05$)of urea x species, which implies that urea had the same effect across the species. There was no effect ($p>0.05$) at 12 and 24 hours. Table 8.2, shows the effect of urea on the gas production. There was a small but significant effect of urea ($p<0.001$) on reducing the fermentation of the substrates at later stages.

Table 8.2: *Effect of urea on the mean gas production (ml) of fodder tree leaves at four incubation times.*

	Incubation Time (hours)			
	12	24	45	70
Without Urea	27.3	52.5	74.2	82.1
With Urea (60mgN/l)	26.0	51.5	72.1	79.5
S.E.D (50 df_{error})	1.007	1.095	0.840	0.778

With regard to the fermentation kinetics, the analysis of variance showed a highly significant difference ($p<0.001$) in the rate constant and gas pool size. There was no effect of urea on the lag phase. In all cases, there was a significant effect of the plant species and there was no significant interaction of urea x species. The effect of urea at 60mgN/l was an increase in the rate constant and a decrease in the gas pool size of the substrates. It did not affect the lag phase (see Table 8.3).

Table 8.3: *Effect of urea on the fermentation kinetics of fodder tree leaves.*

	Curve Parameters		
	Rate Constant ($hour^{-1}$)	Lag phase (hours)	Gas Pool Size (ml)
Without Urea	0.079	13.7	82.3
With Urea	0.082	13.9	79.2
S.E.D (50 df_{error})	$9.451x10^{-4}$	0.334	0.716

In general, the effect of urea was a small but significant reduction in the total gas production and an increase in the rate at which the mixtures were fermented. This increased rate may be the reason why the associative effects were very different with the two nitrogen conditions as discussed below. It is not possible to explain the reasons why urea reduced the gas production from these data. Researchers cannot agree on the level of ammonia required for optimum bacterial growth. Reports vary widely from 50 (Ørskov, 1982) to 200mgNH_3/l (Preston and Leng, 1987) in the rumen. Satter and Roffler (1977) found *in vitro* that, once ammonia starts to accumulate and exceeds 50mg/l, the maximal microbial growth is reached and nothing is gained by further supplementation with non-protein nitrogen (NPN). The level of ammonia at the high level of urea in this experiment was calculated on the basis that 1g of urea produces 0.56g of ammonia. Adding 0.013g of urea per bottle (the amount needed to get 60mgN/l) will produce 0.00728gNH_3/100 ml bottle, this is a level of 72.8mgNH_3 /l, which is well beyond the maximum found by Satter and Roffler (1977). Experiments carried out by the author with hay, straw and sugar cane bagasse (data not published) showed a significant increase in gas production due to the presence of urea at 30 and 60mg/l. Experiments carried out at the Natural Resources Institute have found a positive response to urea at 200mg/l when working with substrates other than fodder tree leaves (C. Wood, personal communication, 1995). It appears that the maximum

limit for the gas production is substrate-dependent and the maximum ammonia level for fodder tree leaves is below $72mgNH_3/l$. One explanation of the present results is that fodder tree leaves have less fermentable carbohydrates than hays or straws to ferment additional urea, or that they are already balanced in terms of the energy-protein ratio.

ASSOCIATIVE EFFECTS

The student's *t* tests showed significant differences between the sum of the individual components and their mixture for some substrates. In these cases, the null hypothesis that gas production of the mixture was equal to the sum of the gas production of its individual components was rejected. In other words, associative effects of mixtures of fodder tree leaves were observed. The difference between the observed and expected values for each mixture is expressed as percentage in Table 8.4.

Table 8.4: *Associative effects (%) of mixtures of fodder tree leaves under two levels of nitrogen.†*

	Time (hours)			
Nitrogen-free medium	**12**	**24**	**45**	**70**
Trichanthera gigantea:Gliricidia sepium	18.1**	12.1***	2.9n.s	0.5n.s
Trichanthera gigantea:Inga sp.	-5.5n.s	4.9n.s	2.0n.s	1.2n.s
Trichanthera gigantea:Leucaena leucocephala	9.0n.s	8.0n.s	3.6n.s	1.2n.s
Trichanthera gigantea:Erythrina edulis	6.0n.s	9.4*	2.9n.s	1.5n.s
Gliricidia sepium:Inga sp.	15.5*	9.3**	7.5**	5.8*
Gliricidia sepium:Leucaena leucocephala	12.3n.s	7.1n.s	2.6n.s	-0.2n.s
Gliricidia sepium:Erythrina edulis	6.3n.s	0.7n.s	-0.9n.s	-1.7n.s
Inga sp.:*Leucaena leucocephala*	0.6n.s	3.3n.s	0.8n.s	-1.1n.s
Inga sp.:*Erythrina edulis*	-6.3n.s	1.3n.s	3.1n.s	2.7n.s
Leucaena leucocephala:Erythrina edulis	-3.0n.s	-2.7n.s	-1.5n.s	-2.3n.s
Level of 60mgN/l from urea	**12**	**24**	**45**	**70**
Trichanthera gigantea:Gliricidia sepium	5.1n.s	5.2n.s	0.4n.s	0.7n.s
Trichanthera gigantea:Inga sp.	0.3n.s	6.5n.s	8.1***	7.9***
Trichanthera gigantea:Leucaena leucocephala	7.2n.s	8.2*	8.0***	6.8***
Trichanthera gigantea:Erythrina edulis	0.0n.s	6.3n.s	4.8**	4.4***
Gliricidia sepium:Inga sp.	10.1n.s	9.4**	9.7***	9.0***
Gliricidia sepium:Leucaena leucocephala	-16.0n.s	-3.3n.s	1.6n.s	1.3n.s
Gliricidia sepium:Erythrina edulis	1.1n.s	1.1n.s	0.6n.s	0.2n.s
Inga sp.:*Leucaena leucocephala*	-17.8*	-6.6n.s	1.3n.s	2.9n.s
Inga sp.:*Erythrina edulis*	-16.4n.s	-6.5n.s	1.0n.s	3.0n.s
Leucaena leucocephala:Erythrina edulis	-1.1n.s	4.3n.s	5.2**	5.2**

†Calculated as: (Observed-expected/observed)x100

The main conclusions drawn from this table are: firstly, there are associative effects of mixtures of fodder tree leaves and secondly that they vary with the level of nitrogen (there were more associative effects when urea was present). These variations with the level of nitrogen are important to have in mind for future experiments and suggest that it may be possible to alter associative effects by manipulation of the level of nitrogen. This is possible both *in vitro* and *in vivo* by the addition of urea or protein (see Chapter 9).

In general, the explanation proposed for associative effects has been a synchronization of the release of nutrients from the two components of the mixture. It has been determined that when proteins are rapidly fermentable/available they show greater associative effects with medium to rapidly fermentable carbohydrates than with slowly fermentable carbohydrates. On the other hand, when the protein is less rapidly fermentable or is available later in the fermentation, the associative effects are greater with slowly fermentable carbohydrates (see Chapter 5). By comparison of these results with those obtained when the fodder tree leaves are fermented with different carbohydrates (see Chapter 6), it was possible to characterise, to a certain extent, the fermentability/availability of the nitrogen from the leaves. This characterization is useful to explain the associative effects of fodder tree leaves with carbohydrates. In this case, as the nitrogen from fodder tree leaves is not as rapidly fermentable/available as purified proteins, the associative effects were greater with medium to slowly fermentable carbohydrates. There were however, important associative effects with rapidly fermentable carbohydrates (see Table 6.5). All plant species exhibited associative effects to different degrees when fermented with carbohydrates. On the other hand, tannins have shown the potential to reduce the rate constant and gas pool size and phenolic compounds have the potential to increase the lag phase of mixed substrates. The different positive and negative effects of the phenolic compounds from the different species when fermented in the presence of carbohydrates have been presented (see Chapter 7).

According to Table 8.4, there were significant associative effects for the mixture *Trichanthera gigantea:Gliricidia sepium* when there was no nitrogen in the medium (see Figure 8.1a). For these conditions, this looks like an ideal combination as it shows the highest associative effects, of all. *Gliricidia sepium* has the highest amount of total and soluble protein whereas *Trichanthera gigantea* is the forage with the highest contents of soluble and total carbohydrates. It was shown in Chapter 4 that these components are the most important contributors to gas production in the early stages of incubation. *Gliricidia sepium* showed associative effects with glucose (greater than those from *Trichanthera gigantea*) in the early stages. It also exhibit associative effects with starch, greater than those with glucose. *Trichanthera gigantea* has the highest content of starch among the selected plants.

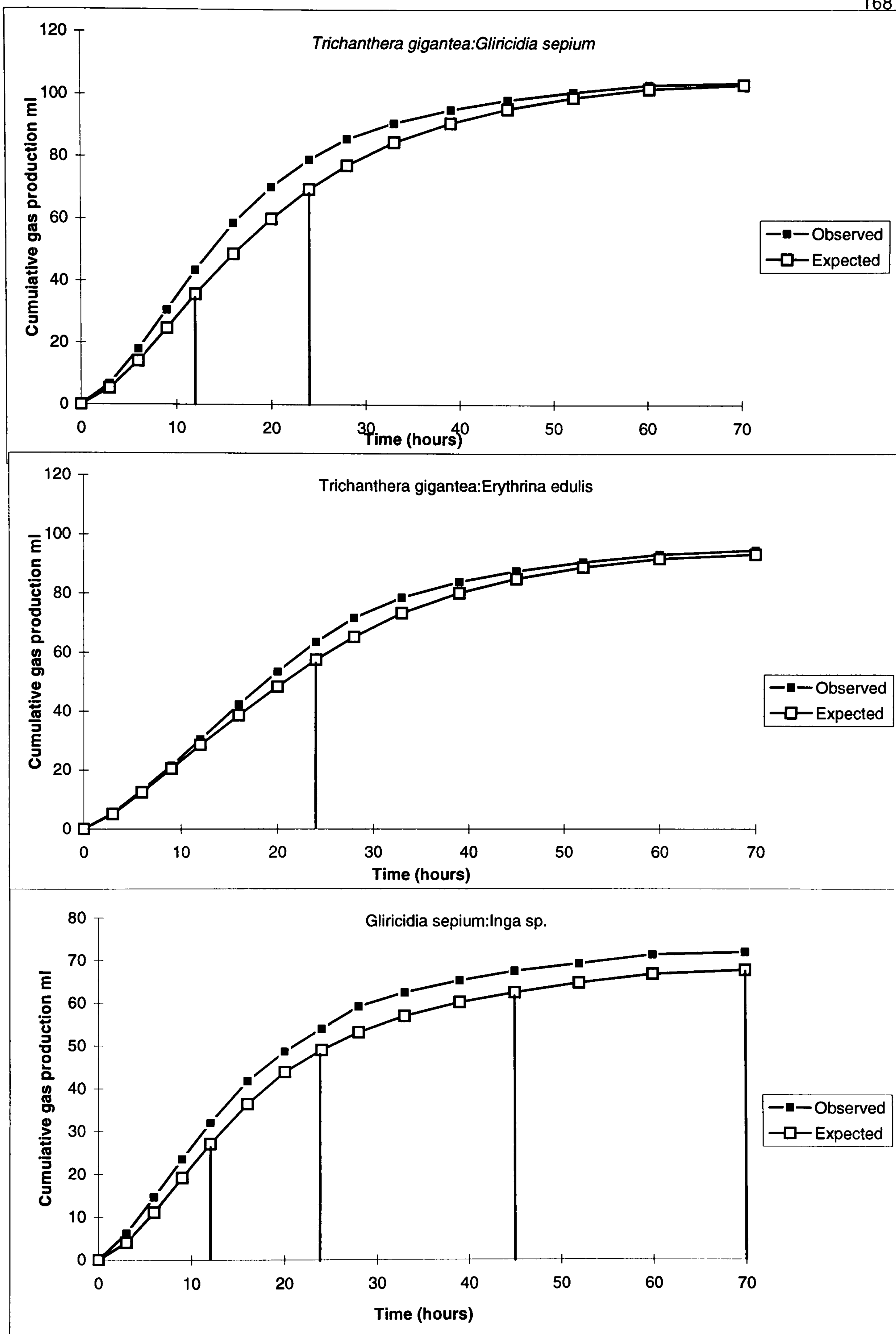


Figure 8.1: *Associative effects on the fermentation of mixtures of fodder tree leaves under nitrogen free conditions. Associative effects were tested at 12, 24, 45 and 70 hours. Vertical lines show the times at which significant differences were found.*

It is also the richest in total sugars (reducing and non-reducing sugars) and in water soluble carbohydrates. Therefore, it is not surprising that the associative effect shown for *Gliricidia sepium* with carbohydrates were replicated with *Trichanthera gigantea*. It was proposed that *Gliricidia sepium* may have at least 2 fractions of protein of different fermentabilities. This would allow it to make more efficient use of the two types of carbohydrates as the first fraction of nitrogen could be used for the fermentation of rapidly fermentable carbohydrates and the second fraction could make use of starch. The fermentation rates of *Gliricidia sepium* when fermented with carbohydrates show how the decay in the rate of gas production of a mixture *Gliricidia sepium:*glucose coincided with the increase in the rate in the mixture with starch (see Figure 6.2).

Another mixture which showed significant associative effects (under nitrogen-free conditions), was *Trichanthera gigantea:Erythrina edulis* (Table 8.4, Figure 8.1b). As with *Gliricidia sepium*, fermentation rates of this plant material with carbohydrates, showed that the decrease and increase in the rates can coincide and this can help to maintain a steady response in a mixture with a source of carbohydrates (see Figure 6.2). This however does not fully explain the associative effect found at 24 hours only. In Chapter 6, the fermentation of *Erythrina edulis* was studied and it was concluded that it is the ideal complement for starch. It was proposed that its protein, or at least one of the fractions of its protein, may exhibit a slow release or slow fermentability. When the fermentation kinetics of chemical entities were studied, (Chapter 5), it was shown that, when fermented with a slowly fermentable/available protein, starch showed a peak in the rate profile at 24 hours. In the case of this mixture, a fraction of the protein of *Erythrina edulis* may coincide with the time at which starch from *Trichanthera gigantea* is made available for the fermentation and this induced a greater gas production that expected. Associative effects of *Erythrina edulis* with starch were among the biggest effects of mixtures of fodder tree leaves and carbohydrates.

The case of the mixture *Gliricidia sepium:Inga* sp. is very interesting as it was the only mixture that showed associative effects at both levels of nitrogen (Table 8.4, Figure 8.1c). It has been proposed that a way to deal with plants high in tannins is to mix them with plants with highly soluble protein, in the hope that the tannins from one plant can protect the protein from the other (Barry and Blaney, 1987; Barry, 1988; Waghorn *et al.*, 1987; Lascano and Palacios 1993). The mixture *Gliricidia sepium:Inga* sp. is an example of such a mixture where *Gliricidia sepium* had the highest contents of soluble and non-soluble protein (Table 4.1) and *Inga* sp. is the plant material tested with the highest amounts of tannins (Table 4.5). If there was a case of some protein being protected, then the associative effect would be negative for the whole period of incubation or negative in the early stages and, with a slower release of protein, it could be positive in the final stages. Instead, the associative effects were positive

and significant during the complete fermentation period. This mixture combined the plant material with the highest and lowest fermentation profiles. A reasonable explanation for these associative effects can be given by examining the chemical composition and the fermentation profiles. *Inga* sp. had twice the amount of starch and the same amount of total sugars as *Gliricidia sepium* (Table 4.2). It also had twice the amount of cell walls and lignocellulose (Table 4.3). Although it was very rich in carbohydrates, it had the lowest fermentation of all forages. The reason for this is that, despite the adequate level of protein compared with other forages, it had the highest amounts of tannins and protein precipitation capacity. It is very likely that its protein is protected by its own tannins and therefore not available to the bacteria. When fermented on its own, it lacks the nitrogen needed to ferment its carbohydrates and therefore the expected value underestimates its real contribution in the mixture. When fermented in the presence of *Gliricidia sepium*, the protein from this plant material (amount and type) may supply the nitrogen needed to maximise the use of the available carbohydrates at a given time This can be supported by the fact that *Inga* sp. showed the highest response to nitrogen of all forages both when fermented in the presence of high and no-nitrogen media (see 4.3) and when the nitrogen protected by tannins was freed by the addition of PEG (Chapter 7).

The proposed explanation for the associative effects when urea was not present is supported by the findings on the fermentation of different substrates under nitrogen-free conditions. They have been also studied at a similar level of nitrogen (80mgN/l as opposed to 60mgN/l) from two different proteins. Urea (at 60mgN/l) increases the rate at which the fermentation occurs and this may affect the synchrony of the release/fermentability of nutrients of any mixture. This is demostrated by the fact that, except for one, the mixtures showed different associative effects when urea was present, and also they tended to be at a later stage. To fully explain the associative effects under these conditions, the whole set of experiments would have had to be repeated with urea as the source of nitrogen. This means that, for any condition in which an associative effect is to be predicted, the methodology carried out in this thesis would have to be followed. This is clearly not feasible for routine or screening analysis. However, a general explanation for an associative effect can be given by studying the dynamics of both the response and the rate profiles. To illustrate this, examples of associative effects observed in this thesis are brought here. There were, in general, four types of responses of the associative effects. They exemplified cases in which the synchronization of the release/fermentability of nutrients occurred with substrates or nutrients of different fermentabilities; from highly fermentable to less fermentable material. (see Figures 8.2 to 8.5). This gives the basis for predicting appropriate mixtures of tree foliage.

Figure 8.2 shows a case in which the synchronization of release/fermentability of nutrients occurs between highly fermentable material and in the early stages. This is shown by the fact that the observed rate profile has moved left and upwards with respect to the expected curve. This effect is a consequence of a combination of a faster rate and shorter lag phase compared to the observed response. The shape of the observed response curve and the sharp decline in the rate profile suggest that the synchronization is a short event which supports the theory that it may be due to highly fermentable material from both components. An example of this is the associative effect of *Trichanthera gigantea* and *Gliricidia sepium* found in this chapter under nitrogen-free conditions.

The graph shown in Figure 8.3 was the most common shape of associative effects found in this work. This sort of response was exhibited by mixtures of glucose and starch in the presence of casein (see Chapter 5) and also by mixtures of all fodder tree leaves with glucose and *Gliricidia sepium* with starch. In these cases, the proposed explanation was early synchrony in the release/fermentability of nutrients (see Chapter 6). In the example, this is shown by the observed rate which was higher than expected.

This response is due to an increment in the gas pool size and in the rate constant of the observed with respect to the expected. The observed rate profile may peak at the same time but is greater than the expected. This is the case of a synchronization in the release/fermentability of nutrients of highly fermentable material that occurs later than in the previous example.

The third case, (Figure 8.4), is also a common response in which the associative effect is negative at the beginning and positive at the end. In this case, the synchrony occurs with material of medium fermentability. This sort of response was found for the mixtures starch:glucose:BSA and glucose:cellulose:BSA, in which the synchrony occurs towards the middle of the incubation period. This response is characterized by an increase in the gas pool size and the lag phase (fermentation starts later) which determine that the observed rate profile moves, to the left. It is lower at the beginning and greater at the end than the expected response.

Figure 8.5, shows the case in which the associative effect is non-existent at the beginning but increases and is important at the end. This is an example of when the synchrony occurs with material of low fermentability. This response was exhibited by most fodder tree leaves when mixed with cellulose, and in mixtures of starch-cellulose-casein. This response is characterised by an increase in the gas pool size, and the lag phase, combined with a decrease in the rate constant of the observed response against the expected. This combination of a late lag phase and low rate makes the rate profile move well towards the end of the fermentation and shows none or a very late point of inflection.

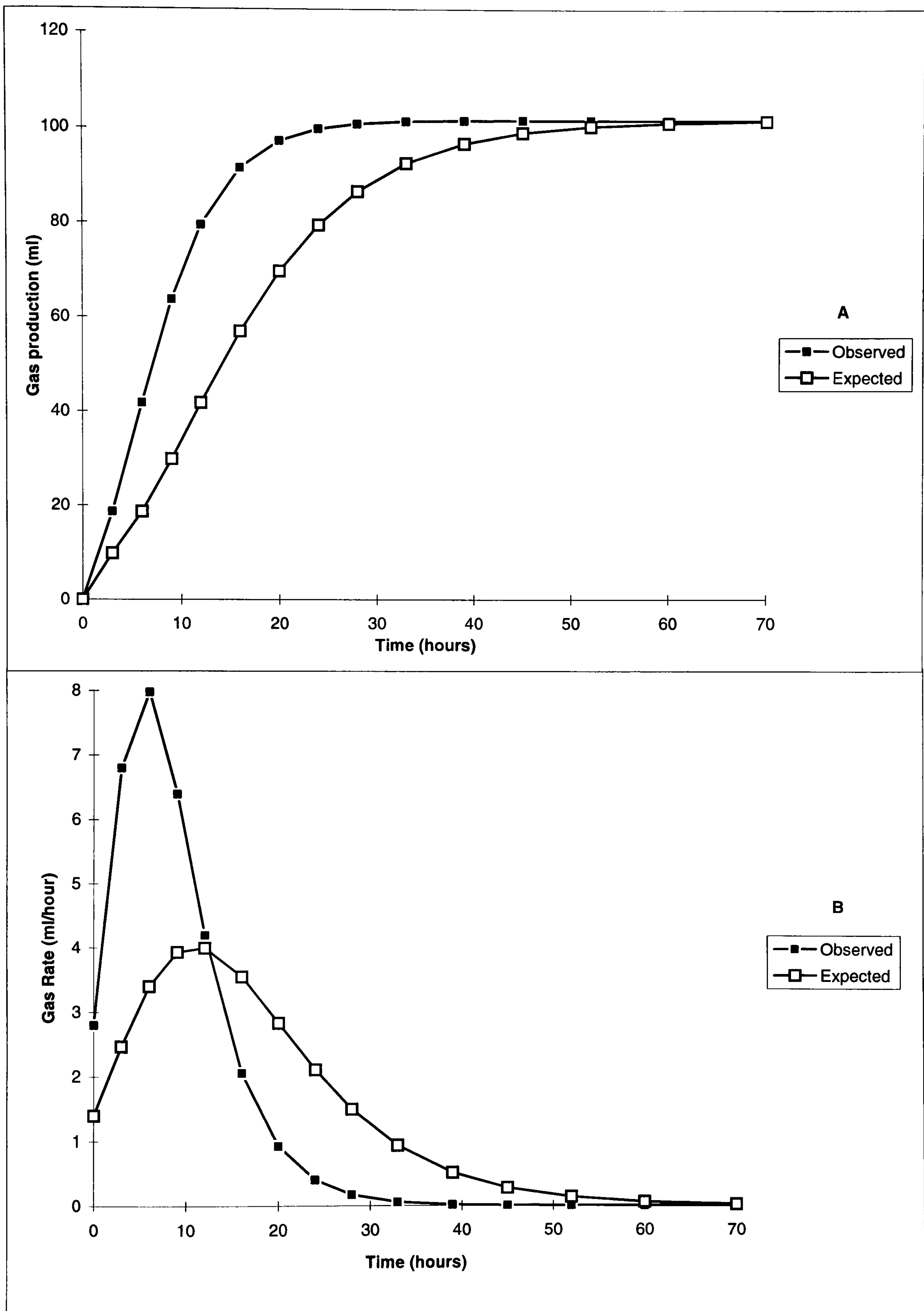


Figure 8.2: *Fermentation dynamics of the associative effects of a mixture when the observed rate > expected and observed lag phase < expected. Figures a and b show curve and rate profiles respectively.*

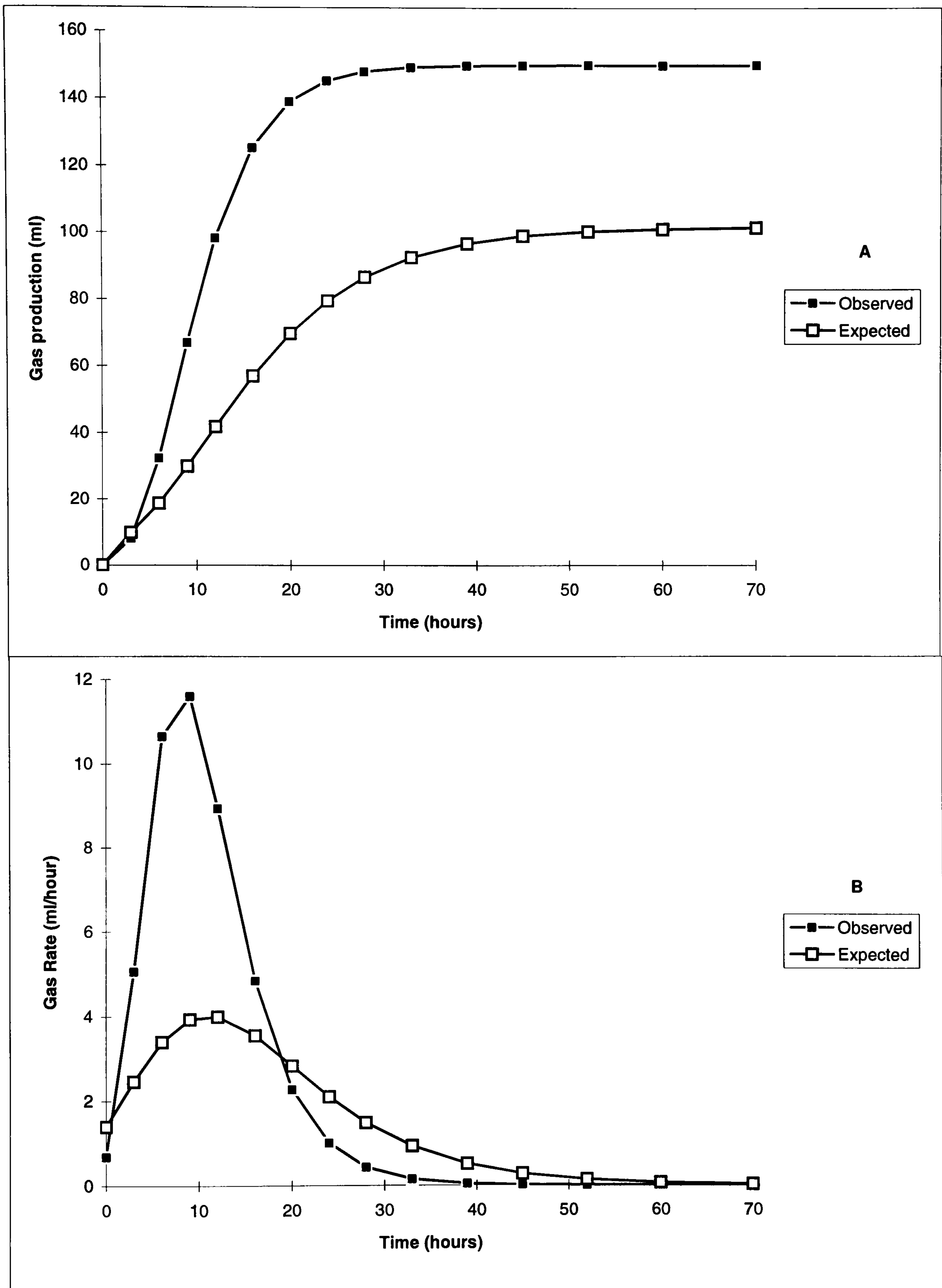


Figure 8.3: *Fermentation dynamics of the associative effects of a mixture when the observed gas pool size > expected and observed rate > expected. Figures a and b show curve and rate profiles respectively.*

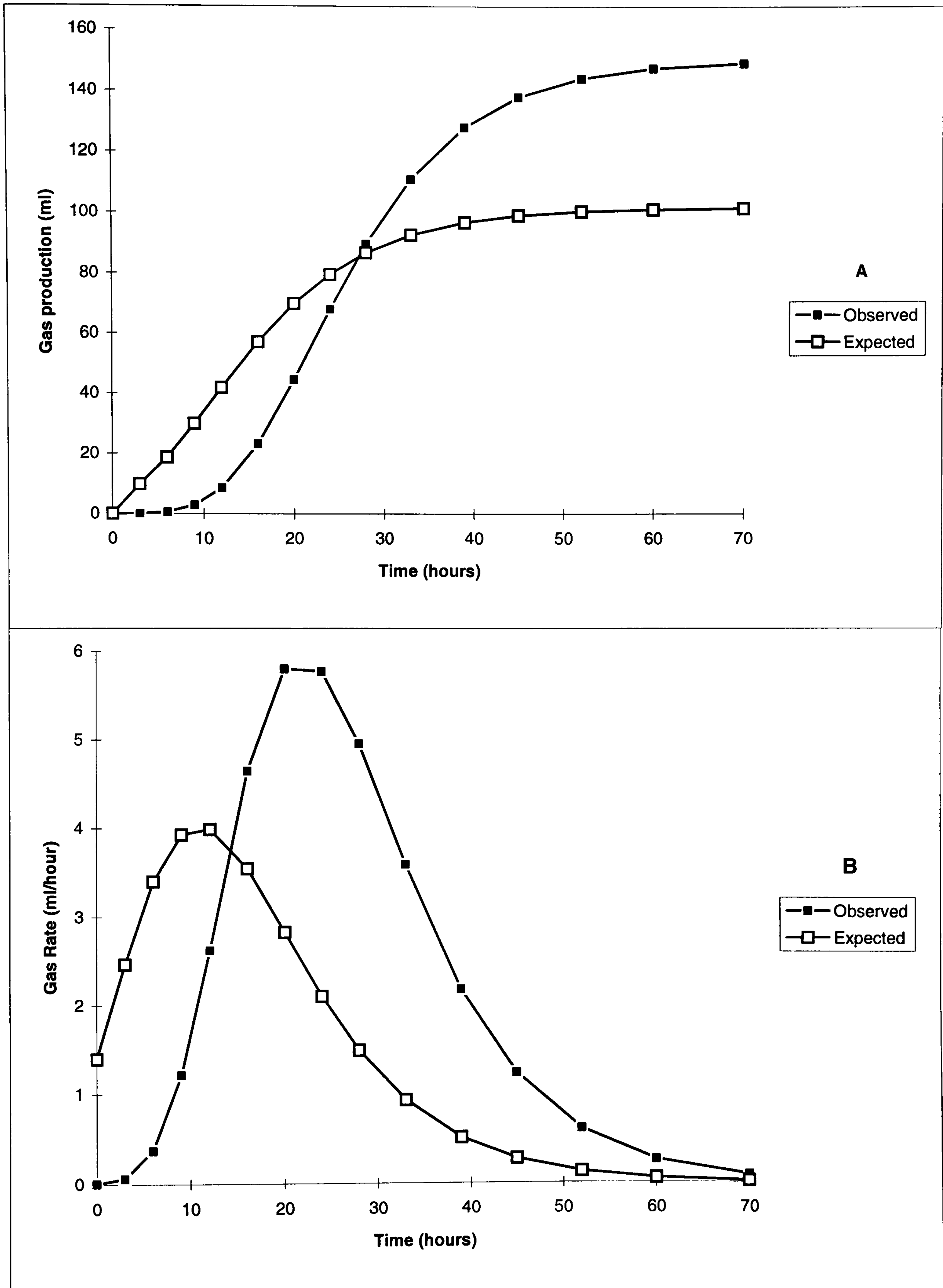


Figure 8.4: *Fermentation dynamics of the associative effects of a mixture when the observed gas pool size > expected and observed lag phase > expected. Figures a and b show curve and rate profiles respectively.*

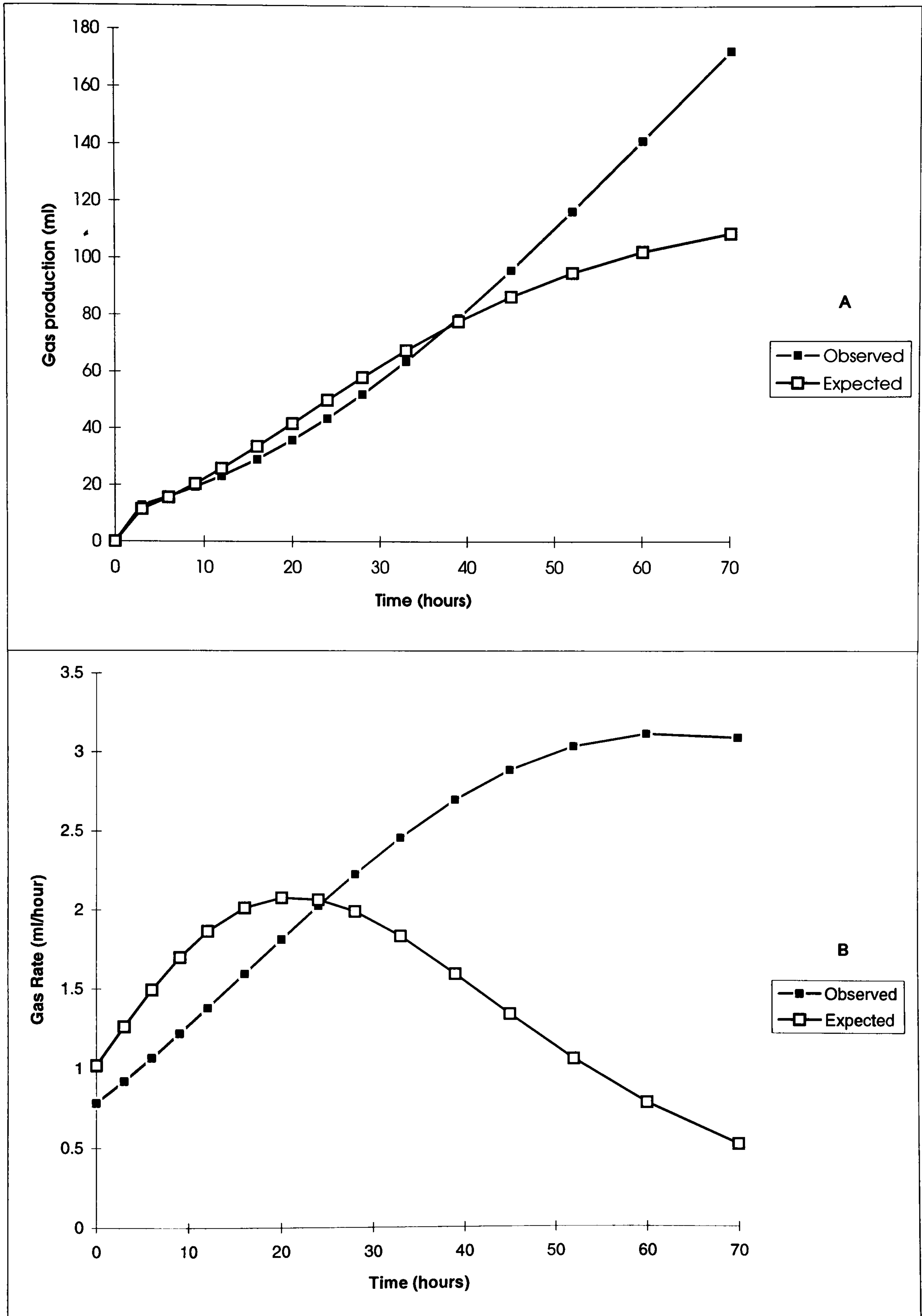


Figure 8.5: Fermentation dynamics of the associative effects of a mixture when the observed gas pool size > expected, observed rate < expected and observed lag phase > expected. Figures a and b show curve and rate profiles respectively.

Chapter 7 showed that the effects of tannins were to reduce the rate constant and the gas pool size. This effect was evident after 12 hours and especially with mixtures of sources of carbohydrates of low fermentability. Given this property, tannin may affect, associative effects, like those of Figure 8.3 especially by reducing them. Phenolic compounds were shown to have a more important effect in increasing the lag phase and reducing the gas pool size. They may affect associative effects like those of Figure 8.4.

ASSOCIATIVE EFFECTS AT 60mgN/l

It is possible to give a general explanation of the associative effects found at 60mgN/l from urea by studying both the response and rate profiles as proposed above. Figures 8.6 to 8.11 show the response and rate profiles of the statistically significant associative effects at 60mgN/l from urea.

Except for the mixture *Inga* sp.-*Leucaena leucocephala* (Figure 8.10), all associative effects showed the same shape of the response and rate profiles. Their profile is similar to that for associative effects produced by synchronisation between highly fermentable material from both substrates (Figure 8.3), although the response is not as dramatic as in the example. Associative effects started to show up from as early as 16 hours in most cases, but they were only statistically significant ($p<0.05$) later. The hypothesis that the associative effect may be due to synchronisation between the fermentation/availability of highly fermentable material from both substrates can be supported by the fact that the same shape of the associative effect was found when all fodder tree leaves were fermented with glucose (see chapter 6).

The case of the mixture *Inga* sp.-*Leucaena leucocephala* is like that from Figure 8.4 in which the synchrony occurs with material of medium fermentability/availability. It may be the case, as was shown in Chapter 6, that *Leucaena leucocephala* favours associative effects with carbohydrates of medium to low fermentability like starch and cellulose which are present in important quantities in *Inga* sp. as discussed earlier. Inga sp. is also high in tannins, phenols and protein precipitation activity.

This general explanation should be taken with caution as similar curves can be produced with very different parameters, but it gives an idea of what can be happening under conditions not previously studied. It also relies on the model describing the fermentation and a good fit to the curves.

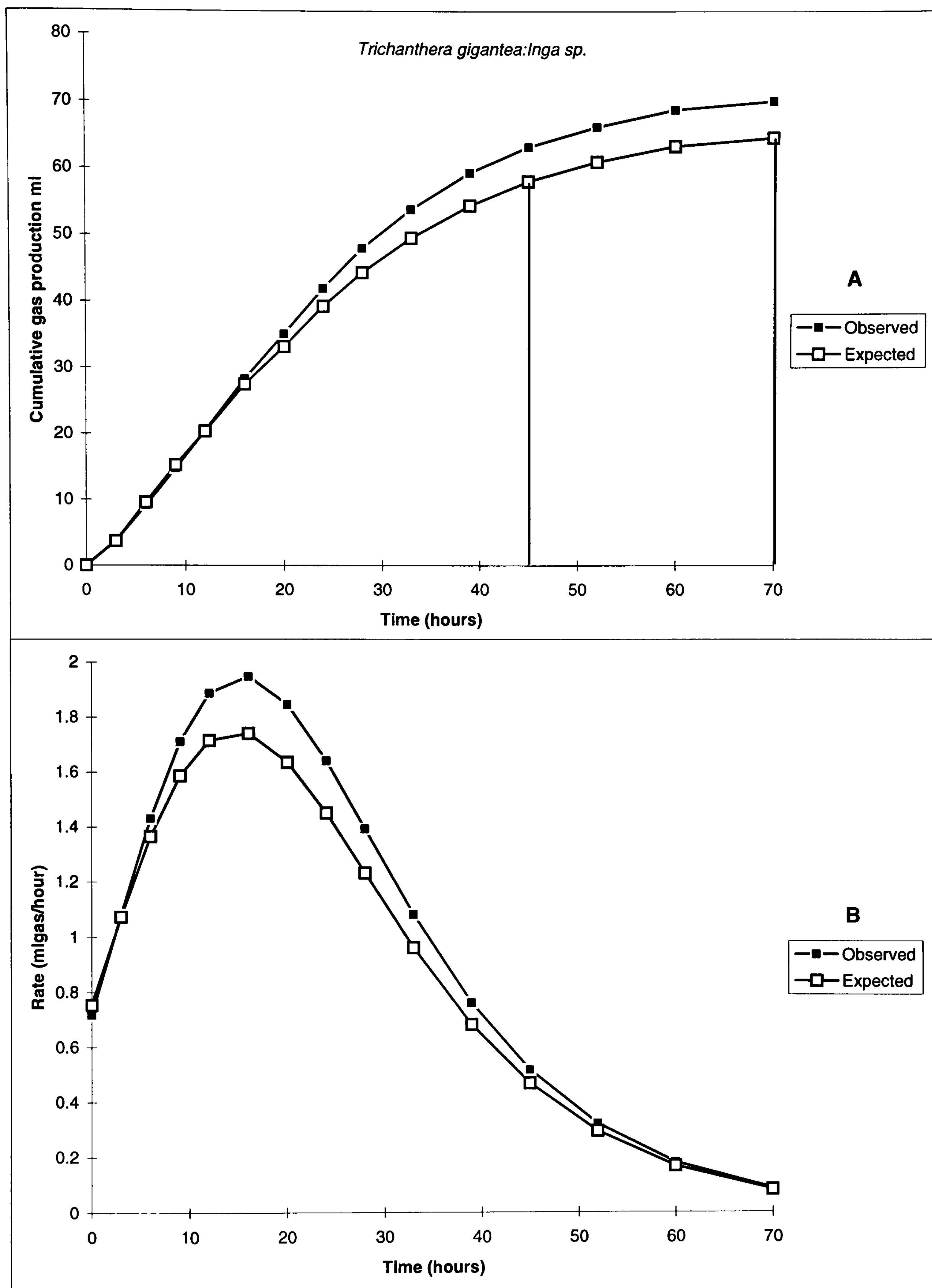


Figure 8.6: *Associative effects on the fermentation of a mixture of Trichanthera gigantea and Inga sp. at 60mgN/l form urea. Associative effects were tested at 12, 24, 45 and 70 hours. Vertical lines show the times at which significant differences were found. Figures a and b show curve and rate profiles respectively.*

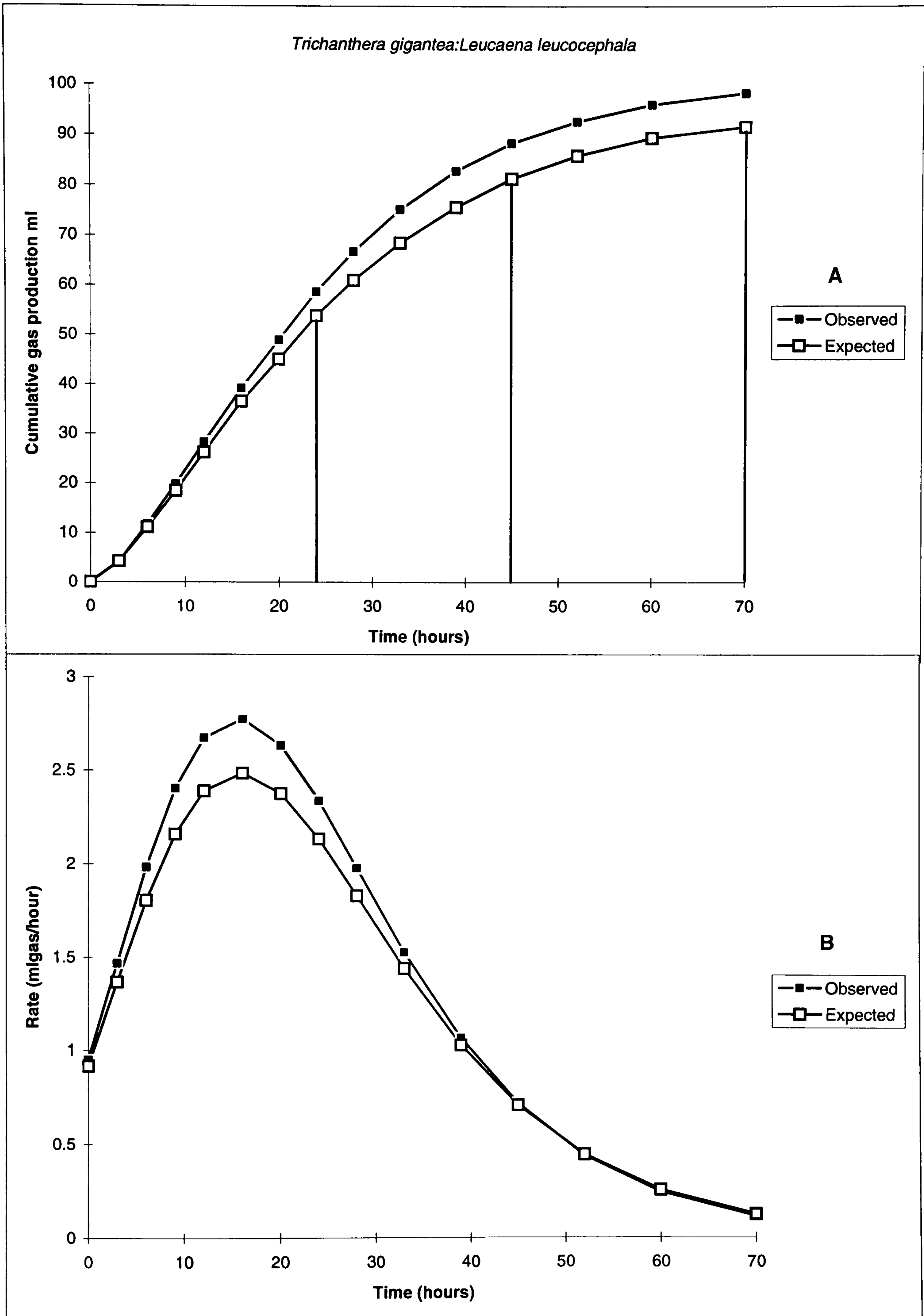
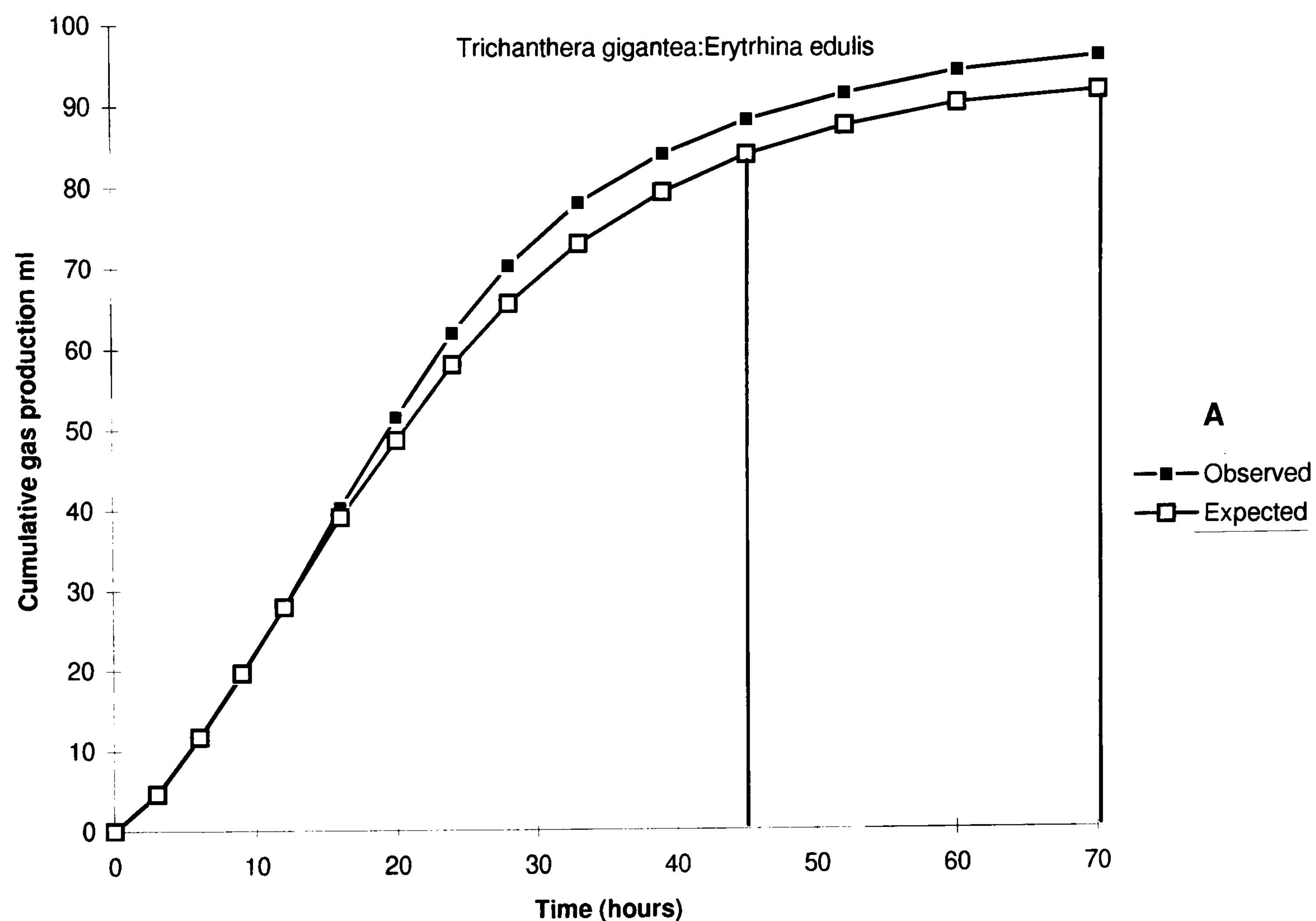


Figure 8.7: *Associative effects on the fermentation of a mixture of Trichanthera gigantea and Leucaena leucocephala at 60mgN/l form urea. Associative effects were tested at 12, 24, 45 and 70 hours. Vertical lines show the times at which significant differences were found. Figures a and b show curve and rate profiles respectively.*



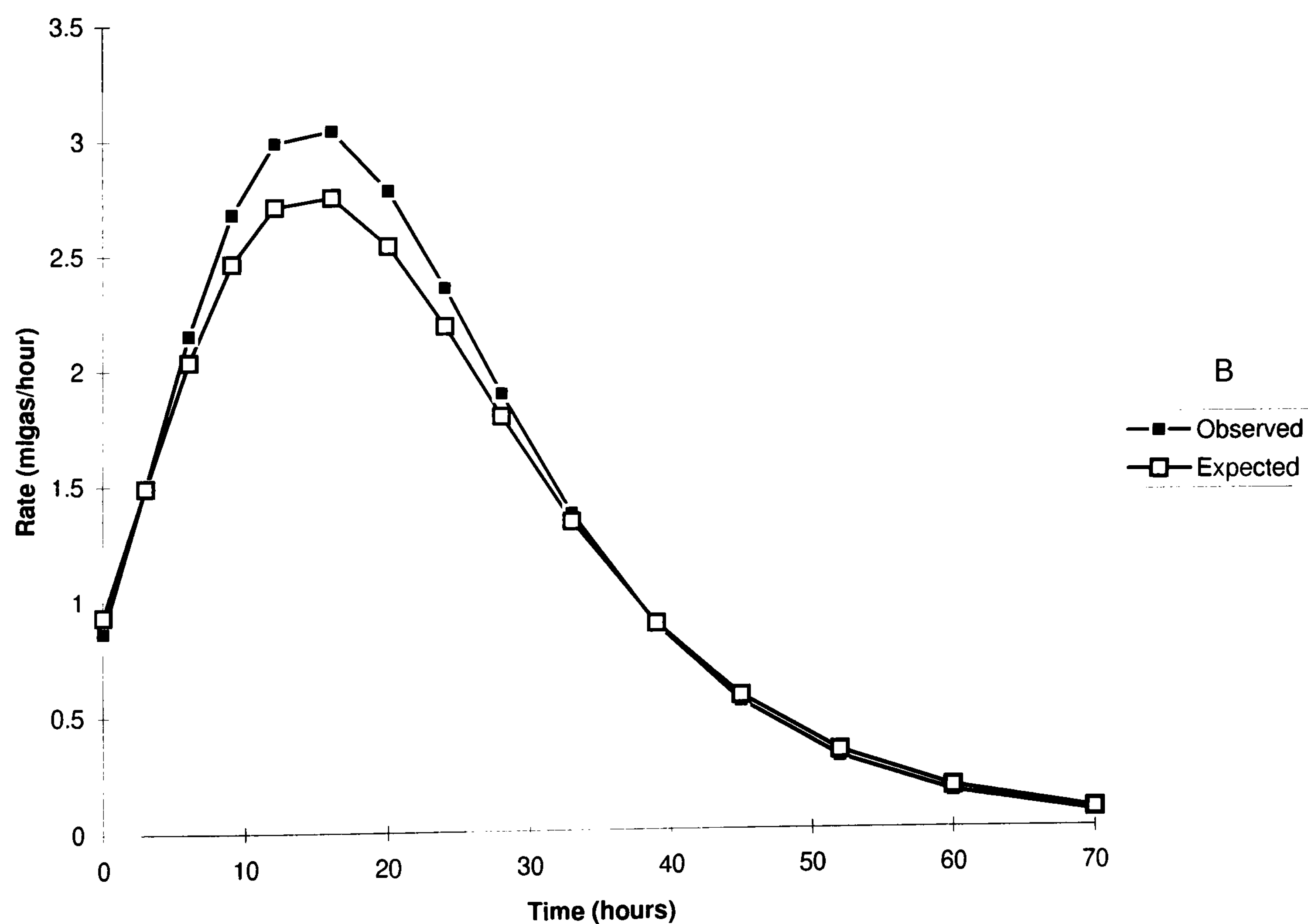


Figure 8.8: *Associative effects on the fermentation of a mixture of Trichanthera gigantea and Erythrina edulis at 60mgN/l form urea. Associative effects were tested at 12, 24, 45 and 70 hours. Vertical lines show the times at which significant differences were found. Figures a and b show curve and rate profiles respectively.*

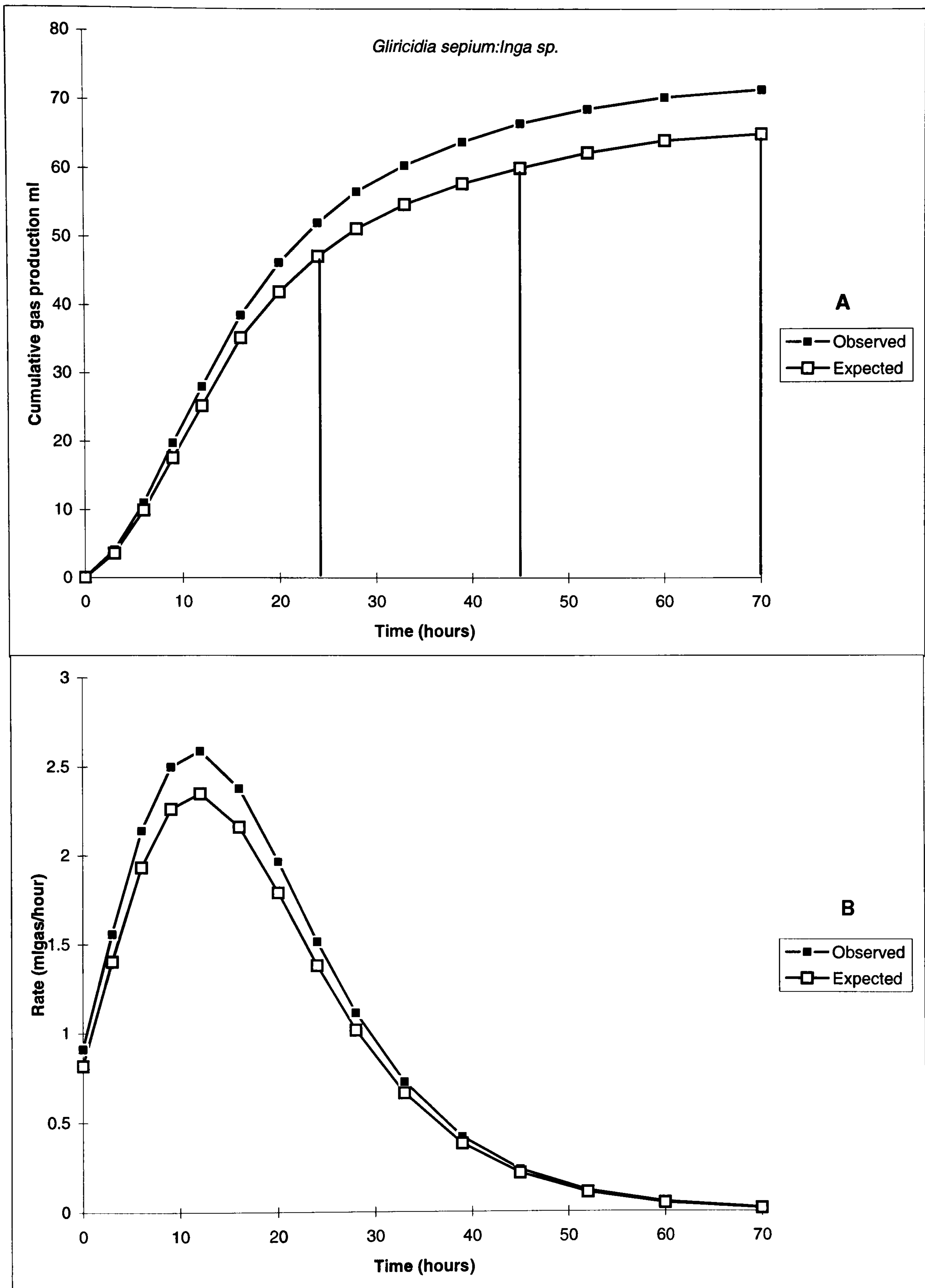


Figure 8.9: *Associative effects on the fermentation of a mixture of Gliricidia sepium and Inga sp. at 60mgN/l form urea. Associative effects were tested at 12, 24, 45 and 70 hours. Vertical lines show the times at which significant differences were found. Figures a and b show curve and rate profiles respectively.*

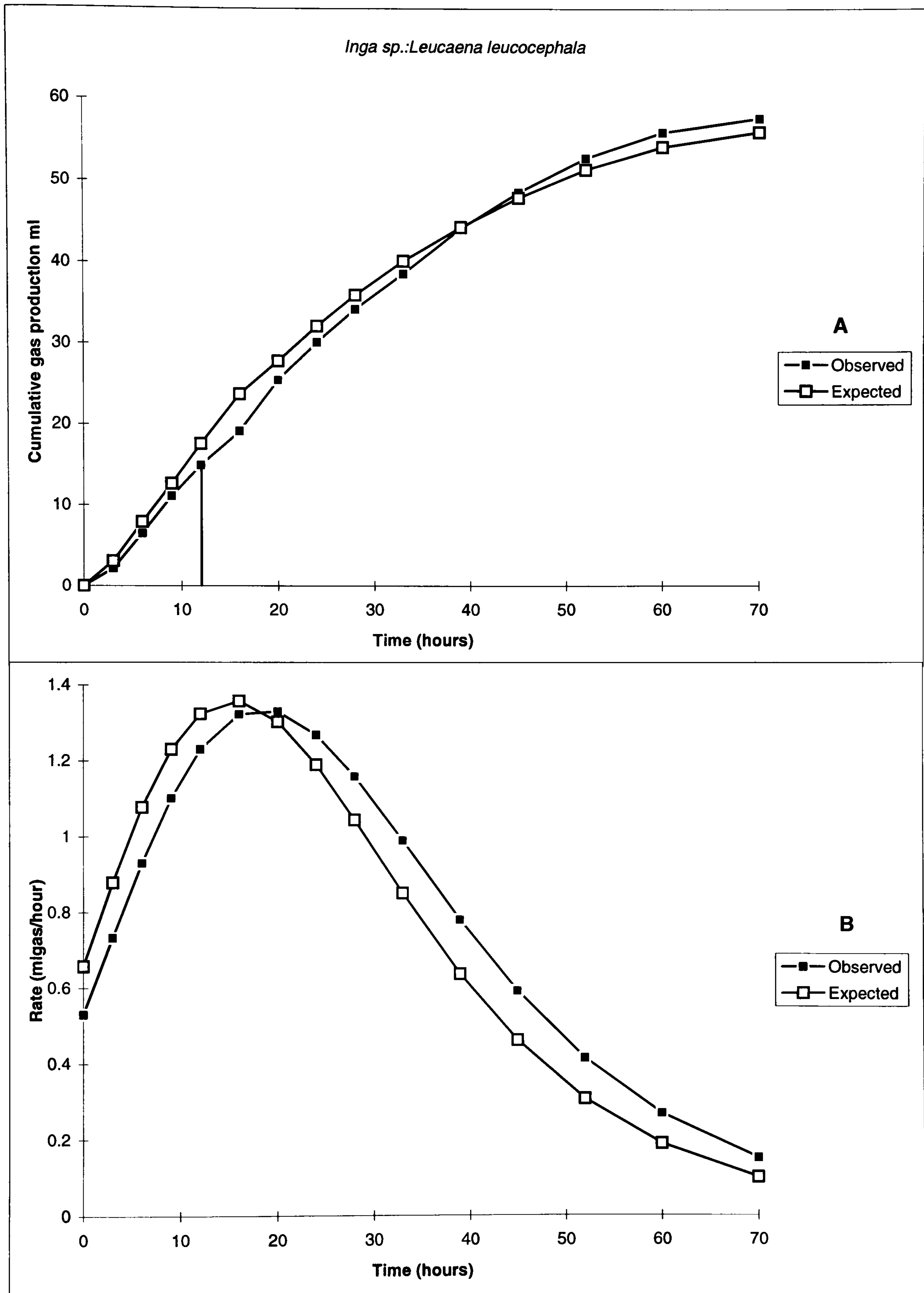


Figure 8.10: *Associative effects on the fermentation of a mixture of Inga sp. and Leucaena leucocephala at 60mgN/l form urea. Associative effects were tested at 12, 24, 45 and 70 hours. Vertical lines show the times at which significant differences were found. Figures a and b show curve and rate profiles respectively.*

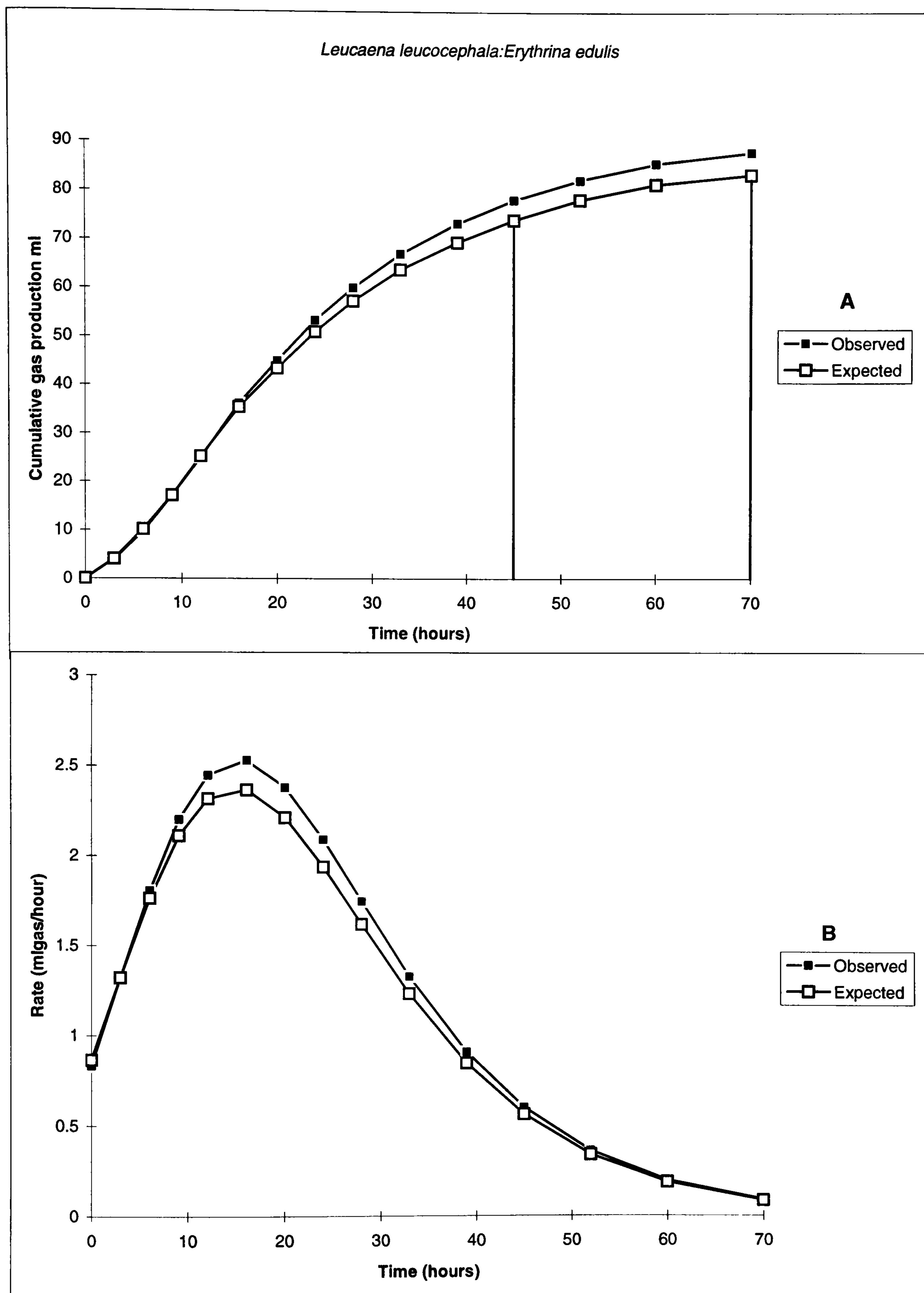


Figure 8.11: *Associative effects on the fermentation of a mixture of Leucaena leucocephala and Erythrina edulis at 60mgN/l form urea. Associative effects were tested at 12, 24, 45 and 70 hours. Vertical lines show the times at which significant differences were found. Figures a and b show curve and rate profiles respectively.*

In general, results proved that there are associative effects of mixing fodder tree leaves in this *in vitro* system. There are some interesting mixtures to carry on the research both *in vitro* and *in vivo*. Among all the mixtures exhibiting negative associative effects, although they may not be significant in terms of gas production, there may be cases of protein bypassing the fermentation process. Other interesting mixtures are *Gliricidia sepium:Inga* sp. as it showed an uniform effect under the two nitrogen conditions; and mixtures with *Trichanthera gigantea* as this plant material tended to show more associative effects than the others.

The mode of action of the associative effects of tree leaves is a synchronisation of the release of nutrients. As was discussed in Chapter 5, appropriate mixtures of carbohydrates and proteins of the same availability/fermentability produced a synchrony in the fermentation rates that explained the associative effects. The same principle was found when mixtures of fodder tree leaves and carbohydrates were fermented. It has been found that the synchronisation of energy and protein supply to the rumen may improve microbial growth efficiency and animal performance (Johnson, 1976; Sniffen *et al.*, 1983; Henning *et al.*, 1991; Sinclair *et al.*, 1993). The importance of this synchrony to the associative effects of mixtures of forages has been proposed. Glenn (1989) suggested that the mode of action of the associative effects in mixtures of lucerne (*Medicago sativa*) and orchardgrass (*Dactylis glomerata*) was a synergism in rumen fermentation of NDF and N from the two species. Findings in this thesis suggest that, although the mode of action is synergism in the fermentation, the components involved are more than those suggested by Glenn. The main chemical components involved in the associative effects are proteins (at least two fractions in each component of the mixture), sugars, starch and cellulose. Phenolic compounds play a role by affecting the synchrony. The difficulty of predicting the associative effect from the chemical composition derives from the fact that the effect is produced by the interplay of all components. The individual fermentability of the chemical components (which varies between species) determines when the synchrony occurs and this determines the type of associative effect produced. Gill and Powell (1993) stressed the importance of characterising the feeds, not only in terms of chemical composition, but also in the availability of nutrients. According to this study, the characterisation of the fermentability of individual nutrients may be more important to predict associative effects than the chemical composition. In the case of associative effects with a nitrogen-free condition for example, these effects can be explained by reference to the chemical composition. Mixtures of leaves of plant material high in rapidly fermentable carbohydrates with material high in soluble and fermentable nitrogen showed the highest effect of all. The mixtures of plant material with the highest phenolic contents and with the highest soluble and insoluble protein showed important associative effects. However, in the case of the high nitrogen conditions, when urea is increasing the fermentation rate, associative effects are more difficult to

predict from the chemical composition as the synchrony depends on the fermentability of the individual components. This highlights the importance of characterising the fermentability of the chemical components from the leaves. The fermentability of the components determined four types of responses from the associative effects discussed previously. This is summarised in Table 8.5.

Table 8.5: *Characteristics of the associative effects in vitro of mixed substrates.*

Fermentability of the components	Time at which synchronisation occur in the incubation period	Effect on fermentation kinetics compared to expected effect
High	Very early	Shorter Lag -Faster Rate
High	Early	Faster Rate- Greater Pool Size
Medium	Middle	Longer Lag- Greater Pool Size
Low	Late	Longer Lag - Slower Rate - Greater Pool Size.

The table shows how the appropriate mixtures determine different associative effects, the time at which the synchrony occur and how the fermentation parameters are affected. Brown and Pitman (1991) found that the mode of action of the associative effects *in vitro* of grass:legume mixtures was a reduced lag phase in the initiation of fibre digestion. According to Table 8.5 it is a combination of effects on the curve parameters which determines the associative effects of the mixtures. Judicious mixtures of fodder tree leaves can be achieved at any time by a synchronisation of the fermentation rates. This can have important consequences for animal production in the tropics. The discussion of how the associative effects found in this work relate to an *in vivo* situation will be addressed in the general discussion.

8.4 CONCLUSIONS

This chapter has shown that there are associative effects of mixing leaves from fodder trees under *in vitro* conditions. Associative effects vary with time and with the level of nitrogen. The mechanism that governs these associative effects is a synchrony in the fermentability/release of nutrients from the leaves. The synchrony can occur at different stages and by means of changes in the fermentation kinetics of the mixture.

GENERAL DISCUSSION

This study has increased our understanding of the mechanisms that determine the associative effects on the *in vitro* fermentation of mixtures of fodder tree leaves. Associative effects are governed by a synchronisation of the fermentation rates of the components of the mixture. These, in turn, are dependent on the fermentability of their chemical constituents. The fermentation of tree leaves was studied with two contrasting media, with zero and high nitrogen contents. As a result, the chemical constituents of fodder tree leaves that had the most effect on the fermentation, and the time at which their importance was greatest, were identified. It also showed that the utilisation of a protein by rumen microbes is a function of its fermentability and not of its solubility, but this is also influenced by the type of associated carbohydrate.

An initial understanding of the associative effects was achieved by studying mixtures of pure chemical entities. Associative effects were found when the mixture had components of similar fermentability. This was observed for both slowly and rapidly fermentable combinations of chemical entities. Associative effects also depended on synchrony in the fermentability of these substrates. Two types of response curves (exponential and sigmoidal) were identified.

Results with mixtures of fodder tree leaves and carbohydrates suggested the presence of different fractions of protein of different fermentability in the tree leaves. The late availability of nitrogen was due to the nature of the protein itself or to the presence of phenolic compounds. Significant associative effects between fodder tree leaves and carbohydrates were shown to occur. Two types of responses were identified: the first was exponential and characteristic of mixtures of high fermentability; the second was sigmoidal and was characteristic of mixtures of low fermentability. These two responses in associative effects were similar to those obtained with mixtures of pure carbohydrates and proteins where the synchronisation in the release of nutrients occurred in the early and late stages respectively.

Associative effects tended to be greater in mixtures with carbohydrates of low to medium fermentability, especially towards the end of the incubation period. The mode of action was a decrease in the fermentation rate and an increase in the lag phase. The associative effects with highly fermentable carbohydrates were higher at the beginning and decreased towards the end of the fermentation. The mode of action was an increase in the rate constant and a reduction in the lag phase.

Phenolic compounds in the leaves were shown to affect the fermentability of both carbohydrates and proteins. The effect was greater with carbohydrates of medium to low fermentability. On the other hand, they were shown to react with both soluble and insoluble protein (shown by the quebracho model). The effect of condensed tannins from the leaves was a depression in the fermentability of their mixtures with carbohydrates. This depression was greater in mixtures of low fermentability.

Forages with phenolic compounds showed positive and negative effects. These effects were possibly due to synchrony or asynchrony in the release of protein.

Associative effects of mixtures of tree leaves themselves were shown to occur. By studying the fermentation rates, it was possible to characterise these effects in terms of the time at which the synchrony occurred and in terms of the fermentability of the components. The synchrony occurred at different times during the incubation period and was characterised by changes in the fermentation kinetics of the mixture. The response was also identified as sigmoidal or exponential in shape, the latter being more common. Associative effects were shown to vary with time and with the level of nitrogen. They were shown to be governed by a synchrony of the fermentation rates of the single components of the mixture.

Various authors (Johnson, 1976., Sniffen *et al.*, 1983., Henning *et al.*, 1991., Sinclair *et al.*, 1993) have proposed that microbial growth efficiency and hence animal performance may be improved by a synchronisation of energy and nitrogen supply to the rumen. The importance of this study is in understanding the associative effects of mixtures of tree leaves *in vitro* as the basis for future prediction, but the questions that remain are how these effects can be related to an *in vivo* situation and what consequences they can have for animal production in the tropics.

9.1 ASSOCIATIVE EFFECTS *IN VIVO*

Gas production is a closed *in vitro* system in which the fermentability of the feedstuff depends on the quantity, quality and availability of nutrients and those supplied by the medium. There are several other factors that can affect the associative effects *in vivo*, such as absorption and recycling of nutrients, pH, rumen retention time (rate of passage), intake and feeding sequencing among others. The role of some of these factors is discussed below.

One of the main assumptions in the gas production system is that the components of the mixture are subject to the same rate of passage. Particle transit is an important factor affecting the associative effects (Sauvant and Giger, 1989; Gill and Powell, 1993). In the rumen, the synchrony that governs an associative effect of a particular mixture is affected by the rate of passage, i.e., a particular associative effect found *in vitro* may not show, or it may change, in an *in vivo* situation because the synchrony did not occur due to differences in the rates of passage. The rate of passage of single components of the mixture may also vary within a day (Gill, 1990). Sinclair *et al.* (1993) have formulated synchronous and asynchronous diets with respect to the hourly supply of energy and nitrogen to the microbes and studied their effects on rumen fermentation and microbial protein synthesis. The results showed that synchronizing the rate of supply of nitrogen and energy can improve protein flow to the duodenum and the efficiency of microbial protein synthesis. The authors developed a "synchrony index" based on the fermentation characteristics of feeds that is calculated from the hourly release of nutrients to the rumen microbes. For example, a rapidly released unit of nitrogen could be used with a slowly released unit of carbohydrates that had been eaten by the animal some hours previously. This index has been developed using feedstuffs rich in energy and protein, like winter barley and fish meal, and the diets are formulated to take account of total DM intake, times of feeding and outflow rate of solids from the rumen (fractional rate of passage calculated from the model proposed by Ørskov and MacDonald (1979)). In the case of tree leaves,which are chemically more complex feedstuffs, the synchronisation of rates may be more critical than that of more homogeneous feeds. Nsahlai *et al.* (1995) calculated the synchronisation indices of the release of nutrients of twenty fodder trees accessions. They found that, from the point of view of the synchronisation of the release of soluble and insoluble nutrients (N and OM), there was generally a moderate to poor synchronisation of the fermentation of N and OM because nitrogen was released in excess. This is a disadvantage if the plant species is fed as a sole food but these indices may be useful to design or predict appropriate mixtures of tree fodders.

Mixtures can be offered to the animal either at the same time (if the components are synchronised in terms of their release of nutrients and rate of passage) or at a different times (feed sequencing). This highlights the importance of feeding behaviour. Garnsworthy *et al.* (1995) suggested that synchrony is also affected by the level of feeding, the number of meals and the timing of meals throughout the day. In the presence of tannins and other astringent factors, it is important to account for the palatability of the components of the mixture which may alter the pattern in which feeds are consumed. When all components of a mixture are offered at each meal, the effect of offering the ration already mixed in the right proportions or offering the components individually should be investigated. Owen (1994) showed that, given the opportunity, small ruminants are able to select for the more

nutritious components of the diet. Therefore, the influence of animal selection and level of offer need to be studied.

Rumen ammonia concentration is another factor that may affect, or may be affected by, the associative effects in the *in vivo* digestibility of mixtures of fodder tree leaves. In this study, it was shown that, in the presence of urea, the associative effects changed both in occurrence and magnitude.

The impact of the associative effect of mixed feeds on the balance of the fermentation end products is an important issue that should also be addressed (Oldham and Emmans, 1990). The molar proportions of rumen VFA produced by an associative effect seems to depend on the type of feedstuffs used (Mould *et al*, 1983a). According to Sinclair *et al.* (1993), molar proportions of VFA in the rumen fluctuated less in animals receiving synchronised diets. These effects should also be investigated for mixtures of tree leaves.

Digestive interactions are affected by rumen pH. Negative associative effects are observed when ruminants are fed high-concentrate diets, where low rumen pH caused by the readily fermented carbohydrates in the concentrate decreases cellulolytic activity (Huhtanen, 1991, Cassida *et al.*, 1994). According to the work reported by Mould *et al.* (1983b) on the influence of pH on associative effects, the depression in digestibility could be avoided if the rumen pH can be maintained above 6.0 - 6.1. However, tree foliage is not likely to depress rumen pH even if fed as the sole food.

Tree leaves are also offered as a supplement to a basal diet, which is rich in carbohydrates. The effects of mixtures of tree foliage on, and associative effects with, the basal diet should also be investigated.

9.2 IMPLICATIONS OF ASSOCIATIVE EFFECTS OF MIXTURES OF FODDER TREE LEAVES ON ANIMAL PRODUCTION

Associative effects of mixtures of tree leaves can have an important impact on animal production. The magnitude of the associative effects found in this *in vitro* study varied from 4.4 to 18.1%. It is difficult to predict accurately what consequences an associative effect of the highest magnitude would have on animal production. The increment on gas production could be related to the *in sacco* degradability (see 4.2) but, even so, animal productivity cannot be predicted by an increment in the degradability of the feed. Prasad *et al.* (1994) studied associative effects in this gas production system and found that they agreed well with *in vivo* digestibility measurements on the same feeds (Prasad *et al.*, 1991). This suggests that *in vitro* fermentation can be used to predict associative effects *in vivo*. If effects of

the same magnitude found in this study are reflected in an *in vivo* situation, the consequences of the associative effects would be important. For example, if the highest associative effect of 18% is found *in vivo,* it means that the animals are receiving almost one-fifth more potentially fermentable material with the mixture than they receive when fed with the single components. This may represent a major benefit for small farmers, especially if they have the forages available. This may also represent important financial earnings for large scale producers.

Another consideration is whether positive or negative associative effects in the rumen should be actively sought. In the case of fodder tree leaves, a negative associative effect could be related to a dietary protein being protected from the fermentation by tannins and thus providing bypass protein. In this case, a negative effect on digestibility can be a positive effect in terms of animal performance. Results in this thesis showed negative associative effects when tannin containing plants were mixed with plant material without tannins but high in soluble protein. Although important in magnitude, these effects were not statistically significant at the levels used. Other levels should be tested. The only case of a significant negative associative effect was found for a mixture of plant materials that there were both high in tannins. This indicates an antagonism of the two components in terms of fermentability but the effect on protein was not evaluated. This needs further study.

9.3 IMPLICATIONS OF THE MIXTURES OF FODDER TREE LEAVES IN FEEDING SYSTEMS FOR THE TROPICS

The use of current rationing systems in the tropics has been questioned from the economic (Jackson, 1980) and nutritional (Preston and Leng, 1987) points of view. According to Gill and Powell (1993), the objective of defining a ration is often to maximise the efficiency with which the most readily available feeds are utilised. Several authors have highlighted the possibility of revising the current rationing systems to account for the associative effect of mixed diets (Oldham and Emmans, 1990, Huhtanen, 1991, Moss *et al.*, 1992, Gill and Powell, 1993). However, this may prove difficult because the extent of the associative effect under different feeding regimes may be variable and unpredictable given the current information (Huhtanen, 1991). The *in vitro* gas production can be used to predict the type of associative effects *in vivo*. Establishing the rate of passage and synchronisation indices of the single components of the mixture may help to improve the prediction. Information on intake of the mixtures is also needed to predict animal performance. Feeding trials should involve the use of separate components and the mixture at differents ratios.

The study of mixtures is particularly important with tropical feeds. The results of this thesis suggest that rationing systems based on additive chemical composition may not be appropriate for mixtures of tropical fodder tree leaves. Given the diversity of fodder trees and their complex chemistry, there is a considerable potential to develop feeding systems based on strategic mixtures that result in added nutritive value.

As was proposed previously, this can be achieved by capitalising on the interactive processes, such as:

- protecting dietary protein with natural tannins in order to increase the amount of nitrogen which by-passes the rumen;
- diluting the effects of deleterious compounds;
- inducing associative effects that result in an increased voluntary intake; and
- inducing associative effects on digestibility between the components of the diet.

Research in these areas is needed as this may lay the foundations for a more comprehensive feeding systems for the tropics.

9.4 SUGGESTIONS FOR FUTURE RESEARCH

The research reported in this study has taken us some way towards understanding the basis for interactions of mixtures of fodder tree leaves and the use of the gas production technique as a research tool. Additional research should be directed towards increasing our ability to predict more accurately the conditions in which associative effects of mixtures of tree leaves occur *in vivo*. The following is a concise but not comprehensive list of the research topics required:

Research on the effects of mixtures of fodder tree leaves *in vitro*.

- The use of the *in vitro* gas production method to establish potential mixtures and ratios and to test a wider range of plant species.
- The effect of fermenting fresh and dry plant material on the associative effects *in vitro*.
- The effect of mixtures of fodder tree leaves on bypass protein (residual protein) and VFA proportions *in vitro*.

- The effect of the use of rumen liquid from animals that have been exposed previously to tannins.
- The effect of fast or slow synchronised mixtures of tree leaves on the fermentation, especially in relation to tannin-protein interactions and bypass protein.
- The effect on gas production of the interaction of mixtures of tree leaves on the basal diet.

Research on the effects of mixtures of fodder tree leaves *in vivo*.

- The relationship between *in vitro* gas production and *in vivo* digestibility in terms of the associative effects.
- Studies of associative effects of mixtures of leaves by feeding increasing proportions of the components including the ratios 0:100 and 100:0.
- The effect of mixtures of tree leaves on the intake of the mixture and its components (feeding behaviour).

Development of synchronisation indices for mixtures of fodder tree leaves.

- Determination of the rate of passage of fodder tree leaves in order to use this information to design synchronous diets.
- Development of a synchrony index for the gas production method in order to enable it to be used in place of the *in sacco* degradability.
- The effect of feeding synchronous and asynchronous mixtures of fodder tree leaves on animal production.
- The effect of fast or slow synchronised mixtures of tree leaves on rumen metabolism and especially in relation to tannin-protein interactions and bypass protein.

The practical applications of these findings requires consideration in the context of sustainable agriculture. Animal production in the tropics is facing new challenges especially the balance between food security and conservation goals. Due to the diversity of fodder trees, there is a potential to develop feeding systems based on mixtures which make better use of the available resources. This will also contribute to improved efficiency in the management and use of natural resources, and take advantage of natural plant diversity in the tropics.

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APPENDICES

1. THE DETERMINATION OF MOISTURE AND DRY MATTER

Principle

Moisture is removed from the samples by heating at 103°C in a forced-draught oven for 4 hours.

Equipment

Glass or metal dishes with lids (air tight).
Forced-draught oven capable of maintaining 103°C ± 2°C.

Procedure

A clean dish was heated (with lid slightly ajar or to one side) for 1 hour in an oven, transferred to a desiccator to cool down and then weighed [A]. Approximately 5 g of sample were weighed accurately into the dish [B] and transferred into the oven for 4 hours with lid slightly ajar. After that period of time, the dish was transferred with the lid on to a desiccator and allowed to cool to room temperature. The weight of the dish plus dry sample [C] was recorded.

Calculations

Moisture (%) "as received" = $\frac{B-(C-A) \times 100}{B}$

Dry matter (%) = $\frac{(C-A) \times 100}{B}$ or 100 - % moisture.

Where:
A = Lid + dish weight
B = Sample weight
C = Dried sample + dish + lid.

2. THE DETERMINATION OF CRUDE PROTEIN BY THE MACRO KJELDAHL METHOD

Principle

The sample is digested in sulphuric acid to break down organic matter and reduce nitrogenous compounds. Ammonia is liberated by boiling with sodium hydroxide, steam distilled into boric acid and determined titrimetrically. Before the digestion, tablets containing potassium sulphate, copper sulphate and titanium oxide are added. The K_2SO_4 + $CuSO_4$ raise and control the temperature of the reaction, while the titanium oxide acts as a catalyst. As the digestion proceeds, some of the sulphuric acid is reduced to sulphur dioxide which in turn reduces the nitrogenous material to ammonia. The ammonia combines with sulphuric acid to form ammonium sulphate. The diluted digest is reacted with sodium hydroxide. The ammonia released is steam distilled, condensed and absorbed into a solution of boric acid to form ammonium borate. The ammonia is then titrated with standardized sulphuric acid.

Reagents

Catalyst tablets	"Kjeltabs"; each tablet containing 5g of potassium sulphate (K_2SO_4), 0.15g copper sulphate ($CuSO_4$) and 0.15g of titanium oxide (TiO_2). 1 tablet per determination.
Boric acid	2% aqueous solution of H_2BO_3, A.R.
Sulphuric acid	H_2SO_4, concentrated (98% w/w, d=1.84).
Sulphuric acid	0.1N (0.05M).
Indicator solution	Screened methyl red add 20 cm^3 of 0.05% w/v methyl red solution in 80% methanol to 4 cm^3 of 0.2% w/v methylene blue solution in water (the mixed indicator solution is stable for about 1 month).
Sodium hydroxide	40% w/v aqueous solution (400 g/litre).

Equipment

Kjeldahl 250 ml digestion tubes and rack.
250 ml conical flasks.
Filter paper - Whatmam 541 or equivalent, 9 cm.
Kjeldahl digestion block.
Kjeldahl distillation unit.

Digestion procedure

One gram of the sample was weighed onto a piece of 9 cm Whatman No 541 filter paper and folded into an "envelope". This was transferred to a labelled tube. In the blank tubes, a single piece of filter paper was placed. One Kjeltab tablet (potassium sulphate, copper sulphate and titanium oxide) and 12 cm^3 of concentrated sulphuric acid (using automatic dispenser) were carefully added to all tubes (including blanks). The tubes were transferred to the digestion blocks, the exhaust caps attached, and vacuum pump turned on. The digester was set up to start from cold and to reach 410°C in one hour. After that, the sample remained for one more hour at that temperature. After this period of time, the tubes were transferred to side trays and allowed to cool for approximately 25 minutes. Then, 75 cm^3 of distilled water were added to each tube.

Distillation procedure

The cooled digestion tubes were attached one at the time to the automated distillation unit. When the distillation started, $50cm^3$ of 40% NaOH were automatically pumped into the digestion tube. A flask $50cm^3$ of 2% boric acid and 4-5 drops of screened methyl red indicator was placed on the Kjeldahl distillation unit to trap the ammonia.

Titration

After distillation, the resultant green solution was titrated to a purple end point using 0.1N (0.05M) H_2SO_4. The sample and blank titres were recorded.

Calculations

$$\%\text{Total nitrogen} = \frac{(\text{titre-blank}) \times \text{normality of } H_2SO_4 \times 0.0014^a \times 100^b}{\text{sample wt.(g)} \times 0.1^c}$$

Where:

a = g of N equivalent to 1.0 cm^3 of 0.1N sulphuric acid.
b = converting to %.
c = Normality of acid is 0.1N, if not, division by 0.1 corrects any variation.

$$\%\text{ N (as received)} = \frac{(\text{titre-blank}) \times 0.1 \times 0.14}{\text{weight(g)} \times 0.1}$$

% Crude protein = % N x 6.25

There is an average of 16% in plants protein, therefore 100/16 = 6.25 gives factor to convert nitrogen to "crude" or generalized protein.

3. THE DETERMINATION OF ETHER EXTRACT

Principle

This method is based on the extraction of crude oils and fats from a prepared sample using petroleum ether with a boiling point in the range from 40 to 60°C.

Reagents

Hydrochloric acid (HCl) 3 N.
Petroleum ether, boiling point 40-60°C.
Blue litmus paper.

Equipment

6 place extraction mantle with condensers.
12 hole water bath with condensers.
250 ml round flat bottomed QF flasks.
Bolton extractors with glass spirals.
Whatman 541 15 cm filter paper.
Whatman extraction thimbles 30 x 80 mm.
Cotton wool.
Drying oven at 103°C.

Procedure

The 250 ml flasks were dried in oven at 103°C for one hour. After cooling, a few anti-bumping granules were added and the flasks were weighed [A].

Five grams of the sample were carefully weighed [B] into individual thimbles and plugged gently with cotton wool. The thimbles were placed in the extractors. 80 ml of petroleum ether were placed in the flasks and assembled with the extractors on a preheated water bath. The samples were extracted until the solvent evaporated from flask and thimble (approximately 5 to 6 hours).

The flasks were dried in oven at 103°C for 1 hour, cooled and weighed [C].

Calculations

Ether Extract % in sample on "as received" basis =

$$\frac{[C] - [A]}{[B]} \times 100$$

4. THE DETERMINATION OF ASH AND ORGANIC MATTER

Principle

The organic matter in a sample is destroyed in a muffle furnace at 500°C.

Equipment

Evaporating basins 45 cm^3, translucent vitreosil, shallow form, round bottomed, with spouts.
Watch glasses, 80 mm diameter.

Procedure

One clean basin and watch glass per sample were heated for 1 hour in a muffle furnace at 500°C, cooled in a desiccator and weighed [A]. (Basins were conveniently marked using a heat-resistant pen). Then, approximately 5 g of the sample were weighed accurately into the dishes [B] and covered with watch glasses. The dishes with watch glasses were placed into the cooled furnace and the temperature was slowly brought up to 500°C and the samples were left to incinerate overnight.

Next day the dishes were remove from the furnace and placed on an asbestos board to cool slightly, then transferred to a desiccator, allowed to cool to room temperature and weighed [C].

Calculations

Ash % on "as received basis"

$$= \frac{\text{wt. of ash (g)} \times 100}{\text{sample wt. (g)}} \qquad \frac{[C]-[A] \times 100}{[B]}$$

On dry basis

$$= \frac{\text{\% ash (as received)} \times 100}{\text{\% dry matter}}$$

The organic matter was calculated by subtracting the value for ash from 100.

Organic matter % = 100 - % ash

5. THE DETERMINATION OF WATER SOLUBLE PROTEIN

Principle

Soluble proteins are extracted with water. The nitrogen content is measured in the liquid fraction obtained after centrifugation by the Kjeldahl analysis (AOAC, 1984) as described in Appendix 3.2.

Reagents

Same reagents as the determination of crude protein (Appendix 3.2).

Equipment

Macro Kjeldahl equipment for the determination of crude protein
Water bath
Centrifuge
Conical Flasks (150 ml) with rubber tops.

Procedure

The sample was accurately weighed out to approximately 0.5 g and transferred into an conical flask. Distilled water (100 ml) was added to the conical flask, which was swirled, and placed in a water bath at 39°C for 1 hour. During that time, the conical flask was not shaken but it was swirled gently every 15 minutes. After an hour, the liquid was transferred into a 150 ml centrifuge tube and centrifuged at 2000 RPM for 10 minutes. The nitrogen content was measured by the Kjeldahl analysis in the liquid fraction obtained after centrifugation. 50 ml of supernatant were transferred to a 250 ml digestion tube and one Kjeltab CT and 12 cm^3 of concentrated sulphuric acid were carefully added to all tubes including the blanks (just water). The tubes were transferred to the digestion blocks. Due to the risk of outburst when digesting liquid samples on the digester at high temperatures, the digestion procedure was altered from that from solid samples. The digester was set up to start from cold to reach 150°C in one hour and to reach 250°C in the next hour. After this, the temperature was raised up to 410°C and the samples were left there for 1 hour and 45 minutes. Once digested, the tubes were transferred to side trays and allowed to cool for approximately 25 minutes. Then, 75 cm^3 of distilled water were added to all tubes. The distillation and titration procedures were exactly the same as those described for the determination of crude protein by macro Kjeldahl (Appendix 3.2).

Calculations

$$\text{\%Total Soluble Nitrogen (as received)} = \frac{\text{(titre-blank)} \times \text{normality of } H_2SO_4 \times 0.0014^{a} \times 100^{b}}{\text{sample wt.(g)} \times 0.1^{c}} \times 2^{d}$$

Where:
a = g of N equivalent to 1.0 cm^3 of 0.1N sulphuric acid.
b = converting to %.
c = Normality of acid is 0.1N, if not, division by 0.1 corrects any variation.
d= Dilution factor: 100 ml (initial volume)/ 50 ml (volume in which the nitrogen was determined).
Then;

$$\text{\% Soluble N (DM Basis)} = \frac{\text{(titre-blank)} \times 0.1 \times 0.14}{\text{weight(g)} \times 0.1} \times 2 \times \frac{100}{\text{\%DM}}$$

This value was corrected by the 6.25 factor as for crude protein (see Appendix 3.2).
% Soluble protein = % Soluble N x 6.25

6. THE DETERMINATION OF SOLUBLE CARBOHYDRATES

Principle

Soluble carbohydrates are extracted in water. The concentration of carbohydrates, expressed as glucose, is determined spectrophotometrically as the blue-green complex which is formed when carbohydrates are heated with anthrone in sulphuric acid.

Reagents

Concentrated sulphuric acid	AR grade (approximately 98% m/m H_2SO_4).
Thiourea	AR grade.
Anthrone	AR grade.
Anthrone reagent	760 ml of concentrated sulphuric acid were added slowly and with constant stirring to 330 ml of distilled water and cooled rapidly. When cold, 1g of thiourea and 1g anthrone were added and dissolved. The solution was stored in a tightly stoppered bottle in a refrigerator and used within 3 days of preparation (See note 1).
D-glucose	AR grade.
Glucose standard solution	A solution of 0.8mg/ml of glucose was prepared (immediately before use) by dissolving 0.400g of D-glucose, previously dried for 1 hour at 120°C, in distilled water and diluted to 500ml.
Glucose working standard solutions	From 0 to 0.2mg/ml. These solutions were prepared from the glucose standard solution (0.8mg/ml) by diluting it to obtain the following concentrations 0, 0.04, 0.08, 0.12, 0.16 and 0.20mg/ml as follows: Dilution 5 - 100 = 0.04mg/ml 10 - 100 = 0.08mg/ml 15 - 100 = 0.12mg/ml 20 - 100 = 0.16mg/ml 25 - 100 = 0.20mg/ml

Equipment

Bottles, 250ml.
Shaking machine.
Test tubes - 150 x 25 mm with stoppers.
Boiling water bath.
Spectrophotometer set at 620nm.

Procedure

Extraction

Approximately 0.2g of dried ground sample were weighed accurately, and transferred into a bottle. 200 ml of distilled water were added. The bottle was capped and shaken for 1 hour. The extract was

filtered through a 125 mm Whatman No. 1 filter paper, rejecting the first few ml of filtrate. Approximately 50 ml of the clear filtrate were retained for the determination of soluble carbohydrates. The determination started within one hour of filtration.

Preparation of standard graph

Two ml of each glucose working standard solutions were transferred into a test tube and the tubes were left standing in a beaker of ice and water for 10 minutes. Each tube was held in the beaker of ice and water and 10ml of anthrone reagent were added slowly from a burette, so that it ran down the side of the tube and formed a layer under the solution. The contents were mixed by gently swirling the tube (keeping it in the ice and water so that at no time did the temperature rise above 25°C (See note 2)). The tubes were loosely stoppered and placed in a boiling water bath for exactly 20 minutes, maintaining the boiling throughout the whole period. The stoppers were removed and the tubes were placed in ice and water to reduce the temperature to ambient as rapidly as possible. The absorbance was measured in a 10mm optical cell at 620nm within 30 minutes. A graph relating absorbance to mg of glucose present was constructed. The absorbances corresponding to 0 and 0.20mg of glucose were approximately 0 and 1.0 respectively.

Examination of extract

Two ml of extract were transferred into a test tube and the tube was left standing in a beaker of ice and water for 10 minutes. Anthrone reagent (10 ml) was added slowly from a burette by letting it run down the side of tube as for the working standard solutions. The contents of the tubes were mixed gently under ice and water to avoid a rise on the temperature above 25°C. The tubes were loosely stoppered and placed in a boiling water bath for exactly 20 minutes, maintaining the boiling throughout the whole period. After exactly 20 minutes the stoppers were removed and the tubes were placed in ice and water to reduce the temperature to ambient as rapidly as possible. The absorbance was read in a 10mm optical cell at 620nm within 30 minutes. A blank determination was also carried out.

Calculations

From the standard graph, the mg of glucose equivalent to the absorbances of the sample were calculated. Water soluble carbohydrates in the sample were calculated by the means of the following formula.

$$\text{\% Water soluble Carbohydrates (as received)} = \frac{\text{mg/ml of glucose} \times 100 \times 100}{\frac{\text{sample wt. (mg)}}{1000}}$$

Notes

1. Despite the presence of anti-oxidant, thiourea, anthrone is slowly, but persistently oxidised. The reagent must be also discarded if it is suspected that atmospheric moisture has been absorbed by the sulphuric acid. The reagent was freshly prepared.

2. Anthrone reacts with many carbohydrates, but the absorbance per unit mass varies with the conditions of the reaction. In particular, the temperature and time of heating are critical. Reactions begin above 25°C and this temperature must not be exceeded at any time during the addition of the anthrone or during mixing of the anthrone and the standard or extract. All the samples were extracted and analysed in one batch.

7. THE DETERMINATION OF STARCH, REDUCING AND NON-REDUCING SUGARS (TOTAL SUGARS)

Principle

Under alkaline conditions reducing sugars reduce ferric cyanide to ferrocyanide, which reacts with ferric ions to form Prussian blue. This reaction can be utilised in a colourimetric assay. This assay is designed for use with sample solutions containing between 0 and 25 mg of sugars (as reducing sugars). To detect high levels of sugar a sample dilution step may be employed. Starch can be determined after acid hydrolysis to reducing sugars.

Reagents

Anhydrous sodium carbonate.
Ethanol.
Glucose.
Hydrochloric acid.
Potassium ferricyanide (potassium hexacyanoferrate(III)).
Sodium hydroxide.

Equipment

Watch glass.
Whatman GF/A filter paper.
Water bath
Drying oven up to 60 °C
pH meter
quartz cuvettes
Soxhlet extractor
Cellulose extraction thimbles.
Glass balls, 3 mm diameter.
UV/VIS spectrophotometer
Glassware and automatic dispensers and pipettes.

Procedure

Extraction of soluble sugars

One gram of sample was weighed into a pre-weighed cellulose extraction thimble and plugged with non-absorbent cotton wool. Flat bottom flasks were labelled with a number corresponding to the thimbles, and 200ml of 85% v/v ethanol and 3 glass balls (3mm diameter) were transferred into each flask. The thimbles were placed into Soxhlet extractors and refluxed for 1.5 to 2 hours.

Once the apparatus had cooled, the thimbles were removed and dried to constant weight at 60°C. The residue was material insoluble in ethanol, and it was stored in a desiccator for subsequent determination of starch (see below). The liquid left in the extraction equipment was a solution of ethanol soluble material. This solution was made aqueous by distilling off the ethanol. This was done by distilling off one extractor full of ethanol, which was allowed to cool and discarded. An equal volume of distilled water was added to the solution in the flask and distilled again. This process was repeated several times until water started to condense on the sides of the condenser. The final solution was made up to volume by washing it carefully into a 250 ml volumetric flask, labelled and used for the determination of starch, reducing and total sugars.

The ferricyanide method

The following reagents were prepared.

Solution A	12.5 g of potassium ferricyanide (potassium hexacyanoferrate(III)) and 10.0 g of anhydrous sodium carbonate were transferred into a 100 ml beaker, dissolved with distilled water and made up to a volume of 250 ml.
Solution B	87.5 g of anhydrous sodium carbonate were transferred into a 500 ml beaker, dissolved with distilled water and made up to a volume of 1000 ml in a volumetric flask.
Ferricyanide reagent	25 ml of Solution A and 100 ml Solution B were placed into a 1000 ml volumetric flask, and make up to volume with distilled water and transferred into an amber glass automatic dispenser.

A calibration curve for glucose was made as follows. A standard solution containing 5mg glucose/ml was prepared. A range of standard solutions, was prepared by adding 1.0, 2.5, 4.0 and 5.0 ml of the 5 mg/ml glucose solution into 50 ml volumetric flasks and made up to volume with distilled water. 8 ml of ferricyanide reagent were dispensed into 50 ml glass stoppered tubes, in duplicate and 2 ml of each standard solution were added. This produced solutions containing 200μg, 500μg, 800μg and 1000μg of glucose in the 10 ml reaction mixture. Blank controls were prepared in duplicate containing 2 ml distilled water and 8 ml ferricyanide reagent. All the tubes were mixed, boiled for 15 minutes in a boiling water bath and cooled rapidly by placing them in a bath of cold water. The absorbance of the mixture was measured at 380 nm with distilled water as the reference/zero, using a quartz cuvette. A graph of weight of glucose in the reaction mixture against absorbance was then plotted.

Determination of reducing sugars

Two ml of the aqueous extract were mixed with 8 ml of the ferricyanide reagent in a labelled, 50ml boiling tube. This was carried out in duplicate for each aqueous extract. For each sample a blank was prepared by using 2 ml of the sample's aqueous extract and 8 ml of water. The tubes were mixed, boiled for 15 minutes in a boiling water bath and cooled rapidly as before. The absorbance of the samples and sample blanks was measured at 380 nm, with distilled water as the reference/zero and using a quartz cuvette. Both the sample blank and sample were read, and the reading for the blank deducted from that for the sample.

The absorbance value obtained when read off the calibration plot gives a value for the amount of reducing sugar present in the reaction mixture, which, after calculation gives a value for the amount of sugar in the original tissue.

DETERMINATION OF TOTAL SUGARS

The total sugar content of the original sample was determined by hydrolysing an aliquot of the aqueous extract prepared and then using the ferricyanide method to determine the sugars.

To hydrolyse the aqueous extract, 25 ml of the extract were placed in a labelled, 100 ml beaker. Five ml of concentrated hydrochloric acid were added and the beaker covered with a watch glass. This was left for 24 hours at room temperature. The solution was neutralised to pH 7.0 by adding 5M NaOH. It was poured into a labelled 50 ml volumetric flask, the beaker was rinsed into the flask with distilled water, and made up to volume using distilled water.

Determination of starch

The starch content was determined in the alcohol insoluble material obtained earlier, by the ferricyanide method, using the same calibration curve. First the starch had to be hydrolysed. This was done by acid hydrolysis.

A solution of 0.7M hydrochloric acid was prepared by pipetting 150ml of 35 % hydrochloric acid into a 2000ml labelled, volumetric flask and made up to volume using distilled water. Another solution of 5M NaOH was prepared by adding 50g of sodium hydroxide pellets into a 250ml conical flask, dissolved by adding distilled water and poured into a 250ml labelled volumetric flask. This was made up to volume with distilled water.

From the thimbles, 200mg of the dry alcohol insoluble material were placed into labelled flat bottomed flasks. To each flask, 110ml of 0.7M HCl and 3 glass balls were added. They were placed over a boiling water bath and boiled for 2.5 hours, shaking the flasks periodically. The level of the liquid was maintained by adding distilled water to the flasks. After that, the flasks were allowed to cool and neutralised to pH 7.0 by adding 5M NaOH solution and using a pH meter. The hydrolysate were poured into 500ml labelled volumetric flasks rinsing and made up to volume with distilled water. An aliquot (50ml) of the hydrolysate were filtered through glass microfibre filter paper (Whatman GF/A) into a 100ml labelled conical flask. The starch in the original sample was determined as reducing sugar, using the ferricyanide method.

Calculations

Calculation of reducing sugars

$$\text{Reducing sugars (mg/g dry weight)} = \frac{(C_1 \times 125)}{D_1} \times 1000$$

Where:
C_1 = Concentration of reducing sugars in the 10 ml reaction mixture (μg/10 ml) read from the appropriate calibration graph.
125 = Conversion factor since the soluble sugars from the original sample were dissolved in 250 ml. From this 250 ml, 2 ml were taken to measure the absorbance.
1000 = Conversion factor to convert final answer from g/g to mg/g.
D_1 = Dry weight of original sample (g).

Calculation of total sugars

$$\text{Total sugars mg/g dry weight} = \frac{(C_2 \times 25 \times 10)}{D_1} \times 1000 \text{ mg/g dry weight}$$

Where:
C_2 = Concentration of reducing sugars in the 10 ml reaction mixture (μg/10 ml) read from the appropriate calibration graph.
25 x 10 = Conversion factor since the soluble sugars from the original sample were dissolved in 250 ml. From this 25 ml were taken, made up to 50 ml, and from this 50 ml, 2 ml were taken to measure the absorbance.
1000 = Conversion factor to convert final answer from g/g to mg/g.
D_1 = Dry weight of original sample (g).

Calculation of starch

As the starch values were obtained by reading off from a reducing sugar calibration curve, the values obtained were for glucose. To convert these values to starch they had to be multiplied by 0.9. This is because as the starch is hydrolysed, water is added to each sub-unit, and the ratio of weights of glucose:water is 9:1. Therefore the starch value will be nine tenths (0.9) of the corresponding value for glucose.

$$S = \frac{0.9 \times C_3 \times 250}{D_3} \text{ g/g alcohol insoluble material}$$

Where:

S = Weight of starch in the alcohol insoluble solids (g).
C_3 = Weight of sugars in the 10 ml reaction mixture (μg/10 ml) from the appropriate calibration graph.
D_3 = Weight of sample of alcohol insoluble solids used (200 mg).
0.9 = Conversion factor from glucose to starch (see above).
250 = Conversion factor since the 200 mg of alcohol insoluble material from the original sample were dissolved in 500 ml; from this 500 ml, 2 ml were taken, to measure the absorbance.

Then;

$$\text{Starch in original sample mg/g dry weight} = \frac{A \times S \times 1000}{D_1} \text{ mg/g dry weight}$$

Where:

A = Weight of alcohol insoluble solids obtained after extraction (g).
S = Weight of starch in the alcohol insoluble solids (g).
D_1 = Dry weight of original sample.
1000 = Conversion factor to convert final answer from g/g to mg/g.

8. THE DETERMINATION OF ACID DETERGENT FIBRE (ADF)

Principle

The acid detergent fibre procedure provides a rapid method for determining lignocellulose in feedstuffs. The residue also includes silica, however. The difference between the cell walls (NDF) and acid detergent fibre is an estimate of hemicellulose, although the difference does include some protein attached to cell walls. The acid detergent fibre is used as a preparatory step for lignin determination.

Reagents

Acid detergent solution	A 1M sulphuric acid solution was prepared by adding 49.04g of H_2SO_4 to approximately 500ml of distilled water and then making it up to 1 litre. The solution was completed by adding 20g of cetyl trimethylammonium bromide (CTAB) to the litre of 1M H_2SO_4, and it was stirred until dissolved.
Decalin	Technical grade (Decahydronaphthalene).
Acetone	

Equipment

Fibretec.	
Sintered glass crucibles	Porosity 2.

Procedure

Approximately 1 gram of sample was accurately weighed into a dried weighed sintered crucible and placed onto the fibretec. 100 ml of acid detergent solution and 2 ml of decalin were added. The fibretec was set to boil for 10 minutes. The heat was reduced when boiling began, to avoid foaming, and then adjusted to an even level. It was refluxed for 60 minutes. After refluxing, the solution was filtered through the crucible and rinsed with hot water (80°C). This was repeated until all fibre was transferred to the crucible. The crucible was washed with acetone in the cold filtration unit until no more colour was removed. The fibre residue and crucible were dried at 103°C overnight, cooled in a desiccator and weighed.

Calculations

Acid detergent fibre ADF % "as received" =

$$\frac{((\text{wt. of crucible + fibre}) - \text{wt. crucible}) \times 100}{\text{wt. of sample}}$$

Adjusting to dry basis =

$$\frac{\%\ \text{ADF (as received)} \times 100}{\%\ \text{dry matter}}$$

9. THE DETERMINATION OF NEUTRAL DETERGENT FIBRE (NDF) (Cell Walls)

Principle

The neutral detergent procedure for cell walls is a rapid method measuring the total fibre in fibrous plant feedstuffs. It appears to divide the dry matter of feeds very near the point which separates the nutritively available and soluble constituents from those which are incompletely available or dependent on a microbial fermentation.

Reagents

Neutral detergent solution	This solution was prepared by adding 30 g of sodium dodecyl sulphate, 18.61 g EDTA, 4.56 g of disodium hydrogen orthophosphate, 6.81 g sodium borate decahydrate (borax) and 10 ml 2-ethoxyethanol to 1 litre of distilled water and then dissolved.
Decalin	Technical grade (Decahydronaphthalene).
Acetone	
Sodium sulphite	

Equipment

Fibretec.
Sintered glass crucibles Porosity 2.

Procedure

Approximately, 1 g of the sample and 0.5 g of sodium sulphite were accurately weighed into a dried weighed sintered crucible and placed onto the Fibretec. 100 ml of neutral detergent solution and 2 ml of decalin were added.

The Fibretec was set to boil for 10 minutes. The heat was reduced when boiling began, to avoid foaming, and then adjusted to an even level. It was refluxed for 60 minutes. After refluxing, the solution was filtered through the crucible and rinsed with hot water (80°C). This was repeated until all fibre was transferred to the crucible. The crucible was washed with acetone in the cold filtration unit until no more colour was removed. The fibre residue and crucible were dried at 103°C overnight, cooled in a desiccator and weighed.

Calculations

$$\text{Cell walls \% as received} = \frac{((\text{wt. of crucible and residue}) - \text{wt. crucible}) \times 100}{\text{wt. of sample}}$$

$$\text{Adjusting to dry basis} = \frac{\text{\% NDF (as received)} \times 100}{\text{\% dry matter}}$$

10. THE DETERMINATION OF TOTAL PHENOLS (Prussian Blue Assay)

Principle

This method is based on the reduction by tannins and other polyphenols of iron (III) to iron (II), followed by the formation of a ferricyanide-iron (II) complex. The coloured product (commonly known as Prussian blue) absorbs maximally at 720 nm.

The major difference between this method and the published method is the use of iron (III) ammonium sulphate instead of iron (III) chloride as the first reagent. Solubility problems are common with iron (III) chloride, but are eliminated by using iron (III) ammonium sulphate.

Reagents

0.1M Hydrochloric acid	This solution was prepared by adding 8.3ml of concentrated HCl to 500ml of distilled water and madding it up to 1 litre.
0.1M Iron (III) ammonium sulphate in 0.1M HCl	48.2g of $FeNH_4(S0_4)_2.12H_2O$ were dissolved in 1 litre of 0.1M HCl. This made a pale yellow solution.
0.008M Potassium ferricyanide	2.63g of $K_3Fe(CN)_6$ were dissolved in 1 litre of distilled water. This made a yellow solution.

Equipment

Spectrophotometer capable of taking absorbance readings at 720 nm.
Erlenmeyer flasks or test tubes of 100 ml capacity.

Procedure

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

50ml of deionised water were dispensed into a 100ml Erlenmeyer flask and 10µl of the acetone extract were added to the water. A solvent-only blank was also included. To each extract, 3ml of $FeNH_4(SO_4)_2$ were added and then swirled. These additions were timed (20 seconds intervals). Exactly 20 minutes after the addition of $FeNH_4(SO_4)_2$, additions of 3ml of $K_3Fe(CN)_6$ were made at 20 seconds intervals. The erlenmeyers were swirled again. Exactly 20 minutes after the addition of $K_3Fe(CN)_6$, the absorbance was read at 720 nm in a 1cm cuvette. Further readings were made at 20 seconds intervals.

Calculations

$$\text{Total phenols OD/g (DM)} = \frac{(\text{Abs Sample.} - \text{Abs. blank}) \times 500}{\text{sample wt. (g)}} \times \frac{100}{\%DM}$$

Where:
Abs: Absorbance
OD= Optical density

11. THE DETERMINATION OF CONDENSED TANNINS (Acid Butanol Method)

Principle

The acid butanol assay is the best assay for selective determination of condensed tannins (proanthocyanidins). In this assay, the flavonoid subunits of the condensed tannin polymer are oxidatively cleaved to yield the anthocyanidin; the reaction does not involve hydrolysis, and colour development is decreased by water.

Reagents

2M Hydrochloric acid	87ml of concentrated HCl were added to 250ml of distilled water and made up to a volume of 500ml.
2% Iron (III) ammonium sulphate in 2M HCl	2g of $FeNH_4(SO_4)_2.12H_2O$ were dissolved in 100ml of 2M HCl.
Acid butanol solution	This solution was prepared by adding 12.5ml of concentrated HCl to 250ml butanol and made up to 500ml with butanol.
Methanol	

Equipment

Scanning spectrophotometer between 520 and 580 nm.
Water bath capable of maintaining 95°C.
8 ml screw top vials.

Procedure

The extraction of the samples was carried out as described on Appendix 3.10.

The vials were filled in by placing 1 ml of methanol into each vial, 5 µl of the acetone extract, 6 ml of the acid butanol solution and 0.2 ml of $FeNH_4(SO_4)_2$. The vials were closed, shacken and placed in a water bath at 95°C. The water level was adjusted so that it was at the bottom of the screw-on caps of the vials. A solvent-only blank was also included. After 40 minutes in the water bath, the vials were removed, mixed thoroughly and allowed to cool. The absorbance was measured at 550 nm in the scanning spectrophotometer, using the blank as reference. The maximum absorbance was recorded.

Calculations

$$\text{Acid butanol OD/g (DM)} = \frac{\text{Abs. max x 1000}}{\text{sample wt. (g)}} \times \frac{100}{\%DM}$$

Where:
Abs. max: Maximum absorbance
OD: Optical density

12. THE DETERMINATION OF PROTEIN PRECIPITATION ACTIVITY (PPA)

Principle

A tannin-containing extract is placed in a well in a protein-containing agar slab. As the tannin diffuses into the gel and complexes with protein, a visible ring of precipitation develops. The area of the ring is proportional to the amount of tannin in the extract.

Reagents

Buffer A	10.6mg of ascorbic acid were dissolved in a small amount of distilled water. 2.9ml of glacial acetic acid were added and made up to just under 1 litre with distilled water. The pH was adjusted to pH 5 using NaOH pellets. This solution was finally made up to 1 litre with distilled water.
Agarose	Type 1, Sigma.
Bovine haemoglobin	Sigma M2500.
70% aqueous acetone	350ml of acetone were made up to 500ml with distilled water.

Equipment

Petri dishes, 8.5cm diameter.
Water bath at 45-50°C.
Incubator at 30°C.

Procedure

The assay was carried out in duplicate on different plates. Samples were extracted in duplicate. Therefore each sample required 2 wells on each of 2 plates.

Preparation of agarose plates

One gram of agarose (Type 1, Sigma) was dissolved in 90ml of buffer A by suspending the agarose in a small volume of cold buffer and adding it to boiling buffer whilst stirring. This solution was transferred to a water bath at 45-50°C and allow to cool down to 38°C. 0.1g of haemoglobin were dissolved in 10ml of cold buffer A (this completed 100ml of buffer) and added to the agarose solution whilst swirling until fully dissolved. Using a plastic open ended pipette, 9.5ml of the warm agarose-haemoglobin solution were rapidly transferred into petri dishes. They were allowed to stand on a flat surface to cool, and stored in the fridge. Before use, the dishes were marked on the underside with a line to mark the origin (so that the numbering sequence of wells can be determined) and dots to mark where the well were to be located. Eight wells were cut using a 4mm cork borer and the gel cores were removed.

Plating out of extract

15µl of extract were placed in each well, using a Hamilton syringe. The extracts were applied in a clockwise sequence around the plate. Duplicate extracts were plated out in duplicate onto two different plates, thus giving four platings per sample. When fully loaded, the plates were left until the extracts had penetrated the gel. Plates were sealed with a strip of parafilm and incubated in the dark for 5 days at 30°C.

After 5 days, the diameters of the rings of precipitated protein formed were measured using a Vernier scale the nearest 0.05mm. Each ring was measured twice, parallel to the circumference of the petri dish and then radially.

Calculations

The average of the diameter (D) of the rings on each plate was calculated including the 2 replicates from each extraction (A and B).

$$\text{Average diameter (D)} = \frac{(DA1 + DA2 + DB1 + DB2)}{4}$$

The proportional area of the ring was calculated by squaring the Average diameter (D) and subtracting from it the square area of the well. (In the original paper, (Hagerman, 1987), the area of the precipitated was mesaured and compare against a standard curve. It was found that the average diameter of precipitated protein was in proportion to the amount of tannins in the extraction. It was decided to use this measurement to simplify the analysis and calculations).

$$\text{Average ring area (a)} = D^2 - 16$$

The radial diffusion was obtained by the following formulae

$$\text{Radial diffusion (RD) } cm^2/g = \frac{\text{area (a)} \times 3.33}{\text{sample wt. a (g)}}$$

Where:
3.33 = Conversion factor since the extraction from the original sample was done in 5 ml. From this, 15 μl were taken for the analysis. This conversion factor is also correcting by the conversion from mm^2 to cm^2 (5000/15x100).

These calculations were repeated for the second plate and a mean of the radial diffusion was obtained and corrected by the dry matter.

$$\text{Protein precipitation activity } cm^2/g \text{ (DM)} = \frac{100}{\%DM} \times \frac{\text{RD/g Plate 1} + \text{RD/g Plate 2}}{2}$$

13. THE DETERMINATION OF DRY MATTER DEGRADABILITY

Principle

In sacco degradability has been used for many years to provide estimates of the rate and extent of disappearance of feed constituents from the rumen. The method requires fistulated animals for the incubation of samples which are suspended in a series of nylon bags in the rumen. The pore size of the bags allows entry of microbes and keeps solid particle losses to a minimum.Bags are removed sequentially at appropriate times after their insertion and particulate residues are subjected to proximate analysis for determination of residual dry matter, nitrogen, ADF, NDF and/or lignin. This technique provides a useful means of estimating the rates of disappearance and potential degradability of feedstuffs, feed constituents and supplements (Ørskov *et al.*, 1980).

Equipment

Nylon bags (6.5x14cm) pore size (30-50μm).
Analytical balance
Forced-draught oven capable of maintaining 103°C ± 2°C.
Dessicator

Procedure

Samples were ground through a 1mm mesh and dried overnight to determine the dry matter. The bags were dried for 30 minutes at 60 - 65°C, allowing to cool to room temperature in a desiccator and their dried weight was obtained. About 3g of dried sample were placed in each nylon bag. The bags were tied tightly using a nylon string and anchored with about 50cm of nylon cord to top of the canula and placed deep in the rumen of a fistulated animal. The animals (two Lucerna cows fitted with rumen canulae) were offered a basal diet of sugar cane tops *ad libitum.*

Samples were incubated for 0, 12, 24, 48 and 72 hours. The 72-hour samples were placed into the rumen on the morning of day 1 of the incubation in each fistulated animal. On the next morning (day 2), the 48-hour samples were placed inside the rumen at the same hour as the day 1 samples. The same procedure, known as sequential addition, was follow until all samples were in the rumen. All the bags were taken out at the same time.

Inmediatelly after removal, all the bags (including the zero hour samples) were washed with cold water under running tap water while rubbing the bags gently with the fingers until the water ran clear. The washed bags were dried at 60 - 65°C for about 48 hours, allowed to cool and weighed.

Calculations

The degradability of the dry matter was calculated by the formula:

$$\text{DM Degradability} = \frac{(\text{SWa-BW}) \times \text{DMa} - (\text{SWb-BW}) \times \text{DMb}}{(\text{Swa-BW}) \times \text{DMa}}$$

Where:
SWa= Weight of the original sample +nylon bag
BW= Weight of the empty nylon bag
SWb= Weight of the sample + nylon bag after incubation
DMa= Dry matter of the feed sample
DMb= Dry matter of residue sample.

14. DERIVATION OF RATE EQUATION FROM GOMPERTZ MODEL *(J. Birks, OFI, Personal communication, 1996).*

Gompertz model:

Y = A + C * EXP(-EXP(-B(X-M)))

Where;
Y= Cumulative gas production
X = Time
A = Constant
C = Gas pool size
B = Rate constant
M = Lag time

Y - A = C * EXP(-EXP(-B(X-M)))

$\text{Log}\,(Y-A) = \text{Log}\,C - e^{-B(X-M)}$

$$\frac{1}{(Y-A)}\ \frac{dy}{dx} = B*e^{-B(X-M)}$$

$$\frac{dy}{dx} = (Y-A)*B*e^{-B(X-M)}$$

$= C*B*EXP(-EXP(-B(X-M)) * e^{-B(X-M)}$

Rate = C*B*EXP(-EXP(-B(X-M)) * EXP(-B(X-M))