Measuring and modelling in-vitro gas production kinetics to evaluate ruminal fermentation of feedstuffs

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Measuring and modelling in-vitro gas production kinetics to evaluate ruminal fermentation of feedstuffs

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STELLINGEN

I

Als de gasproductie van twee heel verschillende voedermiddelen wordt vergeleken, is het belangrijk in de gaten te houden dat VFA verhoudingen de gasproductie kunnen beïnvloeden (*dit proefschrift*).

II

Celwandafbrekende enzymen, zoals die nu op de markt zijn (cocktails), voegen weinig toe aan de pensvertering. Hiervoor zijn meer specifieke enzymen nodig (*dit proefschrift*).

III

Een exponentieel model is niet voldoende om gasproductiecurves te beschrijven (*dit proefschrift*).

IV

De gevonden opbrengsten aan microbiële massa door Blümmel berusten waarschijnlijk op een foute interpretatie van de behandeling met neutraal detergens.
(*M. Blümmel (1992) In: Naturel resource development and utilization. Landbouwuniversiteit Wageningen*).

V

Het toevoegen van kleine hoeveelheden zuurstof aan glazen laboratoriumsilo's is een belangrijke verbetering in de nabootsing van de praktijksituatie (*R. Muck, P. Michel, unpublished results*).

VI

Afbreekbaarheid van een voeder wordt meer beïnvloed door de architectuur van de celwand dan door de concentratie van één enkele celwand component (*C.W. Ford & R. Elliot (1987) J Agric Sci Camb 108: 201-209*).

VII

Niet integratie, maar acceptatie is een eerste voorwaarde voor een multiraciale samenleving.

VIII

De discussie over transgene dieren is schijnheilig zolang er in de moderne fokkerij volop gemanipuleerd wordt met erfelijk materiaal van dieren.

IX

Om in economisch zwakke tijden de concurrentiepositie te kunnen handhaven, is voor elk bedrijf/instelling een goed bijgehouden financiële administratie onmisbaar.

X

Wetenschap heeft die boeiende paradox dat desillusie niet betekent dat je verslagen bent. Het is een stap vooruit (*P.D. James, Death of an expert witness*).

Stellingen behorende bij het proefschrift van J.M.W. Beuvink: Measuring and modelling in-vitro gas production kinetics to evaluate ruminal fermentation of feedstuffs.

SUMMARY

In this thesis, the possibilities of kinetic gas production measurements for the evaluation of ruminant feedstuffs have been examined. Present in-vitro methods were mostly end-point methods. There was a need for a kinetic in-vitro method that described ruminal fermentation, due to new techniques in fabricating additives and new developments in plant breeding.

When a feedstuff is incubated with CO_2/HCO_3^- buffered rumen fluid, the rumen microorganisms ferment the carbohydrates from the feed to volatile fatty acids (VFA) and gas. The VFA also release CO_2 from the buffer. When followed over time, this direct and indirect gas production result in sigmoidal curves. The ratios between the VFA produced (acetic, propionic and butyric acid) depend on the substrate. When rapidly fermentable carbohydrates are available, the relative amounts of propionic acid increased, whereas with slow fermentable carbohydrates, the relative amount of propionic acid decreased at the favour of the acetic and butyric acid. This affects the gas production, because at the production of propionic acid, no gas is produced as a fermentation end product, in contrast to acetic and butyric acid. In other words, the amount of gas produced depends on the fermentation pattern (Chapter 2).
The gas production methods available all required frequent periodical readings. For a frequent use, it was desirable to have an automated method, which was realised through a liquid displacement system with automated registration (Chapter 3). Later this method was replaced by a more sophisticated one, using pressure transducers (Chapter 7).
For comparison of the gas production curves, it was necessary to fit the data to a mathematical model. Several known models were tested, but they did not fit the curves satisfactorily. The fit was especially poor in those cases when rapid early gas production was observed together with a low final gas production rate. Therefore a new model was developed: the "Modified Gompertz Model' (Chapter 4). This model divided gas production in gas produced upon fermentation of rapidly and slowly fermentable material. From this model the following parameters were derived to describe the curves: length of lag phase, total gas production, maximum gas production rate, time at which gas production rate is maximal, and time at which 95% of the gas is produced.
The effects of addition of cell wall degrading enzymes to grass silage was evaluated using the automated method and the model. Addition of those

enzymes that resulted in a significant degradation of cell walls in the silage, resulted in a shortened lag phase and a lower maximum gas production rate, indicating that the enzymes degraded cell wall material to more rapidly fermentable components, leaving a more slowly fermentable fraction. The total amount of gas remained unchanged (Chapter 6).

Another application was the evaluation of the effects of phenolic acids (p-coumaric and ferulic acid), added externally or present in different concentrations in the feed (stems of different maize inbred lines). When added externally, p-coumaric acid inhibited gas production and dry matter disappearance. Ferulic acid was not inhibitory. Maize stems with higher phenolic acid contents showed lower gas production rates and lower total gas production. A causal relationship could not be derived due to interaction with cell wall content (Chapter 7). The present method to determine ruminal degradation kinetics is the nylon bag method, where feed is incubated in the rumen in nylon bags and disappearance of feed components is followed gravimetrically over time. For different maize products a significant relationship was found between the total gasproduction and the unfermentable fraction. The gas production rate and rate of disappearance of organic matter did not agree. This might be caused by the fact that the nylon bag method distinguishes a soluble, an undegradable and a potentially degradable fraction, where degradation kinetics are only determined of the latter two fractions. The gas production method takes the whole sample into account. Future research should reveal the fysiological meaning of the gas production parameters. So far this method can supply a ranking of different feedstufs and/or additives.

SAMENVATTING

In dit proefschrift worden de mogelijkheden van kinetische gasproductiemetingen voor de evaluatie van de fermentatie van veevoeders in de pens beschreven. Mede door nieuwe technieken voor het vervaardigen van additieven en ontwikkelingen in de plantenveredeling was er behoefte aan een laboratoriummethode die het verloop van de pensfermentatie kon voorspellen. De huidige laboratoriummethodes waren veelal gebaseerd op eindpuntsmetingen.

Wanneer een voeder geincubeerd wordt met CO_2/HCO_3^- gebufferde pensvloeistof fermenteren de pensmicroorganismen de koolhydraten uit het voer tot vluchtige vetzuren (VFA) en gas. Bovendien zorgen de VFA voor het vrijkomen van CO_2 uit de buffer. In de tijd gemeten leveren deze directe en indirecte gasproductie sigmoidale gasproductiecurves op. De onderlinge verhoudingen tussen de gevormde VFA (azijnzuur, propionzuur en boterzuur) zijn afhankelijk het soort voer. Bij snel fermenteerbare koolhydraten wordt verhoudingsgewijs meer propionzuur gevormd, terwijl bij langzaam afbreekbare koolhydraten meer azijnzuur gevormd wordt. Dit heeft effect op de gasproductie doordat bij de fermentatie tot propionzuur, in tegenstelling tot bij azijnzuur, geen gas direct als fermentatie-eindproduct gevormd wordt. Met andere woorden, de geproduceerde hoeveelheid gas is afhankelijk van het fermentatiepatroon (Hoofdstuk 2).
De bestaande methoden voor het meten van gasproducties waren allen handmatige methoden. Voor een frequente gebruik van deze methode was het wenselijk om deze te automatiseren. Dit werd gerealiseerd met behulp van een vloeistofverplaatsingssysteem, door het gevormde gas een vloeistof te laten verdringen en deze hoeveelheid vervolgens te wegen en automatisch op te laten slaan (Hoofstuk 3). Deze methode werkte goed, maar was erg bewerkelijk en gevoelig voor lekkages. Gedurende het onderzoek werd dan ook een nieuwe methode ontwikkeld gebaseerd op het meten van gas m.b.v. electronische drukopnemers (Hoofdstuk 7).
Om alle gasproductie-curves goed met elkaar te kunnen vergelijken was het nodig om deze te fitten m.b.v. een wiskundig model. Een aantal bestaande modellen werden getoetst, maar gaven geen van allen een goede fit van de curves. De fit was vooral slecht voor voedermiddelen die een snelle gasproductie aan het begin hadden en langzaam naar hun asymptoot gingen. Hierom werd een nieuw model ontwikkeld: het "Modified Gompertz Model" (Hoofdstuk 4). Dit model deelt de gasproductie op in gas afkomstig uit de fermentatie van snel

afbreekbaar materiaal en gas afkomstig uit de langzaam afbreekbare fractie. Uit dit model werden de volgende parameters afgeleid om de curves te beschrijven: lengte van de lag fase, maximale gasproductie, maximale gasproductiesnelheid, tijdstip waarop de gasproductiesnelheid maximaal is, en tijdstip waarop 95% van de totale hoeveelheid gas is geproduceerd.
Met behulp van de automatische meetmethode en het model werden effecten van toevoegingen van celwandafbrekende enzymen aan grassilages geevalueerd. Toevoeging van díe enzymen die resulteerden in een significante celwandafbraak in de silage, resulteerde in een kortere lag fase en een lagere maximum gasproductiesnelheid. De totale hoeveelheid gas bleef gelijk (Hoofdstuk 6).
Een andere toepassing was het evalueren van effecten van fenolzuren (p-coumaarzuur en ferulazuur), hetzij extern toegevoegd, hetzij in verschillende concentraties in het voer (stengels van verschillende mais inteeltlijnen) aanwezig. Extern toegevoegd werkte p-coumaarzuur remmend op gasproductie en verdwijning van drogestof. Ferulazuur had geen remmend effect (Hoofstuk 7).
De huidige methode om afbraakkinetiek in de pens te bepalen is de nylon zakjes methode, waarbij voer in nylon zakjes in de pens wordt geincubeerd en op verschillende tijden de verdwijning van voercomponenten wordt bepaald. De gas productie methode bleek alleen vergelijkbaar te zijn met deze methode voor wat betreft de totale gas productie en de totale verdwijning van organische stof. De gas productiesnelheid en verdwijningssnelheid van organische stof kwamen niet overeen. Dit is te wijten aan het feit dat de nylon zakjes methode een oplosbare, een onverteerbare en een potentieel verteerbare fractie onderscheid, waarbij alleen van de laatste twee de afbraakkinetiek wordt bepaald. De gas productiemethode heeft echter betrekking op het gehele monster.
Toekomstig onderzoek moet de fysiologische betekenis van de gas productie parameters ophelderen. Tot zover is met deze methode mogelijk een ranking te maken voor verschillende voedermiddelen en/of toevoegmiddelen.

Contents:

CHAPTER 1

General introduction

Roughage feeding

Ruminants can utilize fibrous feedstuffs, indigestible by mammalian enzymes, by microbiological fermentation in the rumen. This fermentation yields many nutritients for the ruminant. However, roughages are never fully digested in the rumen.
From economical and environmental point of view it is important to upgrade the feeding value of roughages. This can be done by using additives, or by plant breeding programmes. The present developments in biotechnology and a better insight in plant genetics provide us with new techniques to improve roughage quality.
To assess the digestibility of animal feedstuffs, the importance of in-vitro feed evaluation has long been recognized. However, due to the developments mentioned above, there is a need for rapid feed evaluation methods that not only give information about the extent to which a feed is digested, but also about the rate and pattern of digestion.

Ruminal digestion of feedstuffs

The ecosystem in the rumen provides a highly reduced environment (oxidation-reduction potential of -250 to -450 mV) where anaerobic microorganisms utilize the carbohydrates from the feed to produce acetate, butyrate, propionate, methane, carbon dioxide and biomass. The volatile fatty acids (VFA) are the major source of energy to the ruminant and the microbial mass is an important source of essential nutrients such as amino acids, and B-vitamins. High producing dairy cows receive concentrates to sustain high production levels. Roughages alone cannot meet their energy demand.
Rumen fluid is well buffered by the copious flow of saliva, of which the main buffering components are bicarbonate, phosphate, VFA produced by fermentation and buffering components from the feed. The pH in the rumen fluctuates between 5.7 and 7.3.
The major microbial populations in the rumen are bacteria, protozoa and fungi. Of these, bacteria are found in the greatest numbers (10^9-10^{11}/g of rumen

contents) and diversity.
Passage from the rumen is influenced by particle size. Retention time for liquids may range from 10 to 24 h, whereas large food particles can stay in the rumen for 2 or 3 days (Hobson and Wallace 1982).
The extent of digestion of a feed is affected, among others, by the retention time in the rumen, or, in other words by the rate of passage (Demeyer 1981). However, the fibre fraction from the feed consisting of plant cell walls is never fully utilized by the animal. The resistance of these fibers to degradation by rumen bacteria is due to the crystalline nature of cellulose and to the lignin-carbohydrate association (Hoover 1986). Procedures (physical, chemical or biological) which would enhance the availability of these feeds would result in an increase of the feeding value. Hereby, not only the extent of feed degradation is important, but also the rate and pattern of degradation.
To predict the effect of treatments it is necessary to have information about the changes in the rumen digestion system as affected by such treatments.

Ruminal digestion of plant cell walls

Plant cell walls consist mainly of carbohydrates of which cellulose, hemicellulose and pectin are the major ones. They are called structural carbohydrates. Starch and fructosans are examples of non-structural carbohydrates.
Cellulose is a polymer of beta-1,4-linked glucose units. In the secondary cell wall, cellulose is generally of a crystalline nature, which makes it less susceptible to enzymatic degradation. In contrast with cellulose, hemicellulose is a highly branched heteropolymer consisting of different monomers: xylose, arabinose, galactose, mannose and glucose are found (McNeil et al. 1984). Pectin is a structural polysaccharide that is mainly found in the middle lamella and primary cell walls of higher plants (Rombouts and Pilnik 1980). It also is a heteropolymer mainly consisting of alpha-1,4-linked galacturonic acid residues with both free and methylated carboxyl groups (Sleath and Mah 1987). In addition to the polysaccharides, plant cell walls contain proteins, small amounts of waxes, organic acids, silica, salts and the complex aromatic compound lignin. The amount of the latter increases with age of the plant.
It is well known that a negative relationship exists between lignin content and digestibility (Demeyer 1981). The presence of bound phenolic acids in the plant cell wall (p-coumaric and ferulic acid) also limits fibre digestion (Hartley 1972; Burrit et al. 1984).
Extent and rate of degradation of different plant cell walls are influenced by a number of factors among which the type of cell wall. Mesophyll cell walls are

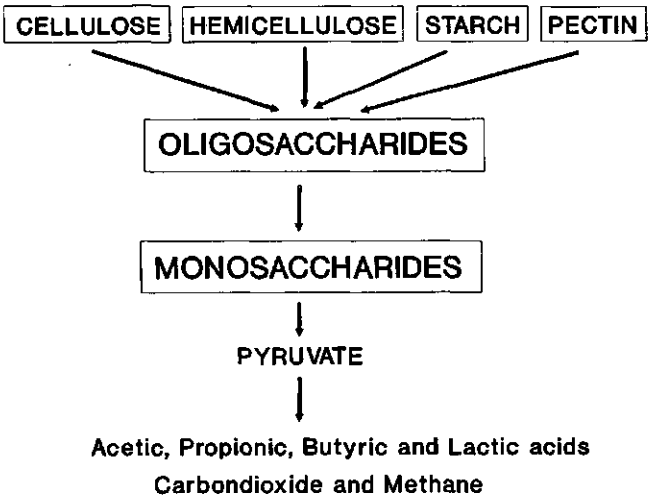

Figure 1. Simplified scheme of fermentation of carbohydrates in the rumen.

almost 100% degradable, whereas xylem cell walls are virtually non-degradable to rumen microorganisms (Akin 1989). Structural carbohydrates are insoluble and therefore attachment of the bacteria is a prerequisite for degradation. After hydrolysis of polysaccharides, the monomers are fermented to VFA (Figure 1).

In-vitro techniques for estimating ruminal digestibility

In-vitro techniques, using rumen contents as an inoculum, can be considered as models of in-vivo rumen digestion, and are applied to obtain a better understanding of the complex ruminal system, or to estimate digestibility of feedstuffs. However, many interactions inherent to the in-vivo system, e.g. passage rates, particle size, and rate of particle size reduction, make interpretation of in-vitro data difficult (Fisher et al. 1989). For estimating digestibility these problems can be avoided by comparison to standard feedstuffs. Many methods of in-vitro feedstuff analysis have been developed. In this section only the most important ones are discussed.

Tilley and Terry (1963) developed an in-vitro method to estimate apparent digestibility by incubating a feedstuff with buffered rumen fluid for 48 h, followed by a pepsin-HCl treatment to remove undigested protein. In-vivo digestibility is estimated from the disappearance of dry matter. Many authors

have used the first step of this method to determine in-vitro digestion kinetics by incubating during different time intervals (e.g. Grant and Mertens 1992).
Menke et al. (1973) described a method to estimate digestibility from the gas produced upon incubation of a feedstuff with buffered rumen fluid, rather than by the disappearance of dry matter. This method has also been used by many researchers to determine fermentation kinetics by measuring gas production at different time intervals (Krishnamoorty et al. 1991; Merry et al. 1991; Beuvink and Spoelstra 1992). Besides gas, other fermentation end products have been measured like heat (Arieli and Werner 1989) and pH (Malestein and van't Klooster 1986).
The Tilley and Terry method and the gas production method have in common that they are both batch fermentations; no inflow of nutrients or removal of fermentation products (except gas) is taking place. In-vitro methods that make use of continuous reactors (e.g. Rusitec described by Czerkawski and Breckenridge 1977) overcome these problems. However, because of their complexicity they are not suitable to perform routine analyses.
At present rumen fermentation kinetics are determined by incubating feedstuffs in nylon bags in the rumen. At various time intervals, bags are analysed for the disappearance of feed components.

Scope of this thesis

To evaluate additives that may improve feeding value, or to evaluate new genetic varieties of plants it is desirable to have an in-vitro system that can measure digestibility as well as digestion rate. In this thesis the possibilities of using in-vitro gas production kinetics, measured during incubation of a feedstuff with buffered rumen fluid have been examined. In **Chapter 2** the principles of gas production of different carbohydrates were investigated, mainly the relationship between gas production and fermentation pattern. Gas production kinetics have been measured manually, but this requires frequent periodical readings over 48 h. This made the method not suitable for every-day use, so it was automated (**Chapter 3**). To facilitate analysis of the obtained gas production curves, a model that described the curves was necessary. The models described in the literature did not satisfactorily fit the curves, so in **Chapter 4** a new model is presented. At present, in ruminant feed evaluation, digestion kinetics are determined by incubating feed in nylon bags in the rumen and following disappearance of dry matter (DM) or feed components over time. In **Chapter 5** a comparison is made between the nylon bag method and the gas production method. The differences between these two methods are discussed.

Recently use of cell wall degrading enzymes to improve animal feedstuffs has gained a lot of interest. The effect of such cell wall degrading enzymes on gas production kinetics has been investigated in **Chapter 6**. Another way of improving animal feedstuffs is to select genetic varieties on digestibility criteria. One of these criteria may be the absence or low concentrations of cell wall components that inhibit digestion, such as phenolic acids. Their inhibitory effects on gas production kinetics have been investigated in **Chapter 7**. In this chapter also stems of five different genetic varieties of maize, differing in their phenolic acid contents, have been examined. **Chapter 8** is a thorough evalulation of the methods and results described in the previous chapters.

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CHAPTER 2

Interactions between substrate, fermentation end-products, buffering systems and gas production upon fermentation of different carbohydrates by mixed rumen microorganisms in vitro

J.M.W. Beuvink & S.F. Spoelstra
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Summary

In animal nutrition, incubation of feed samples with CO_2/HCO_3^- buffered rumen fluid is used to predict the nutritional values of the feed. During fermentation, volatile fatty acids (VFA) are produced, which release CO_2 from the buffer through their H^+-ions. This indirect gas production amounted to 20.8 ml gas per mmol VFA. By incubating glucose, rice starch and cellulose, the relationship between direct and indirect gas production in relation to fermentation kinetics was studied. The total amount of gas formed was found to be dependent on the composition of the fermentation end-products formed.

*there is great interest in measuring the gas production of feedstuffs incubated with buffered rumen fluid (BRF) in vitro. In this work some of the underlying mechanisms were elucidated. This could be described by: ml gas = M_v * mmol HAc + $2M_v$ * mmol HB + $0.87M_v$ * mmol Tot.VFA; where HAc = acetic acid; HB = butyric acid; M_v = molar gas volume. No clear relationship was found between the rate of fermentation and total gas production. From rice starch more total gas was produced than from glucose and cellulose, which were fermented faster and slower, respectively.*

Introduction

Feed ingested by ruminants, is digested by rumen microorganisms which ferment carbohydrates from the feed to gas (carbon dioxide and methane) and volatile fatty acids (VFA). The latter are the major source of energy to the animal (Hungate 1966; Lin et al. 1985). An estimation of degradability can be made in vitro by incubating feed samples with strained rumen contents. The digestibility is derived from the disappearance of dry matter (Tilley and Terry 1963) or amount of gas produced (Menke et al. 1979) after a fixed incubation period. To obtain information about the rate of degradation of a feedstuff, in vitro gas production has been followed over time (van der Meer et al. 1988; Xiong et al. 1990). In these in-vitro gas production measurements, rumen fluid is mixed with a CO_2-bicarbonate/phosphate buffer (Menke 1979). The gas produced, is a result of gaseous fermentation end products (CO_2 and CH_4, direct gas production) and CO_2 released from the buffer by the VFA produced (indirect gas production).

The main end-products of carbohydrate fermentation by rumen microorganisms are acetic, propionic and butyric acid, carbon dioxide and methane. Fermentation of glucose via pyruvate results in the end products listed in Table 1. This table shows that the composition of end products formed, influences the amount of gas produced.

Table 1. Direct and indirect gas production (mol) from 1 mol glucose fermented to different acidic end-products.

Acidic end products (mol)	Direct gas production (mol)	Indirect gas production[a] (mol)	Total gas production (mol)
2 Acetic acid	2 CO_2	2 CO_2	4 CO_2
1 Butyric acid	2 CO_2	1 CO_2	3 CO_2
2 Propionic acid	-	2 CO_2	2 CO_2
2 Lactic acid	-	2 CO_2	2 CO_2

[a]) Assuming that 1 mol organic acid releases 1 mol gas from the buffer.
Stoichiometry according to Hungate (1966) and Demeyer & Giesecke (1973).

Between gas production and total VFA formation in vitro a positive relationship has been reported (O'Hara & Ohki 1973; Naga & Harmeyer 1975; Datta Roy et al. 1976; McBurney et al. 1990). However, no explanation has been given by these authors for different amounts of gas produced upon fermentation of different carbohydrates.

At present, digestibility of feedstuffs is often derived from in-vitro gas production (Menke 1979) and fermentation rates of the rumen microbes derived from (semi) continuous gas production measurements (van der Meer et al. 1988). Measuring gas production is relatively simple (Menke et al. 1979; van der Meer et al. 1988), but interactions between fermentation end-products, buffering system and amount of gas produced are very complex. The goal of the work presented here was to provide more insight in these interactions in order to come to a better evaluation of time-related gas production measurements for estimating rumen degradation rates of animal feeds. Hereto gas production and VFA formation from three carbohydrates (glucose, starch and cellulose) were followed over time in order to explain differences in gas production. The experiments were not set up to imitate the rumen, but only to investigate the limitations and possibilities of the time related gas production test as a simulation technique for in-vitro carbohydrate fermentation.

Materials and methods

Preparation of buffered rumen fluid (BRF)

Rumen fluid was obtained from two rumen fistulated whether sheep kept on a daily ration of 800 g hay and 200 g concentrates fed in two equal meals at 8.00 and 16.00 h. Rumen fluid was taken 2 h after morning feeding and collected in a warm thermostated flask filled with oxygen-free CO_2. Rumen fluid was filtered over two layers of cheese cloth. Filtered rumen fluid was mixed (1:2 v/v) with an anaerobic buffer/mineral solution containing per liter 8.75 g $NaHCO_3$, 1.00 g NH_4HCO_3, 1.43 g Na_2HPO_4, 1.55 g KH_2PO_4, 0.15 g $MgSO_4*7H_2O$, 0.52 g Na_2S, 0.017 g $CaCl_2*2H_2O$, 0.015 g $MnCl_2*4H_2O$, 0.002 g $CoCl_3*6H_2O$, 0.012 g $FeCl_3*6H_2O$ and 0.125 g resazurine (Menke et al. 1979 as modified by Steingass 1983). All handlings were done under continuous flushing with oxygen-free CO_2.

Gas measurements, sampling and analytical procedures

Gas production was measured at 20 °C in a liquid displacement system similar to the one described by Jouany and Thivend (1986). The liquid to be displaced in our system was a saturated NaCl-solution (pH 1.0). Fermentation was carried

out in 100-ml serum bottles closed with rubber septa and placed in a shaking waterbath (50 rev./min) at 39 °C. Solutions of test samples were added by syringe through the septum. In the same way, samples from the BRF were taken for analysis of VFA and lactic acid. These samples were preserved and analysed by gas chromatography as described by Robinson et al. (1986). Total VFA was calculated as the sum of acetic (HAc), propionic (HP), butyric (HB), iso-butyric, 2- and 3-methyl-butyric, and valeric acid. The first three are end products of carbohydrate fermentation; the others are formed through deamination of amino acids in the rumen (Giesecke 1973). Dry matter content of the samples was determined after oven drying (4h at 104°C).

Release of gas upon addition of acid

On five different days, BRF was freshly prepared. On three days, BRF was used immediately after collection, the other two days it was frozen (-18 °C) until use within two weeks. Frozen BRF was thawed at 39 °C under continuous flushing with oxygen-free CO_2. BRF (60 ml) was anaerobically transferred to 100-ml serum bottles and connected to the liquid displacement system. Acetic acid (0.5 ml, 2M) was injected through the septum and gas production read after 30 min. Addition of acid was repeated until no further gas was released. Incubations were done fivefold with parallel incubations of a blank (BRF without substrate). To determine if there was a difference in gas release for different acids, acetic acid, propionic acid or hydrochloric acid (2M solutions) was added to fresh BRF. Acetic acid was also added to 40 ml buffer mixture without rumen fluid.

Formation of VFAs and gas during fermentation of glucose, rice starch or cellulose

Incubated substrates were anhydrous glucose (J.T. Baker, Deventer, the Netherlands), rice starch and crystalline cellulose (both Sigma, St. Louis, MO., USA). Of each substrate, 400 mg (± 10 mg) was incubated in 60 ml BRF in five parallel incubations. Two incubations were used for gas production readings, the other three for periodically sampling of the fermentation mixture and subsequent VFA and lactic acid analysis. Gas production was read every hour for 36 h. After 36 h, experiments were stopped and for all five incubations pH was measured and samples for VFA and lactic acid analyses were taken. Preliminary experiments showed that no significant gas production took place after 36 h. In all series, blanks (BRF without substrate) were measured to correct for gas production from remaining feed particles in the rumen fluid. It was assumed that fermentation of these remaining feed particles was the same with and without substrate. The experiment was repeated with rumen fluid from

another day.
Gas production and VFA concentrations were corrected for the blank and expressed as ml gas per g organic matter (OM) and mmol VFA/g OM, respectively. Since the molar volume for all gases is the same (at the same temperature), no attempts were made to measure methane and carbon dioxide separately.

Results

Release of gas upon addition of acid
Addition of acetic acid to BRF resulted in release of gas as shown in Fig. 1. From 60 ml BRF a maximum of 114 ml (SD = 5.3; n = 25) gas was released upon the addition of acetic acid. After addition of 6 mmol acid no further gas was released. Above 4.5 mmol the relationship between the amount of acid added and gas production was no longer linear. At this point the pH of the BRF had dropped to 6.04.
On a molar basis, the same maximum amounts of gas were released upon addition of acetic acid (114 ml; SD = 5.3; n = 24), propionic acid (114 ml; SD = 0.9; n = 8) or hydrochloric acid (116 ml; SD = 2.6; n = 8). It made no difference wether fresh (113 ml; SD = 5.2; n = 12) or frozen BRF (115 ml; SD = 7.2; n = 12) was used. From 40 ml buffer mixture, the same total amount of gas (116 ml; SD = 2.8; n = 18) was released as from 60 ml BRF, which also contained 40 ml buffer mixture.
On the linear part of the curve (0-4.5 mmol), linear regression was performed, resulting in the following relationship:

$$\text{ml gas} = 0.048 * \text{mmol VFA} \qquad R^2 = 0.989 \qquad (1)$$

Formation of gas and VFAs upon fermentaton of glucose, rice starch or cellulose
The three substrates tested (glucose, rice starch and cellulose), differed in gas production pattern as well as in VFA formation pattern (Fig. 2). The maximum rate of gas production and VFA formation for glucose occurred between 0 and 4 h, for rice starch between 4 and 12 h and for cellulose between 12 and 20 h.
From glucose some lactic acid was produced during the early stage of fermentation. Concentration of lactic acid was highest after 4 h (Fig. 2). After 8 h the lactic acid had disappeared. No lactic acid was formed during fermentation of rice starch, cellulose or in the blank.

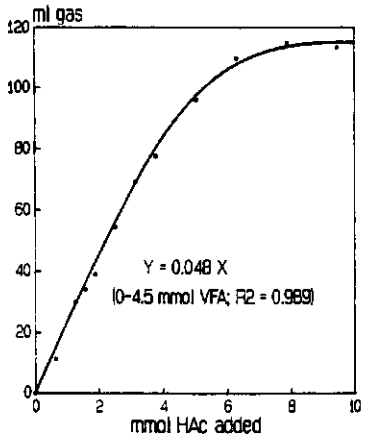

Figure 1. Gas release from 60 ml buffered rumen fluid upon stepwise addition of acetic acid (HAc). The temperature of the gas measured was 20 °C. VFA, volatile fatty acids; Y, ml gas, X, mmol HAc added; R2, coefficient of determination

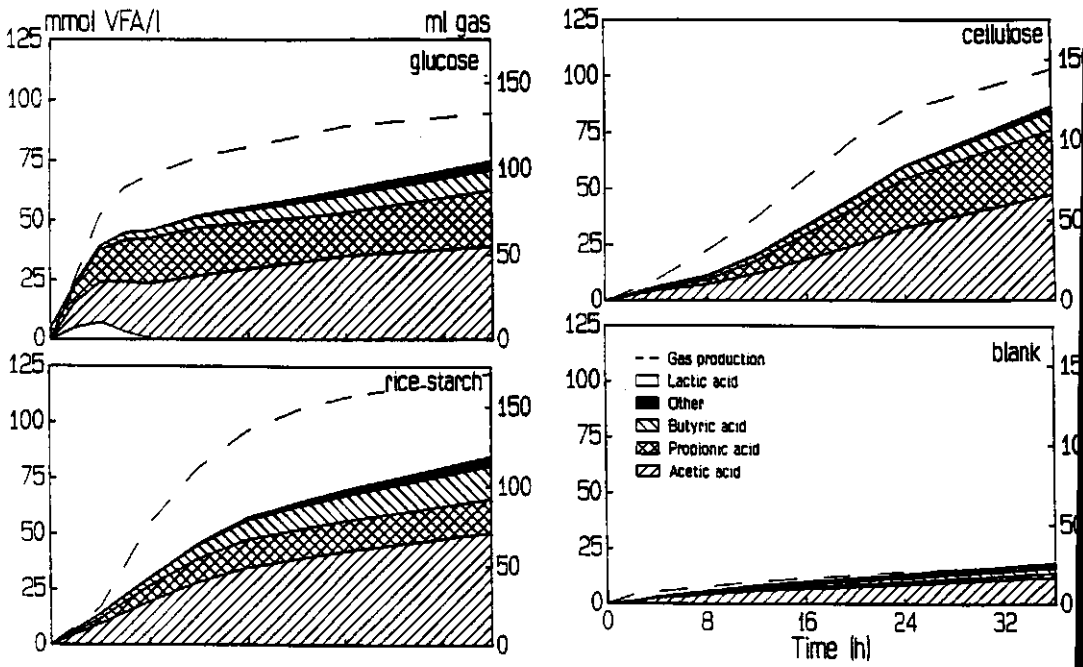

Figure 2. Time course of gas production and VFA concentration for incubations of 60 ml buffered rumen fluid with glucose, rice starch or cellulose (incubated amounts 400 ± 10 mg organic matter) and a blank. The temperature of the gas measured was 20 °C.

Rice starch showed the highest total gas production and cellulose the highest total VFA production (Table 2). Glucose and rice starch differed ($p<0.01$) in absolute amounts of acetic, propionic and butyric acid. Fermentation of glucose and cellulose differed ($p<0.01$) in absolute amounts of acetic and propionic acid formed, but not in butyric acid. There was no difference in percentage composition of VFA between glucose and cellulose. This unlike the fermentation of rice starch showing higher acetic and butyric acid percentages and lower percentage propionic acid (Table 2).

Table 2. Fermentation products (gas and volatile fatty acids; VFA) and pH after 36 h incubation of glucose, rice starch or cellulose with buffered rumen fluid.

	Incubated substrate		
	Glucose	Rice starch	Cellulose
Total gas production[1] (ml/g OM)	286 ± 12.8[a]	375 ± 22.2[b]	304 ± 23.0[a]
Total VFA production (mmol/g OM)	8.3 ± 0.54[a]	8.4 ± 1.15[a]	9.9 ± 1.12[b]
Absolute amounts of VFAs (mmol/g OM)			
Acetic acid	4.1 ± 0.30[a]	4.8 ± 0.49[b]	4.8 ± 0.69[b]
Propionic acid	3.2 ± 0.15[a]	1.7 ± 0.10[b]	3.9 ± 0.43[c]
Butyric acid	0.9 ± 0.14[a]	1.6 ± 0.27[b]	0.9 ± 0.17[a]
Others	0.1 ± 0.01	0.3 ± 0.02	0.3 ± 0.02
pH	6.28 ± 0.064	6.32 ± 0.119	6.19 ± 0.121
VFA percentage composition (mol/100 mol)			
Acetic acid	48.7 ± 0.59[a]	59.2 ± 3.41[b]	48.8 ± 4.56[a]
Propionic acid	38.7 ± 1.58[a]	21.3 ± 2.02[b]	39.9 ± 1.92[a]
Butyric acid	10.6 ± 1.33[a]	19.3 ± 1.33[b]	8.8 ± 1.31[a]

All data are corrected for the blank. VFA production data are means from 10 replicates ± SD, gas production data from 4 replicates ± SD. Results with different letters in one row differ significantly at the 99% confidence level (calculated by analysis of variance using two sided t-test).
OM, organic matter

The initial pH of the BRF was 6.90. The value of the blank remained unchanged during 36 h incubation. On incubations of substrates, pH dropped about 0.6-0.7 units (Table 2).

Discussion

Release of gas from the buffer by acids

VFA contributed, through their H^+-ions, to gas production by the release of 20.8 ml gas per mmol VFA from BRF, in the range 0-4.5 mmol VFA/60 ml BRF) (Fig. 1). Since the molar gas volume at 20 °C is 24.0 l/mol, it can be derived from Equation (1) that addition of 1 mmol VFA releases 0.87 mmol gas.

From the reaction equation of the CO_2-bicarbonate buffer it is expected that 1 mol of acid releases 1 mol of gas. However in the BRF also a phosphate buffer is present, contributing to the total buffering capacity by 18% at pH 6.9, 7% at pH 6.5 and 0% at pH 6.0. The average contribution of the phosphate buffer was calculated to be 13% in the pH-trajectory of the incubations (6.2-6.9). This means that 13% of the H^+-ions formed during fermentation, is neutralized without formation of gas. However, this is an average figure. Figure 1 is the resultant of gas release from the CO_2/HCO_3^- buffer and the titration curve of the phosphate component of the buffer. Since the contribution of the phosphate buffer to total buffering capacity is minor, Figure 1 reflects mainly the release of CO_2 from the CO_2/HCO_3^- buffer.

During incubation, the amount of VFA produced must not exceed 4.5 mmol/60 ml BRF to avoid exhaustion of the buffer. This implies that the sample weight has to be limited to 400 mg fermentable organic matter/60 ml BRF. Raab (1980) exceeding this amount of sample observed a non-linear relationship between sample weight and gas production.

Relation between VFA and gas production

Gas production and VFA formation are closely related processes (Fig. 2). The total gas production after 36 h on rice starch was higher ($p<0.01$) than gas production on glucose or cellulose whereas the same total amounts of VFA were produced on both substrates (Table 2). The extra amount of gas produced on rice starch compared to glucose could not be completely explained by hydrolysation (1 g starch is hydrolysed to 1.1 g glucose) which, if this was the explanation, should also apply to cellulose.

For every millimole of VFA produced, the amount of gas released indirectly was found to be constant, namely 0.87 mmol. The amount of gas produced directly as a fermentation end product, varies with VFA composition (Table 1).

Theoretically, the maximum amount of gas is produced when 1 mmol glucose is fermented to acetic acid, namely 2 mmol directly and 1.74 mmol indirectly. The minimum amount of gas is produced when 1 mmol glucose is fermented to propionic acid (1.74 mmol indirectly).
After 36 h fermentation, rice starch showed higher amounts ($p<0.01$) of acetic acid than glucose and lower amounts of propionic acid compared to glucose and cellulose (Table 2). The total amount of gas produced (directly plus indirectly) could be described by the following equation:

ml gas = V_m * mmol HAc + $2V_m$ * mmol HB + 0.87 V_m * mmol total VFA (2)

where V_m is the molar gas volume

The predicted amounts of gas produced from this formula were (as percentage of measured) 110, 98 and 120% for glucose, rice starch and cellulose, respectively. From Equation 2 can also be seen that the amount of gas produced not only dependends on the total amounts of VFA produced, but also on their percentage composition.
In these systems methane comprises 10-15% of total gas production (Cafantaris 1981). This does not influence the total amount of gas produced because 1 mol of CH_4 replaces 1 mol CO_2 according to

$CO_2 + 8[H] \rightarrow CH_4 + H_2O$ (Hungate 1966).

Methane production from acetate either hardly occurs or does not occur in rumen fluid (Russell and Wallace 1988). The hydrogen produced is mostly incorporated into methane and VFA. Less than 1% can be detected in the gas phase (von Grabe 1978).

Formation of VFA and lactic acid

Most species of rumen microbes are capable of producing various VFA as fermentation end products (Hungate 1966; Giesecke 1973; Lin et al. 1985).
Some rumen microorganisms (e.g. *Selenomonas ruminantium* and *Streptococcus bovis*) can swith between lactate and VFA formation, depending on their growth rate. When easily fermentable sugars are abundant, as was the case in our experiments with glucose (Fig. 2), lactate is directly produced from pyruvate by the enzyme lactate dehydrogenase. With this rapid conversion, the build-up of pyruvate, which is the "bottle-neck" in acetate and propionate production, is avoided (Russell and Wallace 1988). This pattern maximizes the amount of ATP formed per unit of time instead of unit substrate. When carbohydrate is limiting, the fermentation pattern is switched to VFA production, resulting in a higher ATP yield per unit substrate and lower growth rate (Russell 1988; Russell & Wallace 1988).

In our experiments, lactic acid was produced during the first hours of glucose fermentation and then disappeared rapidly (Fig. 2). During fermentation of rice starch and cellulose, no lactic acid was produced. For ruminants adapted to a starch-rich diet, formation of lactic acid is common (Demeyer & Giesecke 1973). The rumen fluid in the experiments described in this paper was from sheep fed mainly on roughage (hay). The observed lag time with this rumen fluid indicates that the microbes were not capable of rapid starch fermentation. A lag time of approximately 3-4 h was observed, which agrees with earlier observations with rumen fluid from a roughage fed animal (Demeyer & Giesecke 1973).

In the experiments described in this paper, cellulose was fermented at the lowest rate, but had the same final VFA percentage composition as glucose which was fermented at the highest rate. Other research groups also found a high propionic level on batch fermentation with cellulose as the only substrate (Barnett & Reid 1957; Senshu et al. 1980). When cellulose-rich feeds were incubated (grass), acetic acid was the predominant fermentation end product (Barnett & Reid 1957). This indicates the development of another microflora when substrate is abundant, with different interactions between the individual microbial species (Scheifinger & Wolin 1973).

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CHAPTER 3

An automated method for measuring time-course of gas production of feedstuffs incubated with buffered rumen fluid

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Summary

A system for automated registration of gas production in time upon incubation of feedstuffs with rumen fluid is presented. The system is based on weighing the amount of fluid replaced by fermentation gas, followed by calculation to gas volume and registration by a data logger. Incubations with glucose, rice starch and crystalline cellulose showed glucose to be fermented at the highest rate and cellulose at the slowest. The major source of variation was rumen fluid from different days. An example of a gas production curve is given for the incubation of grass and its cell wall fraction (obtained after treatment with Neutral Detergent reagent).

Introduction

In vitro incubations with rumen fluid (Tilley & Terry, 1963) or cellulolytic enzymes (McQueen & van Soest, 1975), are widely used to estimate digestibility of ruminant feedstuffs. Yet, these methods do not measure digestibility, but the solid residue after incubation. This gives erroneous results when soluble non-digestible products are formed (Tetlow et al., 1987) and when small particles are lost in the supernatant. To overcome these problems, Menke et al. (1979) proposed to measure gas produced when feedstuffs are incubated with buffered rumen fluid.

In addition to digestibility, the rate of degradation of feedstuffs is of crucial importance to maintain optimum rumen function and is an important feed parameter governing intake (Demeyer, 1981). Rumen degradation rates are now mainly estimated by incubating feed samples in porous nylon bags (Mehrez & Ørskov, 1977) which is a laborious method, and again only the fraction that is solubilised is measured. Alternatively, time related measurements of fermentation end products like pH (Malestein & van 't Klooster, 1986), heat (Arieli & Werner, 1989) and gas (van der Meer et al., 1990; Krishnamoorty et al., 1991; Merry et al., 1991) have been proposed.

Gas production is directly related to rumen fermentation, relatively easy to measure and therefore appears to be a suitable parameter for rumen fermentation rate studies. The methods described so far (van der Meer et al., 1988; Merry et al., 1991) require frequent periodical readings for over 48 h, making them less fit as routine laboratory methods. We describe here a system for automated registration of gas production in time. This automated system allows 24 samples to be measured at the same time.

Materials and methods

Description of the apparatus

A schematic diagram of the apparatus is shown in Figure 1. Gas production was measured by a liquid displacement system connected to a collection vessel placed on a balance. The collection vessel was placed 20 cm above the displacement bottle to achieve an equilibrium between the pressure in the displacement bottle and the sum of atmospheric and hydrostatic pressures. To minimize changes in hydrostatic pressure, an overflow tube was placed in the collection vessel. Gas production caused liquid to stream towards the collection vessel until a new equilibrium was established.

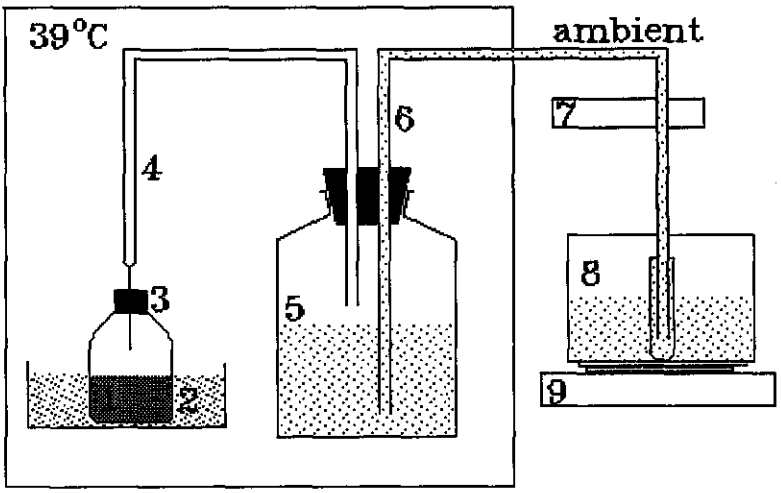

Figure 1. Schematic diagram of the apparatus. 1= Fermentation bottle (100 ml serum flask). 2= Shaking waterbath (50 rev./min; 39 °C). 3= Syringe needle piercing butyl rubber cap (Suba Seal Manuf.). 4= Butyl rubber tubing. 5= Water displacement bottle (1-l) with 700 ml saturated NaCl solution at pH 1. 6= Tygon tubing. 7= 24-way valve. 8= Collection vessel with overflow tube to minimize changes in hydrostatic pressure. 9= Balance with analogue output to data logger.

The system comprised 24 units (fermentation flask plus displacement system) using only one balance. All 24 tubes (Tygon, inner diameter 2.3 mm) were led through a 24-way valve (detail in Figure 2) before entering their own overflow tube in the vessel on the balance. This 24-way valve permitted only one of the tubes to be open. The amount of liquid displaced by the fermentation gas was collected and weighed. On a signal from a camshaft turning synchronously with the rotation disc, this weight was calculated to volume (ml gas) and stored in the memory of a data logger for later calculations. Subsequently, the next tube was opened and the events repeated. The rotation time of the disc was set at 25 min, so every sample was measured once at this time interval.

Gastightness of all the tubes and connections was ensured by closing the butyl rubber tubes with a clamp and letting the apparatus run. In case of leakage, liquid would stream back to the displacement bottle. If the system was gastight, heating up of the air in the displacement bottles caused liquid to flow to the collection vessel until an equilibrium was reached (usually after a few hours). At this moment the apparatus was ready to use. Measurements were started by piercing the syringe needle through the butyl rubber cap of the fermentation bottle and opening the clamp.

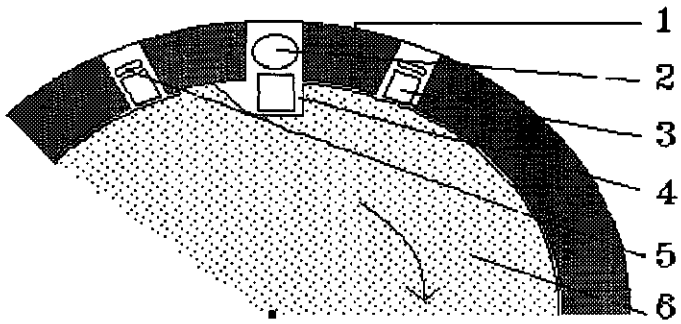

Figure 2. Detail of 24-way valve.
1 = Fixed outer circle. 2 = Open tube. 3 = Metal cilinder. 4 = Notch, permitting one tube to be opened at a time. 5 = Closed tube. 6 = Rotating inner part, arrow indicates direction of movement.

Experimental

Rumen fluid was obtained from two rumen fistulated wether sheep, kept on a daily diet of 800 g hay and 200 g concentrates and fed at 8.00 and 16.00 hours. From the two sheep equal amounts of rumen fluid were taken 2 h after morning feeding and collected in a thermostated flask filled with CO_2. The rumen fluid was filtered through two layers of cheese cloth and mixed (1:2) with the anaerobic medium described by Menke et al. (1979).

Substrate (400 mg $\pm$ 10 mg) was weighed into the fermentation bottles, which were then carefully flushed with oxygen free CO_2 for 2 minutes and closed with a butyl rubber cap (Suba Seal Manuf.). By inserting a syringe needle through the bottle closure, 60 ml of buffered rumen fluid was added to the substrate under continuous flushing with oxygen free CO_2. An extra needle was placed to maintain atmospheric pressure. After removing the needles, the bottle was placed in the waterbath and connected to the water displacement system. The 24-way valve and the clamp on the butyl rubber tube were opened to let the system re-equilibrate for approx. 1 min. After this had been done for all 24 units, measurements were started. Time zero was defined as the moment at which the buffered rumen fluid was added to the substrate.

The following well defined, homogenous substrates were incubated in quadruplicate on four diffent days: glucose (J.T. Baker, Deventer, the Netherlands), rice starch and crystalline cellulose (both from Sigma, St. Louis, MO., USA). Time course of gas production was followed during 48 h. In parallel incubations, gas production from four blanks (buffered rumen fluid without substrate) was measured. For every sample, the gas volumes stored in the memory of the data logger were calculated to cumulative gas productions and corrected for gas

production from the blank. Gas production was expressed as ml gas per g organic matter (OM). Gas production after 24 h from four experiments was used to estimate variation of gas production within and between rumen liquor from different days.

To illustrate the possibilities of the automated system, grass and the cell wall fraction from this gras were incubated as described above. Grass was oven dried (70 °C) and ground over a 1 mm sieve. Cell wall fraction was prepared by boiling 100 g grass for 1 h with 1 litre Neutral Detergent reagent (Goering & van Soest, 1970), subsequent filtration over nylon gaze (mesh 40 μm) and washing three times with hot water. The residue was dried under vacuum (40 °C) and used for gas production measurements.

Results

Cumulative gas production curves for the three substrates are given in Fig. 3 (results of one day). Each curve represents the average of four replicates. Glucose was fermented instantaneously without a lag phase, whereas rice starch was fermented slower and crystalline cellulose the slowest. Highest total gas production was observed upon incubation of rice starch.

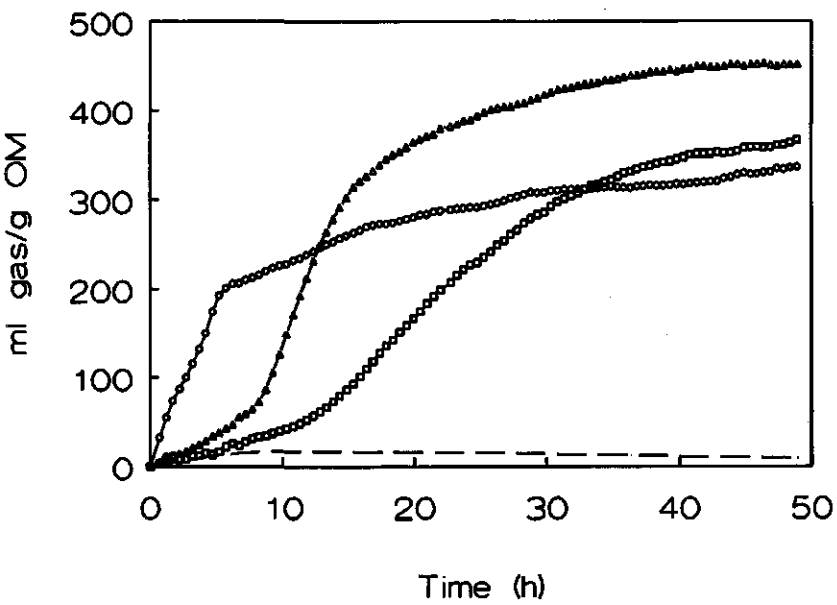

Figure 3. Cumulative gas production patterns for glucose (○), rice starch (△) and cellulose (□) and average SD throughout the curves (- - -). Each point is the average of 3 replicates.

Table 1. Results of analysis of variance of gas production after 24 h incubation, for three substrates and rumen fluid from four days.

	Glucose	Rice starch	Cellulose
DF between days	3	3	3
DF within days	11	12	9
Grand mean (ml gas/g OM)	305	403	257
SED[1]	10.3	15.1	10.2
Between days mean square	1374*	1546	5072*
Within days mean square[2]	181	458	139

1 = Standard Error of Difference of means
2 = Estimated variance of gas production
* = Significant at α=0.01

Table 1 shows the results from analysis of variance of total gas production after 24 h upon incubation of glucose, rice starch or cellulose, on four different days of incubation. For glucose and cellulose the between series mean square was much greater than the within series mean square. This could be ascribed to significantly different gas productions ($p < 0.01$) for rumen fluid from different

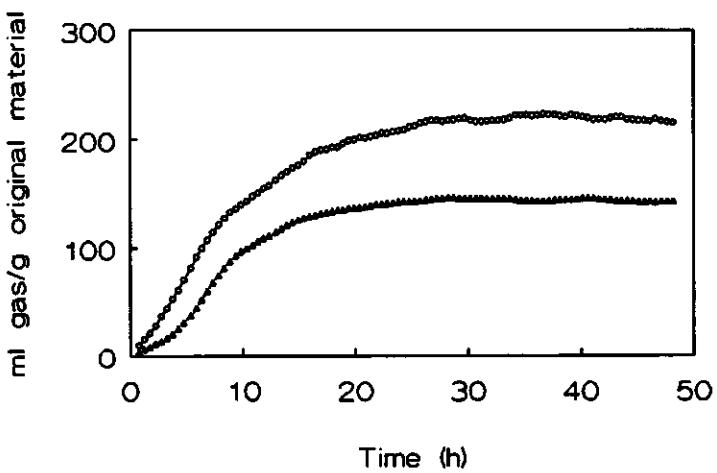

Figure 4. Gas production from 1 g grass (○) and the cell wall fraction (Neutral Detergent Residue) originating from 1 g grass (△). Each point is the average of 3 replicates.

days. For rice starch there was a trend ($p = 0.055$) for gas production to differ with rumen fluid from different days. Differences in total gas production between these carbohydrates can be explained by differences in fermentation pattern (Beuvink et al. 1992). Sometimes, little or no gas production was observed, eventhough there was no evidence for leakage. Such observations were excluded from further calculations. In the experiment described in Table 1, this was the case for 3 out of 48 incubations.
Figure 4 shows results from incubation of grass and cell wall fraction. From 1 g grass, 0.48 g cell walls were obtained. The treatment for cell wall preparation, resulted in a loss of components from wich gas could be produced (probably soluble sugars and polysaccharides like fructosans, and to a lesser extent protein).

Discussion

The use of the automated system described here, may be a valuable instrument in determining rumen fermentation kinetics in vitro. By releasing the pressure every 25 minutes, little build up of pressure occurs in the fermentation bottles.
Giger-Reverdin (1990) suggested corrections for changes in atmospheric and hydrostatic pressure when measuring water displacement by gas in the RUSITEC. Such corrections were not needed in our system. Changes in gas volumes caused by atmospheric and hydrostatic pressure were calculated to be less than 0.5%. Changes in temperature however, must be avoided, because of their immediate effect on gas volumes.
The major source of variation in gas production for incubations with glucose and cellulose was caused by batches of rumen fluid taken at different days. This agrees with observations made by other authors (Mehrez & Ørskov, 1977; Krishnamoorty et al., 1991). For rice starch there was only a trend to differ in gas production with rumen fluid from different days. Variation between rumen fluid from different sheep was avoided by mixing equal amounts of rumen fluid from two sheep. It was found preferable to incubate samples at least triplicate because sometimes, for unknown reasons, a very different fermentation pattern was observed for one of the replicates.
The non-automated method of measuring gas production in time has been used in evaluation of genetic varieties of fodder plants (van der Meer et al., 1988), estimation of starch and protein availability in processed sorghum grain (Xiong et al., 1990) and determining the rate of fermentation of energy supplements (Krishnamoorty et al., 1991). The automated method presented in this paper allows easier measurement of rumen fermentation kinetics in vitro. We intend to

use this system for evaluating enzyme addition to grass silage.

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CHAPTER 4

Modelling gas production kinetics of grass silages incubated with buffered rumen fluid

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Summary

Time course measurements of in-vitro gas production of feedstuffs incubated with buffered ruminal fluid give information about the rate at which the feed is fermented. To compare gas production kinetics from different feeds, a mathematical model was needed to describe the data. We investigated several existing models (exponential, logistic, Gompertz, Richards, Schnute), fitting them to 50 gas production curves obtained with grass silages. None of them gave a satisfactory description of the data. A new model was developed (modified Gompertz model; mGom), that basically divided gas production in two fractions, one arising from rapidly fermentable feed components and the other from slowly fermentable feed components. Residual Mean Squares (RMS) of mGom model were lower ($P < .05$; $n = 50$) than RMS from the other models. A good statistical momparison of kinetic gas production data is made possible with the mGom model.

Introduction

In vivo digestibility of animal feeds can be estimated by measuring in-vitro gas production of feed samples incubated in HCO_3^-/CO_2 buffered rumen fluid (Menke et al., 1979). This method can also be used to measure the degradation rates of feedstuffs by following gas production over time (Van der Meer et al., 1988; Krishnamoorty et al., 1991; Merry et al., 1991).

In such sigmoidal gas production curves three phases can be distinguished: 1) the phase of slow or no gas production (initial phase), 2) the phase of rapid gas production (exponential phase), and 3) the phase in which rate of gas production slows down and finally reaches zero (asymptotic phase).

During the initial phase hydration, attachment and colonization of insoluble substrate by rumen microbes take place (Cheng et al., 1980). 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, nondegradable material is left and gas production rate reaches zero.

To evaluate the results of such kinetic gas production studies, a suitable model that describes gas production over time is essential. In this paper we try to facilitate data analysis by modeling the data, thus reducing them to a few parameters. Several existing models were fitted to the gas production curves of grass silages. We will show that neither of these models describes the gas production satisfactorily, and we will present in this paper a new model for the description of gas production curves.

Material and methods

Models

France and Thornley (1984) described several sigmoidal growth models for biological systems of which some are listed in Table 1. We used these models to connect gas production (Y) to time (t) through a function (f): $Y = f(t)$.

The only non-sigmoidal model is the exponential model, which is frequently used in feed evaluation by the nylon bag technique (Ørskov and McDonald, 1970). This model describes simple first-order reaction kinetics with or without a lag phase. In the case of gas production, gas production rate (dY/dt) is proportional to the amount of gas yet to be produced, which is a reflection of the substrate concentration. The logistic model assumes gas production rate to be proportional to microbial activity, represented by the amount of gas already

Table 1. Mathematical description of evaluated models

Model	No. of parameters	Equation[a]
Exponential	3	$Y = Y_\infty \exp[-\mu(t-\lambda)]$
Logistic	3	$Y = \frac{Y_\infty}{1+\exp[-\mu(t-t_i)]}$
Gompertz	3	$Y = Y_\infty \exp\{-\frac{\mu_0}{D}\exp(-Dt)\}$
Richards	4	$Y = \frac{Y_\infty}{1+v\exp[-\mu(t-t_i)]^{1/v}}$
Schnute	4	$Y = \left\{Y_1^b + (Y_2^b - Y_1^b)\frac{1-\exp[-a(t-t_1)]}{1-\exp[-a(t_2-t_1)]}\right\}^{1/b}$
Modified Gompertz	5	$Y = Y_\infty \exp\left\{-\frac{\mu_{0r}}{D_r}\exp(-D_r t) - \frac{\mu_{0s}}{D_s}\exp(-D_s t)\right\}$

[a] Y_∞ = upper asymptote, representing total gas production, t_i = point of inflection, = lag phase, v, a and b = shape parameters, μ = specific gas production rate, μ, μ_{0r}, μ_{0s} = specific initial gas production rates, D, D_r and D_s = fractional constants governing the decay, t_1, t_2 = fixed time points, Y_1, Y_2 = expected Y at t_1 and t_2, respectively.

produced, and to substrate concentration. The Gompertz model assumes that gas production rate is proportional to the microbial activity, but the proportionality parameter decreases with time, according to first-order kinetics, which can be ascribed to loss of efficiency in fermentation rate with time. The Richards model, which is a generalisation of the logistic model, introduces a

fourth parameter (v) allowing some flexibility in the shape of the curve. For v = -1, 0 and 1 the Richards model is reduced to the exponential, Gompertz and logistic model, respectively (Richards, 1959). The Schnute model also is a comprehensive model that includes many other equations as special cases. This model is more flexible and its parameters are statistically more stable than is the case with the Richards model (Schnute, 1981).

Data Set and Gas Production Measurements

Twenty different grass silage samples were obtained from different ensiling experiments using laboratory or farm silos. Samples were oven dried (70°C) and ground through a 1-mm sieve. Samples were selected to obtain variability in stage of maturity, dry matter content, chemical composition and use of silage additives. DM content at ensiling varied from 162 to 491 g/kg DM, NDF content from 228 to 569 g/kg DM, reducing sugars from 11 to 137 g/kg DM, lactate content from 0 to 221 g/kg DM and crude protein from 103 to 280 g/kg DM. The silage with 0 g lactate was obtained by adding formic acid at a rate of 8 ml/kg. Silage samples (.5 g) were incubated with 60 ml HCO_3^-/CO_2 buffered ruminal fluid (Menke et al., 1979). Gas production was followed over time with an automated system based upon the weighing of the amount of liquid (saturated NaCl solution at pH = 1) displaced by the fermentation gas produced. A detailed description of the system and the experimental procedure is given in Beuvink et al. (1992). Samples were incubated in duplicate or in triplicate, resulting in 50 individual gas production curves.

Statistical Comparison of Models

Because for a given gas production curve the residuals from fitted models were not independent, the models could not be compared by F-test or t-test (e.g. Zwietering et al., 1990). We used residual mean squares (RMS) to discriminate between models. Data were fitted to exponential, logistic, Gompertz, Richards, Schnute and modified Gompertz (mGom; see next section) models. The RMS were compared by weighted analysis of variance. Weight for each model was the reciprocal of the variance of RMS. Models were fitted using the statistical program Genstat (Payne et al., 1987).

Description of Modified Gompertz Model

Applying the Gompertz model to gas production, gas production rate is assumed to be proportional to microbial activity, projected as gas production (Y) with a proportionality parameter (μ) :

$$\frac{dY}{dt} = \mu\ Y \qquad [1]$$

The parameter μ, representing the specific gas production rate is governed by a constant (D) describing the decay in specific gas production rate (caused by diminishing growth rate of microorganisms and increasing substrate limitation as reflected in gas production):

$$\mu = \mu_0 \exp[-Dt] \qquad [2]$$

with μ_0 being the value of μ at t=0.
This did not describe our data satisfactorily, especially for the samples that showed a rapid gas production during early stages of fermentation and a slowly increasing asymptote. To obtain a better fit, the parameter μ was modified and divided into two parts, one for rapid early gas production rates (μ_r) and one for the slower gas production rates during later stages of fermentation (μ_s), each with their own fractional decay constants D_r and D_s, respectively:

$$\mu = \mu_{0r} \exp[-D_r t] + \mu_{0s} \exp[-D_s t] \qquad [3]$$

with $\mu_{0r} > \mu_{0s}$ and $D_r > D_s$
Substituting Equation [3] into Equation [1] and integrating gives:

$$Y = Y_0 \exp[\frac{\mu_{0r}}{D_r} \{ 1-\exp(-D_r t)\} + \frac{\mu_{0s}}{D_s} \{ 1-\exp(-D_s t)\}] \qquad [4]$$

However as with $t \to \infty$, the asymptote $Y_\infty = Y_0 * \exp(\mu_{0r}/D_r + \mu_{0s}/D_s)$ is reached, it is more convenient to work with:

$$Y = Y_\infty \exp[-\frac{\mu_{0r}}{D_r} \exp(-D_r t) - \frac{\mu_{0s}}{D_s} \exp(-D_s t)] \qquad [5]$$

From this mGom model (Equation [5]), the following degradation characteristics can be derived. Gas production rate is maximal in the point of inflection at

which the second derivative is zero. Maximum gas production rate is the slope of the tangent through the point of inflection. In the computer program the parameters are approached numerically because they cannot be explicitly calculated from Equation [5]. Length of the initial phase is defined as the t-axis intercept of the tangent line through the point of inflection, and can be calculated after the point of inflection and maximum gas production rate are known. Further parameters to be derived are total gas production (asymptote, Y_∞) and a parameter providing information about the asymptotic phase (arbitrary chosen as the time at which 95% of the asymptote is reached, $t_{.95Y_\infty}$).

Results and discussion

The RMS for exponential, Gompertz, Schnute and mGom models for 50 gas production curves, are depicted in Figure 1. In all cases, exponential fit had the highest RMS. Fitting mGom resulted in RMS lower than or similar to Gompertz fit, which was caused by the fact that the Genstat computer program used Gompertz parameters as starting values for mGom fit and tried to lower RMS.
For the Gompertz, Schnute and mGom models, RMS were generally low; mGom had the lowest RMS for 70% of the curves. Because variation of RMS for the exponential model was much higher than variation of RMS obtained with the other models, RMS were compared by weighted analysis of variance. The RMS

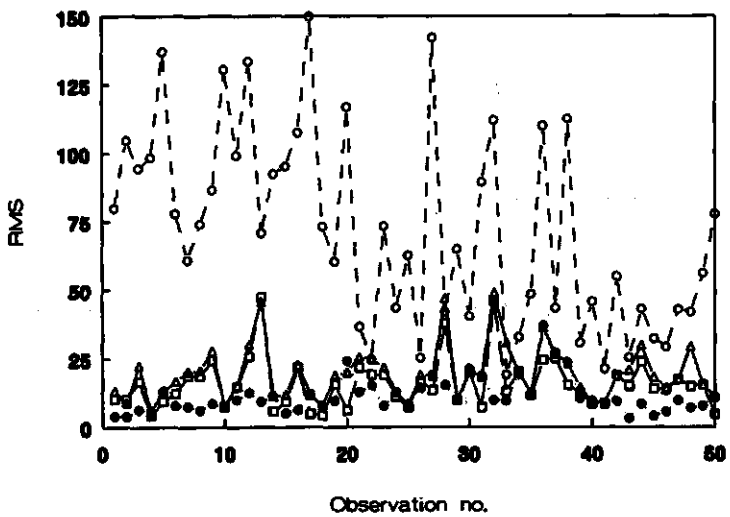

Figure 1. Residual mean square (RMS) of 50 gas production curves of grass silages fitted to exponential (○), Gompertz (△), Schnute (□), and modified Gompertz (●) models.

Table 2. Mean of residual mean squares (RMS; n = 50) obtained after fitting gas production data exponential (Exp), logistic (Log), Gompertz (Gom), Richards (Rich), Schnute (Sch) and modified Gompertz (mGom) models. The LSD, obtained after weighted analysis of variance followed by two-way t-test, are given for comparison of all mean RMS.

MODEL	Mean RMS	LSD Exp	Log	Gom	Rich	Sch
Exp	71.3	-	-	-	-	-
Log	29.8	11.19*	-	-	-	-
Gom	19.7	10.27*	5.83*	-	-	-
Rich	18.2	10.33*	5.93*	3.94	-	-
Sch	15.8	10.24*	5.78*	3.71*	3.86	-
mGom	12.2	10.19*	5.68*	3.55*	3.71*	3.47*

* = *Mean RMS for models in horizontal and vertical rows differ (P < .05).*

for the exponential model were highest (P < .05) followed by logistic RMS (Table 2). Gompertz and Richards models had the same RMS, whereas RMS for Schnute model were lower (P < .05) than those for the Gompertz model, but they did not differ from the RMS for Richards model. The RMS for the mGom model were lower (P < .05) than the RMS for the other five models (Table 2).

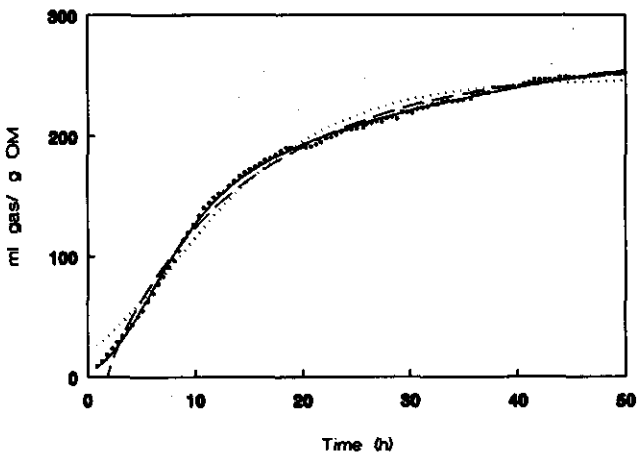

Figure 2. Time course of gas production of grass silage (• ; ml gas/gram of organic matter) and fit to Gompertz (....), Schnute (----), and modified Gompertz (—) models.

Figure 2 illustrates the fit provided by the Gompertz, Schute and mGom models to gas production of one grass silage sample. The Gompertz model underestimated the asymptote and did not give a succesful fit for the exponential part of the curve. The Schnute model did not fit the first part of the curve correctly but fitted the asymptotic phase quite well. However, the mGom model overcame these problems, and gave a good fit of the first stage of fermentation as well as the asymptotic phase. This pattern was obvious for samples having rapid gas production during early stage of fermentation together with a slower development in the asymptotic phase.

The exponential model is widely used in ruminant feedstuff evaluation to describe degradation kinetics as measured with the nylon bag technique (Ørskov and McDonald, 1970) but has also been used to describe kinetic gas production data (Krishnamoorty et al., 1991). Our data show that the fit of an exponential model to gas production data is inferior to all other (sigmoidal) equations discussed here. This might be explained by the following argumentation. Histologically, grass cell walls can be divided into three types: 1) the rapidly fermentable material (mesophyll cells), 2) the slowly fermentable material (bundle sheath cells and sclerenchymal tissue), and 3) the unfermentable fraction existing mainly of lignified vascular tissue (Akin, 1979; Cheng et al., 1980; Harbers et al., 1981; Chesson et al., 1986). Part of the substrate, mainly soluble sugars, may be fermented immediately, but this is only a small part of the potentially fermentable material. As more substrate is hydrated and colonized by microorganisms, the fermentation rate will increase (Van Milgen et al., 1991). From this point of view it is obvious that an exponential model, as often used in feed evaluation systems (Ørskov and McDonald, 1970; Krishnamoorty et al., 1991), shows high RMS when fitted to gas production data because it assumes that after a discrete lag time (in which no apparent activity occurs) the feed is fermented instantaneously at maximum rate.

Gas production is proportional to the amount of metabolic end products (Beuvink and Spoelstra, 1992) produced during bacterial growth. Therefore it is not surprising that gas production curves resemble the sigmoidal growth curves of bacteria grown in batch culture (Zwietering et al., 1990).

Biological Interpretation of the Modified Gompertz Equation

For small t, the terms within square brackets of Equation [5] behave like $-\mu_{0r}/D_r \exp(-D_r t) - \mu_{0s}/D_s$ and therefore

$$Y \approx Y_r = Y_{\infty r} \exp\left[-\frac{\mu_{0r}}{D_r} \exp\left(-D_r t\right)\right] \qquad [6]$$

where $Y_{\infty r} = Y_\infty \exp(-\mu_{0s}/D_s)$. Y_r can be identified with the gas production arising from rapidly fermentable components and $Y_{\infty r}$ with its total gas production.

Therefore $Y_s = Y - Y_r$ must represent the gas production from the slowly fermentable fraction. Only now, Y_s is not a Gompertz function as Y_r is. At this point an interesting feature of our model can be derived. The following Gompertz function has the same asymptote and maximum gas production rate as Y_s:

$$Y_{s,pot} = Y_{\infty s} \exp\left[-\frac{\mu_{0s}}{D_s} \exp\left(-D_s t\right)\right] \qquad [7]$$

where $Y_{\infty s} = Y_\infty - Y_{\infty r}$. The $Y_{s,pot}$ can be interpreted as potential gas production from the slowly fermentable fraction. However, Figure 3 shows that during the first 15 h of fermentation $Y_{s,pot} > Y_s$, indicating that during this period not all the slowly fermentable material is available for fermentation. After 15 h of fermentation $Y_{s,pot} = Y_s$. This feature distinguishes the mGom model from the other models described in this paper. They assume all substrate to be available for fermentation at t = 0. The mGom model takes into account that the slowly degradable fraction is not entirely available for fermentation during early stage

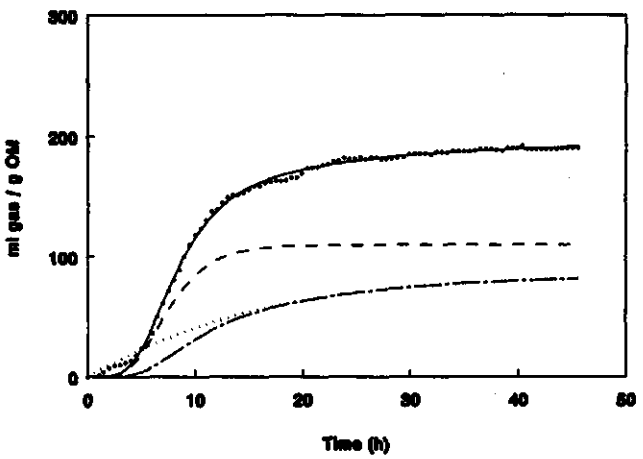

Figure 3. Time course of gas production of grass silage (•) and fit to modified Gompertz model (—). Gas production can be divided by this model into gas production arising from rapidly and slowly fermentable components of the feed (Y_r (--) and Y_s (·--), respectively). The $Y_{s,pot}$ (·····) represents the potential gas production from the slowly fermentable fraction.

of fermentation and that the fermentation of slowly fermentable components is suppressed by fermentation of rapidly fermentable components. For substrates such as grass and grass silages the assumption that all substrate is available for fermentation at t = 0, is clearly not biologically correct. Despite grinding the incubated samples through a 1-mm sieve, particles varied probably in shape, size, and chemical composition; only the outside of the particles was available for hydration, colonization and degradation by microbes.

Rapid gas production was usually completed within 10-15 h, whereas slow gas production took much longer to complete. Chesson et al. (1986) incubated isolated grass cell walls in the rumen of sheep. Loss of DM from mesophyll and epidermis cell walls was completed within 12 h, whereas fibre cell walls showed only a 40% loss of DM over the same period. These findings can be very well described by our model.

From fitted curve parameters, other fermentation characteristics can be derived. The length of the initial phase indicates the time needed for hydration, attachment and colonization by the microbes. The maximum gas production rate gives information concerning the availability of the substrate when the limitations of the initial phase have been dealt with (France et al., 1990). Total gas production (asymptote) has been shown to have a close relationship with digestibility (Menke et al., 1979). Five parameters of the mGom model are sufficient to obtain a complete picture of the gas production curve, namely length of initial phase, maximum gas production rate, total gas production, the time at which gas production rate is maximal and the time at which 95% of the asymptote is reached.

Applying the mGom Model to Other Feeds

The mGom model was developed for grass and grass silages, but its potential for describing gas production curves from other feeds was also examined. Gas production curves (triplicates) of whole crop maize silage (MS), corn cob mix (CCM), chopped maize ear silage (MES), ensiled brewers grains (EBG) and palm kernel expeller (PKE) were fitted to mGom model and compared with Gompertz fit.

Because the comprehensive mGom model encompasses the Gompertz model, these models could be compared by F-test (e.g. Zwietering et al., 1990). The mGom residuals have a normal distribution, but are not independent, so this comparison is at best an approximation. Gom and mGom models were fitted to gas production data and Residual Sum of Squares (RSS) was calculated. The following F-test was performed

$$f = \frac{(RSS_2 - RSS_1) / 2}{RSS_1 / (df_1)}$$ tested against F^2_{df1}

with the null hypothesis that the simpler Gompertz model already gives an adequate fit of the data. RSS_1 and RSS_2 are RSS from mGom and Gompertz model respectively. Df_1 is the number of degrees of freedom for the mGom model (equalling no. of observations minus 5).
For MS, CCM, MES and PKE fitted curves were similar (results not shown, but for EBG a closer fit ($P < .05$) was obtained with the mGom model. The Gompertz model gave an underestimation of the asymptote and higher RMS for the exponential part of the curve (Figure 4).

Implications

The modified Gompertz (mGom) model gives a good description of the gas production curves obtained by incubation of different feedstuffs with buffered ruminal fluid. It describes gas production as the sum of gas production from rapidly and slowly fermentable components. From this mGom model fermentation parameters can be derived, that give information concerning ruminal fermentation kinetics as simulated in vitro. In this way statistical comparison of gas production curves is made possible. Five parameters can provide a complete description of the gas production curve. More research is needed to relate these parameters to biological and physiological processes.

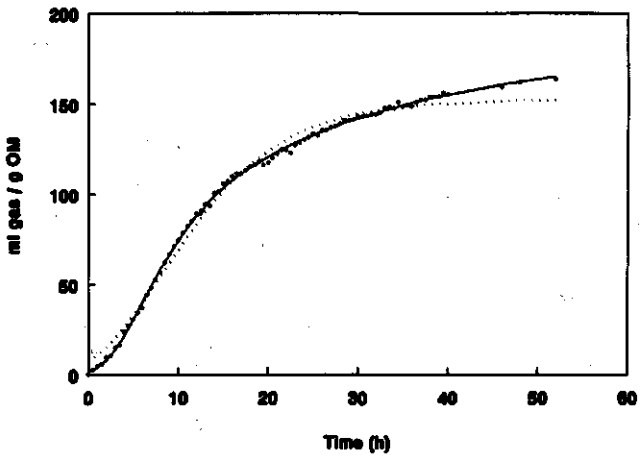

Figure 4. Time course of gas production of ensiled brewers grains (•; ml gas / gram of organic matter) and fit to Gompertz (---) and modified Gompertz (—) models.

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CHAPTER 5

In-vitro gas production kinetics of different maize products: A comparison to nylon bag degradation kinetics

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(submitted for publication)

Summary

In-vitro gas production kinetics of maize silage, chopped ear corn silage and corn cob mix were compared to the disappearance of organic matter from nylon bags. Gas production data were fitted to a modified Gompertz model from which the following parameters were derived: length of lag phase, total gas production, maximum gas production rate, time at which gas production rate was maximal and time at which 95% of the gas was produced. With the nylon bag method, the instantly degradable soluble fraction (S) was determined and the degradation of organic matter in the residues of the nylon bag incubations fitted to an exponential model, including an undegradable fraction (U) and a potentially degradable fraction (D) degraded at constant rate (k_d). The gas production curves showed a sigmoidal shape, whereas the shape of the nylon bag curves was exponential. Total gas production showed a significant relationship with the size of the U-fraction ($p < 0.001$; $R^2_{adj} = 0.936$). The gas prodution parameters showed no relationship with the size of the D-fraction and k_d.

Introduction

In animal feed evaluation, predicting the energy and protein value of feedstuffs is an important issue, but also predicting the rate, pattern and site of digestion becomes more important in high yielding dairy cows. Increasing the rate of digestion in the rumen may result in an increased growth of rumen microorganisms as well as a higher dry matter (DM) intake (Hoover 1986).
In ruminant nutrition the feed degradation process is followed over time by incubating a feedstuff in nylon bags in the rumen of a cannulated animal. Bags are removed periodically and analyzed for disappearance of different feed components (Verité et al. 1979; van Vuuren et al. 1989). However this method is very laborious and expensive. An alternative way of measuring ruminal fermentation kinetics is by following gas production over time of a feedstuff incubated with buffered rumen fluid (Xiong et al. 1990; Pell and Schofield 1993). In the present paper, gas production kinetics of 21 different samples of maize products (whole crop maize silage (MS), chopped ear corn silage (ES), and corn cob mix (CCM)) were compared to the disappearance of organic matter (OM) from nylon bags.

Experimental

Samples and chemical analyses
Samples were taken from eleven whole crop maize silages (MS-1 to MS-11), five chopped ear corn silages (ES-1 to ES-5) and five corn cob mix (CCM 1 to CCM-5). Samples for nylon bag incubations were homogenized in a cutter. Samples used for gas production measurements were oven dried (70 °C) and ground to pass a 1 mm screen.
Dry matter (DM) content of the samples (previously dried at 70 °C) was determined after ovendrying (4h at 104 °C). Neutral detergent fibre (NDF) content was determined according to Robertson and van Soest (1981). In vitro digestibility of organic mater (IVDOM) was measured as described by Steg et al. (1990). Starch was determined enzymatically as with amyloglucosidase on an autoanalyzer (Breda Scientific, Breda, the Netherlands). Before analyzing, sugars were extracted with 40% ethanol and starch was denatured by autoclaving at 121 °C for 2h.

Gas production measurements
Each sample (0.500 g) was incubated in triplicate with 60 ml CO_2/HCO_3^- buffered rumen fluid (Beuvink et al 1992). Rumen fluid was obtained from two

fistulated wether sheep, kept on a daily diet of 800 g hay and 200 g concentrates (low in starch), and fed twice daily. Samples of rumen fluid were taken 2 h after morning feeding. Gas production was measured with a liquid displacement apparatus and automatically registrated every 30 min for 48 h (Beuvink et al. 1992). Gas production was corrected for a blank (buffered rumen fluid without substrate) and calculated to milliliters of gas/ g OM. Gas production data were fitted to a modified Gompertz model, (Beuvink and Kogut 1993):

$$Y = Y_{\infty} \exp\{-\frac{\mu_{0r}}{D_r}(-D_r t) - \frac{\mu_{0s}}{D_s}\exp(-D_s t)\}$$

where Y = gas production at time t, Y_{∞} = total gas production, μ_{0r} and μ_{0s} are specific initial rates, D_r and D_s are degradation constants. From this equation the following parameters were derived. Length of lag phase and maximum gas production rate were calculated by numerical approach. Also calculated was the the time at which the rate of gas production was maximal (T_i). After 48 h, for the incubations with MS-1, MS-2, MS-3, ES-1, ES-2, CCM-1 and CCM-2, VFA concentrations in the medium were determined. Samples were preserved and analysed for volatile fatty acids (VFA) by gas chromatography as described by Robinson et al. (1986b). All concentrations were corrected for VFA present in the blank to obtain the production of VFA.

Nylon bag experiments

Nylon bag incubations were carried out as described by van Vuuren et al. (1989) with four rumen cannulated cows. Bags were removed after 0, 2, 4, 8, 12, 24, 48, 72 and 336 h. The instantly degradable fraction of OM (S) was estimated as the fraction disappearing from the bags during washing (zero incubation time). The residues present in the nylon bags after incubation were fitted by a first order model, including an unsoluble potentially degradable fraction (D), degraded at a constant rate (k_d), and an undegradable fraction (U, calculated from the residu after 336 h), without a discrete lag time (Robinson et al. 1986a).

Statistical evaluation

We chose to compare the two methods the way they are normally carried out at IVVO-DLO, even though this meant differences in sample pre-treatment and modelling of the data . Since gas production reflects degradation of OM, gas production kinetic parameters were compared to disappearance of OM from

nylon bags. Correlations between parameters obtained with the two methods were determined by linear regression. It was assured that all the residuals were equally distributed among zero.

Results

Chemical composition and IVDOM are given in Table 1. The samples represented a wide range in starch and NDF contents and in IVDOM. Higher starch contents were positively correlated with lower NDF contents and in general with a higher IVDOM.

Table 1. Chemical composition (g/kg DM) and in vitro digestibility (IVDOM) of samples used. DM = DM content at harvesting (g/kg); NDF = Neutral Detergent Fibre; IVDOM = % OM digested in vitro.

Sample	DM	Starch	NDF	IVDOM
MS-1	373	303	418	73.8
MS-2	312	211	451	74.6
MS-3	283	260	422	73.0
MS-4	223	212	455	75.4
MS-5	276	242	448	74.1
MS-6	353	273	477	73.9
MS-7	421	346	407	74.2
MS-8	349	313	386	72.8
MS-9	258	180	470	71.0
MS-10	258	251	448	75.0
MS-11	302	280	426	75.4
ES-1	547	535	235	84.0
ES-2	445	536	223	84.2
ES-3	516	543	218	84.2
ES-4	579	560	193	83.9
ES-5	611	575	488	82.3
CCM-1	630	650	100	89.9
CCM-2	574	644	87	88.1
CCM-3	493	576	148	87.6
CCM-4	610	587	113	88.8
CCM-5	661	685	93	89.1

Figure 1a and 1b show examples of gas production curves and disappearance of OM from nylon bags for MS, ES, and CCM. The gas production curves showed a sigmoidal shape, whereas the nylon bag curves had a more exponential shape. In general CCM had highest total gas production followed by ES and MS (Table 2). Maximum gas production rate was highest for the CCM samples and lowest for the MS samples. ES and CCM samples showed a longer lag phase than MS. With the nylon bag experiments, the U-fraction was lowest for CCM and highest for MS. The opposite was true for k_d, were CCM had the highest and MS the lowest values. Within a product group the S-fraction varied at the cost of the D-fraction, whereas the U-fraction was fairly constant.

Table 2. Results of curve-fitting of samples

Sample	Gas production parameters				Nylon bag parameters			
	Tot	Mrate	Lag	T_i	S	D	U	k_d
MS-1	219	14.8	2.0	9.0	33.7	50.6	15.7	2.77
MS-2	187	12.3	1.7	7.3	34.0	49.7	16.3	2.76
MS-3	176	12.6	2.3	8.6	36.8	45.6	17.6	2.63
MS-4	176	14.1	3.6	7.7	30.5	49.8	19.7	2.80
MS-5	173	12.7	2.4	7.4	24.8	56.3	19.0	2.81
MS-6	154	14.4	4.8	8.8	17.3	62.9	19.8	2.45
MS-7	162	12.8	6.3	8.9	15.3	64.8	19.9	2.66
MS-8	171	13.8	1.7	6.1	36.8	45.3	17.9	2.18
MS-9	177	12.4	1.6	6.6	26.5	53.5	20.2	2.57
MS-10	177	11.6	1.2	6.2	28.1	54.6	17.3	2.84
MS-11	180	14.7	1.8	6.3	23.1	58.6	18.4	2.93
ES-1	257	18.0	4.9	9.8	21.4	71.0	7.6	3.66
ES-2	252	23.2	5.2	8.2	61.1	32.2	6.7	2.42
ES-3	272	21.5	6.3	9.3	41.8	50.9	7.2	2.94
ES-4	215	21.6	5.5	9.2	34.2	55.5	10.3	3.46
ES-5	266	18.1	6.3	8.7	26.7	63.2	10.1	3.72
CCM-1	289	18.5	5.5	10.9	34.9	63.5	1.6	3.95
CCM-2	284	26.6	6.1	8.3	73.9	23.9	2.2	4.59
CCM-3	298	26.9	7.1	10.2	73.0	22.2	4.8	2.39
CCM-4	311	24.2	6.3	9.9	58.8	39.1	2.2	4.56
CCM-5	313	20.6	7.3	11.9	42.7	55.0	2.3	3.61

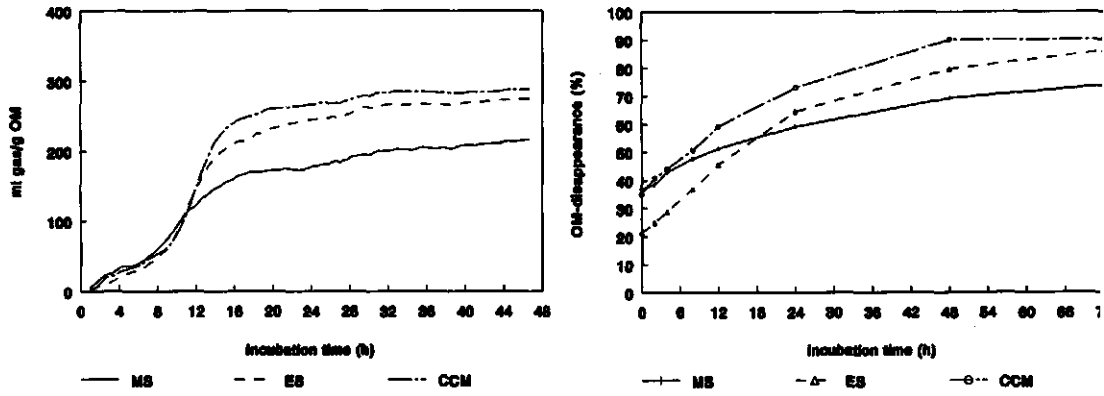

Figure 1A, 1B. Gas production curve (A) and disappearance of OM from nylon bags (B) for one sample of whole crop maize silage (MS), chopped ear corn silage (ES) and corn cob mix (CCM).

Table 3 gives the adjusted R^2 (R^2_{adj}) for the linear regression between the fitted parameters obtained with the two methods. Total gas production showed a significant negative relationship ($p < 0.001$; $R^2_{adj} = 0.936$) with the U-fraction (Figure 2). Since S + D = 1-U, a similar positive relationship therefore existed

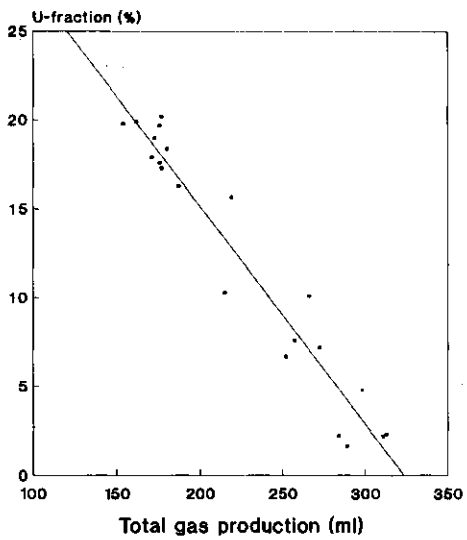

Figure 2. Relationship between total gas production and U-fraction.
$U = 39.8 - 0.123\ Y_\infty$; $p < 0.001$; $R^2_{adj} = 0.936$.

between total gas production and total OM-disappearance from nylon bags. The U-fraction, was negatively correlated to the maximum gas production rate (U = 33.7 - 1.23 max.rate; $p < 0.01$; $R^2_{adj} = 0.757$). The D-fraction and k_d were poorly correlated to kinetic gas production parameters.
The kinetic gas production parameters generally had good correlations with chemical composition and with IVDOM (Table 4). For nylon bag parameters, U was the parameter that correlated best with chemical composition and IVDOM. Degradation rate (k_d) showed no correlation with chemical composition.
Between the different product-groups, differences in VFA composition were observed in the gas production incubation mixture after 48 h (Table 5). ES and CCM samples had somewhat lower acetic and propionic acid production and higher butyric acid production than MS samples.

Discussion

The nylon bag degradation curves (disappearance of OM from the D-fraction) could very well be described by the mGom model, with residual mean squares equal to or lower than those obtained with the exponential model (results not shown). However, gas production curves obtained with maize fodders could not be fitted by the exponential model as was also shown for grass silages by

Table 3. Adjusted R^2 for comparison of fitted parameters for disappearance of organic matter from nylon bags and gas production kinetics. For explanation of the parameters see text.

Fitted parameter	S-fraction	D-fraction	U-fraction	k_d
Lag	0.142	#	0.522^{***}	0.197^{*}
Max. rate	0.667^{***}	-0.322^{**}	-0.757^{***}	0.238^{*}
Y_∞	0.425^{***}	-0.074	-0.936^{***}	0.416^{***}
T_i	0.036	#	0.508^{***}	0.180^{*}

*, **, *** = significant relationship at $p<0.05$, $p < 0.01$ and $p < 0.001$ respectively; # = Residual variance exceeded variance of y variate.

Table 4. Adjusted R^2 for comparison of fitted parameters for disappearance of organic matter from nylon bags and gas production kinetics to chemical data and in vitro digestibility. For explanatation of the parameters see text and Table 2.

Fitted parameter	DM	Starch	NDF	IVDOM
Lag	0.684***	0.707***	-0.636***	0.641***
Max. rate	0.556***	0.734***	-0.776***	0.777***
Y_∞	0.734***	0.827***	-0.878***	0.883***
T_i	0.602***	0.555***	-0.500***	0.543***
S-fraction	0.012	0.286**	-0.394**	0.376**
D-fraction	#	#	0.053	-0.039
U-fraction	-0.775***	-0.894***	0.938***	-0.954***
k_d	0.492***	0.416***	-0.469***	0.461***

Beuvink and Kogut (1993). Sauvant et al. (1985) concluded that a Gompertz model described the in sacco dry matter digestion of various concentrates and by-products better than an exponential model.

The different shape of the curves obtained with the two methods (Figure 1) is caused by the principles on which these methods are based. The nylon bag method is a gravimetrical method, based on the disappearance of feed components from nylon bags. The gas production method is based on microbiological principles, namely measuring gas, being a fermentation end product of the rumen microbes. With the latter method and in modelling the data, the whole sample is taken into account, whereas for the nylon bag data, only degradation of the U+D-fractions is modelled, because it is assumed that the S-fraction is degraded instantly at an infinite rate. The lag period observed with the gas production method indicates that this assumption might be a false one, though the lag phase might partially be ascribed to the fact that the microflora of the rumen fluid was not fully adapted to the degradation of starch. However, for the parameters based on the total material (Y_∞ compared to U) the two methods agreed very well.

Tamminga et al. (1990) found no relationship between solubility and the rate of disappearance of starch from nylon bags for various concentrates. In the present work, the S-fraction was positively correlated with the maximum gas production rates of ES and CCM (S = -73.0 + 5.47 max. rate; $p < 0.001$; $R^2_{adj} = 0.908$), but was not correlated with those of MS. The size of the S-fraction is influenced by factors such as the stage of maturity of the plant and

harvesting and sample (pre-)treatments (e.g chopping and grinding). The same factors influence availability of substrate for the rumen microorganisms, which at its turn determines the maximum gas production rate. The more substrate is available, the higher the maximum gas production rate will be. We found no relationship between the rate of disappearance of organic matter (k_d) and the maximum gas production rate. This supports the idea that disappearance of feed components from nylon bags does not necessarily mean that this fraction is also fermented at the same time. The fraction disappearing from the nylon bags consists of substrate that is truly solubilized and of small substrate particles smaller than the pore size of the bag. The latter fraction still needs further degradation before it can be fermented by rumen microorganisms.

The ES and CCM gas production curves resembled the curves obtained with pure starch by Beuvink et al. (1992), indicating that availability of starch was responsible for their shape. With MS, the shape of the curves was also determined by fermentation of NDF, which played a minor role with ES and CCM.

After 48 h of fermentation, the relative amounts of propionate were lower for ES and CCM than for MS (Table 5). Beuvink and Spoelstra (19920 previously observed low relative amounts of propionate after 48 h incubation with rice starch, compared those with glucose and cellulose. Starch fermenting microorganisms are known to be propionate producers (Russell and Wallace 1988). In our gas production system, buffering capacity is high, which ables the pH to stay above a value of 6.2 (Beuvink and Spoelstra 1992). In in-vitro systems, at relatively high pH values (6-7), the proportions of propionic acid are decreased compared to lower pH values (5-6) (Erfle et al. 1982).

Table 5. Volatile fatty acids (VFA) produced after 48 h incubation in gas production experiments. All figures are corrected for VFA contents in the blank and are average of three incubations. Figures in parentheses represent standard deviations. HAc = acetic acid; HP = propionic acid; HB = butyric acid.

Sample	Total mmol/l	%HAc	%HP	%HB
		----------mmol/100 mmol------		
MS-1	48.1 (4.39)	62.9 (0.68)	18.5 (0.07)	18.5 (0.65)
MS-2	45.5 (0.20)	61.8 (0.05)	22.0 (0.10)	16.3 (0.15)
MS-3	47.4 (4.46)	61.5 (0.23)	21.0 (0.16)	17.5 (0.37)
ES-1	51.5 (1.76)	58.3 (0.46)	14.0 (0.55)	27.7 (1.01)
ES-2	51.6 (3.05)	62.5 (0.61)	13.1 (0.84)	24.3 (0.92)
CCM-1	49.7 (1.76)	55.8 (0.35)	13.0 (0.59)	31.3 (0.94)
CCM-2	51.2 (5.25)	59.7 (1.51)	12.2 (0.55)	28.1 (1.72)

Because of the small amount of sample to be incubated in the gas production method, drying and grinding of the samples was necessary to obtain a homogenous sample. The consequences of this were smaller particle sizes and probably some damaging of the starch (Rooney and Pflugfelder, 1986). Other possible factors causing errors might be the donor animals (cow or sheep) or their diet. Degradation of starch in rumen fluid from a hay-fed cow was significantly lower than in rumen fluid from a concentrate fed cow (Cone et al. 1989). However, it seemed that ranking of the degree of degradability between feedstuffs was not determined by the ration of the donor cow, but merely by the proporties of the starch (Cone et al. 1989).

Both methods, conducted the way they are performed at IVVO-DLO, show little agreement. Many factors can cause this, like composition of the inoculum (depending on the donor animal), pH of the buffer or pretreatment of the samples. However, for the feed samples used in this paper, a quick estimate of the size of the U-fraction can be obtained by measuring the gas production after 48 h. With the nylon bag method, disappearance of different feed components (like NDF, starch, protein) are determined after different time intervals. The gas production method only gives information about the fermentation of carbohydrates, but the method can be extended to measure disappearance of feed residues, by terminating the incubations and analyzing the residue at various time intervals.

Future research should supply more information about the physiological meaning of in-vitro gas production kinetics.

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CHAPTER 6

In-vitro gas production kinetics of enzyme treated grass silages

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Summary

Silages were treated with six different enzymes and ensiled under different conditions (dry matter (DM) content, stage of maturity). Silage samples were dried, ground and incubated with buffered rumen fluid. Gas production was followed in time and gas production curves were fitted to a modified Gompertz equation.

In general, enzyme treated silages showed a shorter initial phase and lower maximum gas production rate, indicating that the enzymes degraded cell wall material to more rapidly fermentable components, leaving a more slowly fermentable fraction. Total amount of gas was not altered but only a shift towards quicker fermentable material was observed. These changes in gas production kinetics went hand in hand with a change in chemical composition (more degradation of neutral detergent fibre and higher production of lactic acid in enzyme treated silages than control silages). Enzyme preparations that did not contain cellulase showed no difference with control silages, nor in gas production kinetics nor in chemical composition.

Wilting up to 300 g DM kg^{-1} had no influence on gas production kinetics whereas wilting up to 488 g DM kg^{-1} caused longer initial phase, lower maximum rate and lower total gas production. With increased maturity, lower total amounts of gas and lower maximum rate were observed.

Measuring changes in gas production kinetics of enzyme treated silages compared to control silages is a powerfull and rapid tool in prescreening effects of enzyme addition.

Introduction

Addition of cell wall degrading enzymes to grass silage may serve two purposes. Firstly the liberation of extra fermentable sugars to enhance silage fermentation, and secondly, predigestion of cell walls in the silo resulting in an improved intake or digestibility.

Enzymes used as silage additives are mostly from fungal origin and contain many activities of which cellulases and hemicellulases are the major ones. A number of workers have shown that addition of such enzyme mixtures to grass silages leads to reduced cell wall contents and increased lactic acid concentrations compared to untreated silages (Henderson et al., 1982; McHan, 1986; van Vuuren et al., 1989).

Literature on animal performance is not uniform. No effect on intake (Kennedy, 1988; Jacobs et al., 1991) as well as an increase in intake (Steg et al., 1989; Jakkola, 1990; Chamberlain and Robertson, 1992) have been reported. Similarly, digestibility of organic matter (OM) has been found to be improved (Chamberlain and Robertson, 1992), to remain unchanged (Jaster and Moore, 1988) or to be decreased (Jaakkola and Huhtanen, 1990; Jacobs and McAllan, 1991).

In vitro experiments show a higher OM digestibility for enzyme treated silages (McHan, 1986; Forwood et al., 1990) or unchanged digestibility (van Vuuren et al., 1989).

To investigate the effects of enzyme treatment on the rate of digestion in the rumen, time course studies have been made by incubating samples in nylon bags in the rumen. Results show a higher directly soluble fraction and lower rate of digestion of the remaining cell wall components (van Vuuren et al., 1989; Jacobs and McAllan, 1991). Similar results have been obtained by measuring in vitro dry matter (DM) disappearance at different time intervals (McHan, 1986; Forwood et al., 1990).

For the evaluation of ruminal behaviour of feedstuffs, these methods show some drawbacks. They are laborious and measure DM disappearance rather than fermentation of DM. Nevertheless, in vitro methods imitating rumen digestion appear to be the method of choice for a first judgement of the potential of new and well defined enzyme activities for use as silage additives.

We feel that measuring the time course of in-vitro gas production as described by Beuvink et al. (1992) provides relevant information about fermentation and rate of fermentation of feedstuffs by rumen microorganisms (Beuvink and Spoelstra, 1992).

In this paper we investigated the effects of different cell wall degrading enzymes, applied to grass ensiled under different conditions, on fermentation

kinetics by measuring in-vitro gas production.

Materials and methods

Enzyme preparations

The following enzyme preparations were used: Maxazym (C) a solid cellulase preparation from *Trichoderma viride* containing 170 g ECP kg^{-1}; Hemicellulase (H) a hemicellulase preparation from *Aspergillus niger* containing 334 g ECP kg^{-1}; Rapidase (P) a pectinolytic preparation from *Aspergillus niger* containing 34 g ECP kg^{-1}. These enzyme preparations were obtained from Gist-brocades, Delft, The Netherlands. The following mixtures of these enzyme preparations were used: HC (47% (w/w) H and 53% C), HP (22% H and 75% P), CP (20% C and 80%) and HCP (13% H, 8% C and 71% P).

In addition the following commercially available enzyme based silage additives were used: Rohacent 7056 (Röhm Gmbh, Darmstadt, Germany; here referred to as enzyme E, containing 125 g ECP kg^{-1}) and Clampzyme (Finnish Sugar Co. Ltd., Finland; here referred to as enzyme F, containing 99 g ECP kg^{-1}).

Unwilted silage (163 g DM kg^{-1}) of the youngest material (224 g CF kg^{-1} DM) treated with the above mentioned enzymes at a level of 0.2 g ECP kg^{-1} grass and the untreated control silages were evaluated through in vitro gas production kinetics.

Silages

One third of a 0.3 ha plot, of predominantly perennial ryegrass, was mown on May 9th, May 25th and June 14th 1988, thus obtaining different stages of maturity as indicated by crude fiber (CF) content. The grass was sampled directly after mowing (about 200 g DM kg^{-1}) and, for the material mown on May 9th, after wilting to about 300 and 450 g DM kg^{-1}. Each combination of mowing date and DM was treated with 0 (control), 0.2 g enzyme crude protein (ECP) of enzyme F kg^{-1} grass. Unwilted material of May 9th was also treated with 0 and 0.2 g ECP kg^{-1} grass of the enzymes HC, HP, CP, HCP and E. The grass was ensiled (in duplicate) in 1-l laboratory silos which were stored for three months at ambient room temperature before opening and analyzing.

Sample preparation

Silage samples were oven dried at 70 °C for 24 h, ground over a 1 mm screen and stored in air tight glass flasks at ambient temperature. For gas production measurements the duplicate silo samples were pooled.

Determination of gas production kinetics

Gas production upon incubation of samples with buffered rumen fluid was monitored during 48 h in the automated system described earlier (Beuvink et al., 1992). Samples were incubated triplicate within a serie and every series was conducted twice. Gas production was expressed as ml gas g^{-1} OM.

Gas production was also calculated as ml gas g^{-1} Fermentable Organic Matter (FOM), with FOM being OM minus lactic acid content, thus correcting for the general higher lactic acid contents of the enzyme treated silages. This correction is desirable because fermentation of lactic acid does not result in gas production (Counotte, 1983). Other fermentation products in the silo were not taken into account, since their concentrations were low compared to lactic acid.

Gas production curves were fitted to a modified Gompertz equation, basically dividing the material fermented into a quickly and a slowly fermentable fraction (Beuvink & Kogut, 1992):

$$Y = Y_{\infty} \exp\left\{-\frac{\mu_{0r}}{D_r}\exp(-D_r t) - \frac{\mu_{0s}}{D_s}\exp(-D_s t)\right\}$$

where Y = the amount of gas produced at time t, Y_{∞} = the upper asymptote representing the total amount of gas produced, μ_{0r} and μ_{0s} are specific initial gas production rates for the rapidly and slowly fermentable fraction, respectively, D_r and D_s are the fractional degradation constants for the two fractions.

From this modified Gompertz equation the following parameters can be numerically approached: length of initial phase, maximum gas production rate and time point at which gas production rate is maximal (T_i). Also calculated were total gas production and time point at which 95% of the gas had been produced ($T_{0.95}$).

Chemical analyses

Oven dry matter, ash, crude fibre and total sugars were determined according to the procedures of the Netherlands Normalization Institute (NEN 3332, NEN 3329, NEN 3327 and NEN 3571, respectively). Lactic acid was determined as aceet aldehyde by gas chromatography (Spoelstra, 1983). Neutral Detergent Fibre (NDF) content was determined according to Robertson & van Soest (1981).

Oven DM was corrected for loss of volatile fermentation products assuming that ethanol, acetic acid, propionic acid and butyric acid were completely volatilized during oven drying.

Chemical composition of the silages is given in Table 1.

Table 1. Chemical composition of silages (g kg^{-1} DM), ensiled with addition of different enzymes and under different ensiling conditions.

	Grass			Silage		
Treatment	DM	CF	NDF	NDF	Sugars	Lactate
Control	163	224	470	401	12	108
Enzyme HC				228	17	178
Enzyme HP				373	13	87
Enzyme CP				234	14	176
Enzyme HCP				268	15	165
Enzyme E				288	13	117
Enzyme F				289	17	221
None	163	224	470	386	11	93
Enzyme F	163	224	470	289	17	221
None	300	224	470	498	16	101
Enzyme F	300	224	470	434	25	115
None	488	224	470	569	35	17
Enzyme F	488	224	470	555	43	16
None	210	254	515	394	15	92
Enzyme F	210	254	515	354	18	121
None	247	308	586	432	18	91
Enzyme F	247	308	586	416	17	104

Statistical procedures

The significance of addition of enzymes and influence of ensiling conditions (DM content and stage of maturity) on kinetic gas production parameters (length of initial phase, maximum gas production rate, total gas production, T_i and $T_{0.95}$) was determined using analysis of variance followed by two way t-test. Since the greatest source of variation in gas production is caused by different batches of rumen fluid (Beuvink et al., 1992), data were divided into two blocks, each block comprising the measurements within one series (GENSTAT, 1987).

Results

Enzyme preparations

Silages treated with enzymes HC, CP, HCP and F showed a shorter initial phase ($p < 0.001$) than the untreated control silage (Table 2). For enzyme E initial phase tended to be shorter but the difference with control was not significant. Compared to the control, maximum gas production rate was lower for the

Table 2. Contents of NDF and lactate and gas production kinetics of grass ensiled with different enzymes. Grass was ensiled at 163 g dry matter kg^{-1} and 224 g crude fiber kg^{-1}. Gas production kinetics were calculated using a modified Gompertz equation (see text).

	Enzyme treatment[b]							
	Control	HC	HP	CP	HCP	E	F	sed
NDF (g kg^{-1} DM)	440	281[a]	439	298[a]	310[a]	346	331[a]	47.5
Lactate (g kg^{-1} DM)	104	226[a]	103	221[a]	197[a]	140	253[a]	24.9
Initial phase (h)	1.9	0.1[a]	1.8	0.2[a]	0.2[a]	1.0	0.1[a]	0.45
Max. rate (ml h^{-1} g^{-1} OM)	13.5	9.2[a]	11.9	10.2[a]	9.6[a]	12.0	9.3[a]	1.09
Total gas (ml g^{-1} OM)	145	125	153	119[a]	115[a]	163	139	11.9
T_i (h)	5.8	5.2	6.5	4.4[a]	4.0[a]	6.0	5.5	0.49
$T_{0.95}$ (h)	17.2	20.8	20.9	18.4	22.8[a]	21.6	26.1[a]	2.33
After correction for lactic acid								
Max. rate (ml h^{-1} g^{-1} OM)	15.1	11.6[a]	13.2	12.7	11.8[a]	13.9	12.4[a]	1.33
Total gas (ml g^{-1} FOM)	165	159	170	149	141	189	186	13.8

OM = Organic Matter; FOM = Fermentable Organic Matter

a) different from control ($p < 0.05$); b) DF = 34

Table 3. Contents of NDF and lactate and gas production kinetics of grass ensiled without (Control) and with enzyme preparation F (Treated) at different dry matter (DM) contents (DF = 29). Grass was ensiled at 224 g crude fiber kg^{-1}. Gas production kinetics were calculated using a modified Gompertz equation (see text).

	163 g DM kg^{-1}		300 g DM kg^{-1}		488 g DM kg^{-1}		SIGNIFICANCE		
	Control	Treated	Control	Treated	Control	Treated	DM	ENZ	DM*ENZ
NDF (g kg^{-1} DM)	449	331	419	371	460	433	***	***	**
Lactate (g kg^{-1} DM)	109	253	107	121	18	17	***	***	***
Initial phase (h)	3.5	2.6	3.4	2.7	3.6	3.2	*	***	NS
Max. rate (ml h^{-1} g^{-1} OM)	20.8	14.2	21.5	17.8	16.1	16.8	***	***	***
Total gas (ml g^{-1} OM)	175	138	171	162	153	163	NS	**	***
T_i (h)	6.5	6.2	6.1	6.0	6.8	6.6	***	NS	NS
$T_{0.95}$ (h)	19.0	17.4	19.2	17.4	29.0	20.6	NS	NS	NS
Max. rate (ml h^{-1} g^{-1} FOM)	23.2	18.9	24.3	20.4	16.4	17.5	***	***	***
Total gas (ml g^{-1} FOM)	198	184	193	186	155	164	***	NS	NS

NS = not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 4. Gas production kinetics of grass ensiled without (Control) and with enzyme preparation F (Treated) at different stages of maturity (as indicated by crude fiber (CF) content; DF=29). Grass was ensiled at 163 g dry matter kg^{-1}. Gas production kinetics were calculated using a modified Gompertz equation (see text).

	224 g CF kg^{-1}		254 g CF kg^{-1}		308 g CF kg^{-1}		SIGNIFICANCE		
	Control	Treated	Control	Treated	Control	Treated	CF	ENZ	CF*ENZ
NDF (g kg^{-1} DM)	449	331	585	500	648	610	***	***	**
Lactate (g kg^{-1} DM)	109	253	108	140	104	115	***	***	***
Inital phase (h)	2.2	1.2	2.7	0.2	1.4	0.2	*	***	*
Max. rate (ml h^{-1} g^{-1} OM)	17.5	11.9	12.3	10.9	10.6	8.2	***	***	*
Total gas (ml g^{-1} OM)	186	152	162	174	154	143	NS	NS	NS
T_i (h)	6.0	5.9	7.5	5.7	6.6	6.3	NS	*	*
$T_{0.95}$ (h)	31.3	21.5	22.6	30.0	23.9	27.6	NS	NS	NS
Max. rate (ml h^{-1} g^{-1} FOM)	19.5	15.7	13.7	12.6	11.8	9.2	***	***	NS
Total gas (ml g^{-1} FOM)	208	200	181	202	171	161	**	NS	NS

NS=not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

silages treated with enzymes HC, F ($p < 0.001$), CP and HCP ($p < 0.01$), but not with HP and E. Addition of the enzymes CP and HCP resulted in a lower ($p < 0.05$) total gas production. Point of inflection (T_i) was earlier with CP ($p < 0.01$) and HCP ($p < 0.001$). After correction for lactic acid, silages treated with enzymes HC, CP and F still showed lower maximum gas production rates ($p < 0.05$), but total amounts of gas produced from control and treated silages were similar.

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DM content

Addition of enzyme F shortened length of initial phase ($p < 0.001$) (Table 3). Maximum gas production rate and total gas production showed strong interaction ($p < 0.001$) with DM content and enzyme addition. At higher DM contents, the effect of enzyme addition on maximum gas production rate and total gas production diminished. Silages with 488 g DM kg^{-1} had a longer initial phase ($p < 0.05$) than silages with 163 and 300 g DM kg^{-1}. T_i was only influenced by DM content and $T_{0.95}$ was not influenced by DM content or enzyme addition.

After correction for lactic acid, total amount of gas produced was only influenced by DM content and no longer by enzyme treatment as was the case for the non corrected data. Total gas production was lower at 488 g DM kg^{-1} ($p < 0.001$) than at 163 and 300 g DM kg^{-1}.

Stage of maturity

There was interaction ($p < 0.05$) between the effects of stage of maturity and enzyme addition on length of the initial phase (Table 4). Maximum gas production rate diminished with increasing maturity and the effect of enzyme addition was larger at lower CF contents. No effects of either treatment on total gas production or on $T_{0.95}$ were observed.

After correction for lactic acid, maximum gas production rate was lower upon enzyme addition ($p < 0.001$) and with increasing maturity ($p < 0.001$). Total gas production was lower with increasing maturity ($p < 0.01$).

Discussion

Enzymes

All enzymes, except HP, reduced NDF content in the silo and increased lactic acid concentration. These effects on silage composition appeared to be closely related to effects on gas production kinetics. Changes in gas production kinetics were most obvious for enzymes that had effect on silage composition namely HC, CP, HCP, E and F (Tables 1 and 2).

In the rumen, lactic acid is fermented mainly to propionic acid via the acrylate pathway at which no gas is being released (Counotte, 1983). Since a great part of the material liberated by the enzymes had been fermented to lactic acid in the silo, total amount of fermentable, potentially gas producing material was lower in the enzyme treated silages. This resulted in a lower maximum gas production rate and lower total gas production for enzyme treated silages compared to their accessory controls. Lactic acid is nevertheless of energetic value to the ruminant, so correction of OM for lactic acid, resulting in FOM, was necessary to get a fair comparison of the samples. This correction resulted in a stretching out of the gas production curve along the Y-axis, but not along the X-(time) axis, thus giving corrected values for maximum gas production rate and total gas production.

The enzymes HC, HP, CP and HCP were all composed from the same original enzyme preparations. However, only enzyme HP showed no difference with control, neither in gas production kinetics nor in chemical composition. This enzyme was the only one of these four that did not contain cellulase, so apparently the cellulase component was necessary to establish changes both in gas production kinetics and chemical composition.

Of the structural cell wall components, cellulose is most easily degraded by enzymes (Henderson et al., 1982; van Vuuren et al., 1989; Jacobs and McAllan, 1991). The lower maximum gas production rates of enzyme treated silages indicated a lower rate of digestion of the remaining cellulose (van Vuuren et al., 1989).

The synergism between cellulases, hemicellulases and pectinases observed by Tengerdy et al. (1991) could not be detected in our experiments.

Gas production kinetics

During the initial phase several processes take place like fermentation of soluble sugars, hydratation, attachment and colonization of the insoluble substrate by microorganisms (Cheng et al., 1980). Enzyme addition shortened the length of this initial phase indicating that enzymic degradation of cell walls resulted in the liberation of quickly fermentable material. The sum of residual sugars and fermentation end products (lactic and acetic acid and ethanol) could not account for all of the NDF that had disappeared during ensiling. Therefore enzymes must have degraded cell wall material to readily fermentable fragments that were too large to be analyzed as sugars and too small to be analyzed as NDF.

Nylon bag incubations (van Vuuren et al., 1989; Jaakkola et al., 1991) also showed an increase in the content of soluble cell wall fractions of grass silages upon enzyme treatment. Our results show that the enzymically liberated

material was available for (immediate) fermentation, a conclusion that could not be drawn from in-sacco incubations measuring DM disappearance.

The faster initial fermentation took place at the cost of maximum fermentation rate, indicating that the enzymes degraded cell wall material to more rapidly fermentable fragments, leaving a more slowly degradable fraction. This agrees with the lower rate of cell wall degradation found in nylon bags (van Vuuren et al., 1989) and with reduced crude fibre digestibility in vivo (Jaakkola, 1990).

With increased maturity, the total amount of gas produced decreased. Menke et al. (1979) used total amount of gas produced to estimate in vivo digestibility. So our data show lower digestibility with the older grass which is in agreement with well known effect of maturity on digestibility. In addition, maximum gas production rate also decreased showing that in the older grass less fermentable material was avialable at all stages of fermentation. This might also reflect rate of fermentation in the rumen.

Enzyme treatment had no effect on total gas production so addition of enzymes did not influence the total amount of fermentable material but caused a shift towards quicker fermentable material.

Wilting up to 300 g DM kg^{-1} had no influence on gas production kinetics, but wilting up to 488 g DM kg^{-1} caused lower maximum gas production rate ($p < 0.05$), lower total gas production ($p < 0.001$) and longer initial phase ($p < 0.05$). Wilting took place under ideal weather conditions (dry, sunny), so length of the field period (less than 24 h) and subsequent losses were kept to a minimum.

It might well be that wilting up to high DM contents causes high temperatures in the field, resulting in a diminished availability of nitrogen and carbohydrates to microorganisms (due to Maillard reactions) but the precise reason is unclear.

Measuring in vitro gas production kinetics appeared to be a good method to discriminate between effects of cell wall degrading enzymes on rumen fermentation. Differences between treated and untreated silages and also between enzymes were demonstrated.

Enzymes that clearly improve animal performance have not been described so far. The future might be in new enzymes with well defined activities. We think that measuring changes in gas production kinetics of enzyme treated silages in comparison to control silages is a powerfull and rapid tool in prescreening the effects of such enzymes. However, definite effects on animal performance remain, as allways, to be demonstrated by animal experiments.

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CHAPTER 7

Influence of free and esterified phenolic acids on in-vitro ruminal fermentation kinetics of maize stems

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Summary

Maize stems were incubated with buffered rumen fluid to which p-coumaric acid (PCA) or ferulic acid (FA) was added at 0, 0.1, 0.5, 1.0 or 2.0 g/l. Degradation of organic matter was determined by measuring gas production over time. During the first 8 h of fermentation, gas production was not influenced by the addition of phenolic acids. However, after this period the 1.0 and 2.0 g/l PCA incubations showed reduced gas production and reduced dry matter disappearance. No inhibitory effects of FA addition were observed. Recovery of PCA and FA was low (less than 12%, and trace amounts, respectively). PCA appeared to be metabolized to phloretic acid and to a lesser extent to 3-phenylpropionic acid. In the FA incubations, no phenolic metabolites could be detected after 48 h.

To determine the effects of naturally occurring phenolic acids on fermentation kinetics, samples of stems of five different maize inbred lines, differing in phenolic acid content (extractable with 1.0 M NaOH) were incubated with buffered rumen fluid. The ratio PCA:FA in the solid residue increased during incubation due to a more extensive removal of FA than of PCA. The absolute

amounts of PCA and FA released during fermentation were different for the five maize varieties, but all samples had the same PCA and FA concentrations in the undigested residue. This suggested that the phenolic acids, extractable with 1.0 M NaOH could be divided into two pools. One that could be removed by rumen microorganisms during 48 h incubation and onother pool that could not be removed. Although there was a relationship between PCA and FA contents and gas production kinetics, it was not clear wether this relationship was causal because of the interaction with cell wall content.

Introduction

Phenolic acids are common constituents of plant cell walls with p-coumaric (PCA) and ferulic acid (FA) being the major ones (Jung and Fahey 1983; Eraso and Hartley 1990). They are known to crosslink lignin to hemicellulose through ester-bonds, thereby limiting feed degradation (Hartley 1972). Phenolic acid contents differ for different plants and plant parts and so does the ratio PCA:FA (Eraso and Hartley 1990). For maize, highest concentrations of esterified phenolic acids (27 mg/g cell walls) are found in the stems (Eraso and Hartley 1990).

Free phenolic acids can inhibit the growth of cell wall degrading bacteria (Borneman et al. 1986; Varel and Jung 1986) and fungi (Akin and Rigsby 1985) in the rumen. PCA inhibits growth of isolated single strains of rumen bacteria by increasing lag time and reducing the growth rate when added at concentrations above 1.0 g/l (Akin 1982; Chesson et al. 1982). Also in vitro dry matter digestibility of cellulose (Varel and Jung 1986) and maize stem cell walls (Theodorou et al. 1987) was reduced when 1.0 g/l PCA ws added.

The effect of PCA and FA on in vitro fermentation kinetics of mixed rumen microbes (as measured by gas production) has not been examined. In this field, the fate of added phenolic acids is also important. They might be metabolized or adhere to substrate or microbes (Chesson et al. 1982), which would reduce their concentration.

This paper describes the effects of added PCA and FA on gas production kinetics, measured upon incubation of maize stems with buffered rumen fluid. In addition, gas production kinetics of stems of five different maize inbred lines, differing in phenolic acid content, were studied as well as the removal of PCA and FA from these stems during incubation.

Materials and methods

Phenolic acids

Trans p-Coumaric acid (PCA) was purchased from Merck (Darmstadt, Germany); trans ferulic acid (FA) and phloretic acid (PA; p-hydroxyphenyl-propionic acid) were from Sigma (St. Louis, MO, USA). All solutions containing phenolic acids were shielded from light with aluminium foil to prevent cis-trans isomerisation.

Extraction of phenolic acids

Esterified phenolic acids were extracted from forage, undigested residues, or bacterial pellets with 1 M NaOH under N_2 atmosphere at 39 °C for 24 h in the dark (Jung and Shalita-Jones 1990). Samples were filtered over 40 μm nylon gauze and washed three times with 10 ml water for each washing. The filtrate was acidified to pH 2.6 with concentrated phosphoric acid and the volume adjusted to 100 ml with water. Samples were frozen and stored at -18°C until HPLC analysis.

Free phenolic acids were extracted from clear rumen fluid (see below) which was acidified to pH 2.6 with concentrated phosphoric acid. Twentyfive ml fluid was extracted three times with 25 ml diethylether (Jung and Fahey 1983). The ether was evaporated and the remains redissolved in 3 ml methanol and kept at -18 °C until HPLC analysis.

Determination of phenolic acids by HPLC

Prior to injection, all samples were filtered through a 0.2 μm filter. Separation of phenolic acids was accomplished using a LKB HPLC with a reversed phase Superpher 60RP-8 (125 x 4 mm) column (Merck, Darmstadt Germany) at room temperature. Gradient elution was carried out with solvent A (0.2% (w/v) NaAc and 0.2% (v/v) HAc in water), and solvent B (0.2% (v/v) HAc in methanol). The gradient was 12% B from 0 to 4 min, 12-25% B from 4 to 20 min, 25% B from 25 to 30 min, 25-40% B from 30 to 40 min, and 40-95% B from 40 to 45 min to remove all impurities. Prior to the next injection, the column was equilibrated with 12% B for five minutes. Flow rate was 1.00 ml/min. Detection was at 285 and 254 nm using a LKB-UV dual wave length detector. Identification of the peaks was by comparing the elution profile of authentic standards and by their absorption ratios at 285 to 254 nm. The peak area was calculated by a Hewlett Packard LAS integrator using an external standard.

Gas production measurements

Fermentation was carried out in 250-ml bottles containing 500 mg feedstuff and 60 ml buffered rumen fluid (for composition see Beuvink and Spoelstra 1992). Rumen fluid was obtained from rumen canulated wether sheep, 2 h after morning feeding. The custom build gas production measurement system comprised twelve fermentation units, placed in a shaking waterbath at 39 °C, an electric control unit and datataker connected to a personal computer. Fermentation bottles were closed with screw caps on which electronic pressure meters were mounted, which were connected to an electronic valve. The pressure build-up in a bottle was measured by a pressure transducer (Microswitch 164 PC, Honeywell, USA) with a range of 0 to 2500 Pa. When the fermentation gas produced caused the pressure to equal the preset upper value, the electronic valve opened, allowing the pressure to fall back to the preset lower value (nearly atmospheric) resulting in closing of the valve. The time during which the valve was opened was only a few microseconds. Every valve-opening represented a known amount of gas (set at about 1 ml). So, the number of valve openings, periodically stored in a data logger (every thirty minutes), was proportional to gas production.

Samples were incubated in duplicate within one serie and every series was duplicated on another day. Gas production was corrected for a blank (buffered rumen fluid without substrate) and expressed as ml gas / g dry matter (DM). Gas production curves were fitted to a modified Gompertz model (Beuvink and Kogut 1993) from which the following parameters were derived : length of initial (lag) phase, maximum gas production rate, time at which gas production rate was maximal (T_i), total gas production, and time at which 95% of the gas was produced ($T_{0.95}$). Data were fitted to the model using the statistical program Genstat (Genstat 1987).

Analysis of incubation mixtures

After 48 h the incubation mixture was centrifuged at 200 x g for 10 minutes, washed (20 ml water). This was repeated twice. The final pellet was dried at 40 °C under vacuum and weighed. The weight, corrected for the blank, was regarded as the undigested residue from which dry matter disappearance (DMD) was calculated. The supernatants were combined and centrifuged at 8,500 x g for 30 min. The pellet was dried at 40 °C under vacuum and regarded as the loosely adherent bacterial fraction and the supernatant as clear rumen fluid. The volume of the latter was adjusted to 100 ml and samples were preserved for VFA analysis by GC as described by Robinson et al. (1986). In the undigested residue, the loosely adherent bacterial fraction and in the clear rumen fluid PCA,

FA and phloretic acid concentrations were determined. Metabolites of the added phenolic acids were qualitatively determined by analyzing the clear rumen fluid using NMR. ^{1}H-NMR spectra were recorded in CD_3OD on a Bruker AMX 400 WB-NMR spectrofotometer. Typically 32 free induction decays (45° pulse = 6 μs; 10 s relaxation delay) were accumulated and averaged in 16K memory. Spectra were compared to reference spectra.

Experiment 1: addition of free phenolic acids to the incubation mixture
Stems of fieldgrown maize (cv Scana) were harvested at 22 september 1992, ovendried (70 °C) and ground to pass a 1 mm screen. Stems contained 603 mg cell walls/ g DM, 13.39 (±0.280; n=3) mg PCA/g DM and 6.52 (±0.1-07; n=3) mg FA/g DM. Samples of 0.500 g were incubated with buffered rumen fluid to which 0, 0.1, 0.5, 1.0 or 2.0 g/l PCA or FA was added. Gas production was monitored for 48 h after which the fermentation mixture was analyzed as described above. Only the incubations to which 0.0 or 2.0 g/l PCA or FA was added were analyzed by NMR.

Experiment 2: Stems of different maize inbred lines
Five stem samples of different maize inbred lines (A, B, C, D and E) were analyzed for cell wall content (Goering and van Soest 1970) and phenolic acids (Table 1). These samples were incubated with buffered rumen fluid as described above. Gas production was monitored for 48 h after which the fermentation mixture was analyzed as described above.

Table 1. Contents of cell walls, p-coumaric acid (PCA) and ferulic acid (FA) in stems of five inbred lines of maize Data are means of four replicates.

Inbred line	cell walls (% DM)	total phenol. mg/g DM	PCA	FA	Ratio PCA/FA	
	-----		-----			
A	51.1		16.09^{a}	12.28^{a}	3.81^{a}	3.22
B	54.4		16.85^{a,b}	12.85^{a}	4.00^{a}	3.21
C	56.5		18.27^{b}	12.67^{a}	5.60^{b}	2.26
D	58.4		18.64^{b}	14.09^{b}	4.53^{a,c}	3.11
E	70.3		21.21^{c}	15.01^{b}	6.20^{d}	2.43
sem			0.687	0.540	0.257	0.205

^{a,b,c} = Means with different superscripts within one column differ significantly ($p < 0.05$)

Statistical procedures

The significance of treatments (addition of PCA or FA and different stem samples) on fermentation parameters was determined using analysis of variance followed by two way t-test. Since the major source of variation is the rumen fluid (Beuvink et al. 1992), data were divided into two blocks, each block comprising the measurement within one series. Differences in phenolic acid contents were determined by analysis of variance by two way t-test.

Results

Experiment 1

When 1.0 g/l PCA or more was added to the medium, a significant decrease of DMD, total gas production, maximum gas production rate, as well as total VFA production was observed (Table 2). In addition an earlier $T_{0.95}$ and an increase in the relative amount of popionic acid were found (Table 2).

The influence of PCA on fermentation kinetics is shown in Figure 1. The inhibitory effect became visible after 5-8 h of incubation. When 2.0 g/l PCA was present, 95% of the gas was produced within 17 h, whereas at 1.0 g/l PCA, 95% of the gas was produced after 41 h (Table 2).

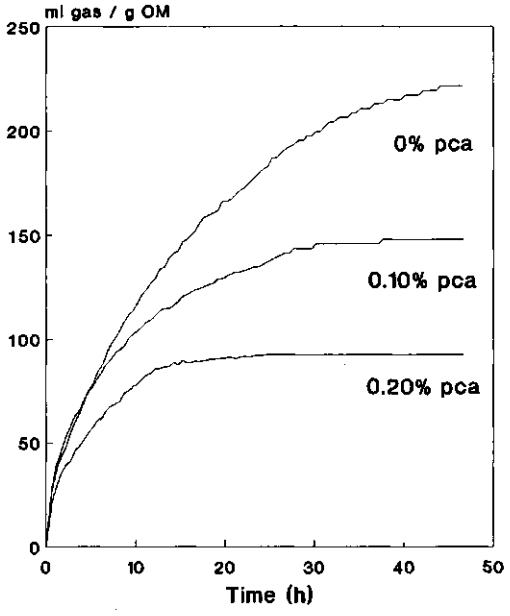

Figure 1. Time related gas production of maize stems incubated in buffered rumen fluid to which 0, 1.0 or 2.0 g/l PCA was added.

Only at the highest concentration tested (2.0 g/l), FA addition resulted in a decrease of DMD by 11%, and a lower relative amount of butyric acid. The lower levels of FA did not influence fermentation (Table 3).
For none of the incubations a lag phase was observed.
To investigate to which extent added PCA and FA were metabolized by the rumen microorganisms, the amounts of phenolic acids and some metabolites present in the medium and bacterial pellet after 48 h of incubation were quantified. Only traces of PCA and FA could be detected in the clear rumen fluid after 48 h incubation, except for the 2.0 g/l PCA addition of which 12% could be recovered. In the microorganism fraction, only traces of phenolic acids could be detected, possibly originating from contamination with undigested residue.
In the incubations to which 2.0 g/l PCA was added, the main phenolic metabolites as detected by NMR were PA and to a lesser extent 3-phenyl-propionic acid (3-PPA). Also some PCA could be detected. The ratio PA:PCA:3PPAA was 11:2:1. The amount of PCA in the incubation residue was 0.25 g/l (as determined by HPLC). Concentrations of these components in the blank were neglectable compared to the 2.0 g/l PCA incubations. In the incubations with FA, no phenolic constituents could be detected after 48 h fermentation.
Reduced disappearance of dry matter upon PCA incubation after 48 h showed a good linear relationship with decreased VFA production ($R^2 = 0.991$) and total gas production ($R^2 = 0.976$). Also between VFA production and gas production a close linear relationship existed ($R^2 = 0.946$).

Experiment 2

The kinetic fermentation parameters of the five maize stem samples are shown in Table 4. In general, varieties D and E were fermented slowest and to the smallest extent. In contrast, variety B was fermented at the highest rate and to the highest extent. Variety E had the highest relative amount of HAc and lowest of HP and HB.
The content of phenolic acids in the undigested residue after 48 h fermentation was slightly higher than in the starting material (Table 5). DMD was negatively correlated to PCA content of the stems ($R^2 = 0.882$; $p < 0.05$) and positively correlated to the relative amount of PCA released from the stems ($R^2 = 0.905$; $p < 0.01$). Such relationships were not found for FA. Although the varieties were degraded to various extents, the contents of PCA and FA in the undigested residue of all five samples were the same. Due to a more extensive removal of FA than of PCA, the ratio PCA:FA increased during the fermentation process.
Total contents of PCA plus FA in maize stems increased with cell wall content ($R^2 = 0.947$; $p < 0.01$), but the contents on a mg cell wall basis did not differ.

Table 2. Influence of different amounts of added para-coumaric acid (PCA) on fermentation characteristics of maize stems. Data are means of four replicates and corrected for fermentation occuring in the blank.

	PCA-concentration (g/l)						
	0.0	0.1	0.5	1.0	2.0	sem	SIGN
DMD (%)	64.9	65.5	63.2	49.4	32.5	1.31	***
ml gas / g DM	214	222	222	146	98	6.2	***
Max. rate (ml/h)	10.6	11.5	9.7	10.1	13.0	1.94	**
$T_{0.95}$ (h)	54.2	50.9	62.8	40.7	16.5	6.37	***
T_i (h)	4.4	3.9	5.3	1.0	0.8	1.98	NS
Total VFA (mmol/l)	46.4	49.9	46.0	35.8	21.0	2.22	***
% HAc (mmol/100 mmol)	62.9	63.0	62.7	60.7	56.0	1.55	**
% HP "	28.2	28.0	28.4	31.3	43.3	1.52	***
% HB "	8.2	7.9	8.0	7.1	3.7	0.89	**

NS= No significant effect of PCA addition; ***; ** and * = significant at $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively. DMD=dry matter disappearance; HAc=acetic acid, HP=propionic acid; HB=butyric acid.

Table 3. Influence of different amounts of added ferulic acid (FA) on fermentation characteristics of maize stems. Data are means of four replicates and were corrected for fermentation occuring in the blank.

	FA-concentration (g/l)						
	0.0	0.1	0.5	1.0	2.0	sem	SIGN
DMD (%)	64.3	64.9	64.1	63.4	57.3	1.35	***
ml gas / g DM	202	227	202	218	218	14.7	NS
Max. rate (ml/h)	10.8	11.7	10.2	11.2	11.2	0.98	NS
$T_{0.95}$ (h)	38.8	41.9	34.3	41.0	31.8	5.16	NS
T_i (h)	2.8	3.5	3.2	3.4	2.7	1.41	NS
Total VFA (mmol/l)	43.6	42.7	44.8	38.3	36.4	3.43	NS
% HAc (mmol/100 mmol)	63.3	64.8	64.2	64.5	65.8	1.48	NS
% HP "	27.1	26.6	26.3	27.6	28.7	1.21	NS
% HB "	7.9	7.57	7.9	6.9	5.6	0.34	***

NS= No significant effect of FA addition; ***; ** and * = significant at $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively. DMD=dry matter disappearance; HAc=acetic acid, HP=propionic acid; HB=butyric acid.

Table 4. Fermentation characteristics of stems of different maize inbred lines. Data are means of four replicates.

	Inbred line					
	A	B	C	D	E	sem
DMD (%)	$70.9^{a,b}$	71.6^{b}	68.2^{a}	63.8^{c}	57.1^{d}	1.46
ml gas / g DM	206^{a}	223^{a}	206^{a}	185^{b}	171^{b}	9.4
Max. rate (ml/h)	$11.3^{b,c}$	12.4^{b}	10.7^{a}	8.5^{a}	8.0^{a}	0.71
$T_{0.95}$ (h)	42.7	48.9	48.2	48.2	48.1	5.61
T_i (h)	2.8	2.3	2.3	2.3	5.7^{a}	0.82
Total VFA (mmol/l)	35.9^{a}	$32.8^{a,b}$	32.3^{b}	31.7^{b}	27.3^{c}	1.48
% HAc (mmol/100 mmol)	65.6	64.5	65.1	65.7	67.6^{a}	0.89
% HP "	23.2	24.3^{a}	23.9	23.8	22.8^{b}	0.61
% HB "	9.6^{a}	10.1^{a}	9.8^{a}	9.2^{b}	8.3^{c}	0.34

^{a,b,c} = Means with different superscripts within one row differ significantly ($p < 0.05$)

DMD = dry matter disappearance; HAc = acetic acid, HP = propionic acid; HB = butyric acid.

After 48 h of incubation, the undigested residues all contained the same concentration of PCA and FA, the PCA content being higher than in the starting material (Table 5). The ratio PCA:FA of the maize stem varieties ranged from 2.3 to 3.2 and had no relationship with cell wall content. After 48 h incubation, the ratio had increased to on average 5.6; no differences were found between the varieties. In the clear rumen fluid no PCA, FA, or phloretic acid could be detected by HPLC. These samples were not subjected to analysis by NMR.

Discussion

Addition of PCA or FA

PCA was more inhibitory than FA which is in agreement with findings of others (Akin 1982; Borneman et al. 1986; Martin 1988). For the observation that no initial inhibition of fermentation by PCA occurred, there are three possible explanations. 1) PCA inhibits the microbial population that is active at the later stages of fermentation (fibre degrading micoorganisms), and does not affect the microbial population that is active during early stages of fermentation when easily degradable substrates like free sugars, oligosaccharides, mesophyll and epidermis cells are fermented (Akin, 1982). 2) PCA atttaches to the fiber and thus inhibits digestion. 3) A temporarily accumulating intermediate is responsible for inhibition by mechanism 1 or 2.

The added PCA could not be detected in the undigested residue, so explanation 2 is very unlikely. When explanation 3 would be true, a biphasic gas production curve would be expected due to temporary inhibition of the fermentation. This leaves us with option 1. PCA has been shown to inhibit the growth of cellulose

Table 5. Phenolic acids in undigested residues of stems of five different mazie inbred lines after 48 h incubation, corrected for the blank.

Inbred line	----- mg/g DM ----- total phenol.	PCA	FA	Ratio PCA/FA	% disappeared PCA	FA
A	18.83	16.04	2.79	5.92	61.9	78.9
B	20.51	17.07	3.44	5.38	58.2	74.4
C	20.06	16.87	3.19	5.44	61.7	82.6
D	19.40	16.31	3.09	5.38	58.2	75.1
E	20.04	16.96	3.08	5.71	51.5	78.6
sem	0.864	0.114	0.303	0.932		

and xylan degrading bacteria (Akin 1982; Jung and Fahey 1983). Since cellulose is fermented at the later stages of fermentation (Beuvink et al. 1992), it might be that PCA is more inhibitory to these bacteria than to other microorganisms at concentrations higher than 1.0 g/l. Assuming that cellulose degradation started after 10 h, it is obvious that not only the extent of the cellulose degradation was inhibited, but also the rate. The shift in VFA molar ratios also indicated a shift in the microbial population when high amounts of PCA were added. However, since the inhibitory effect was observed only after 5-8 h, is can not be ascertained that PCA still present as such.
Akin (1982) describes a growth delay for single strain rumen microbes grown on filter paper in the presence of PCA. In our experiments the microbial population, as well as the substrate composition were very diverse, which could explain the absense of a lag phase.
No effect of FA on maize stem fermentation was observed. This compound is known to have little or no effect on cellulose utilizing microorganisms, nor on the degradation of mesophyll and epidermis tissue (Akin 1982).
Our results show a low recovery of PCA and FA after 48 h incubation. The main metabolites of PCA were PA and 3-PPA. For FA, no phenolic constituents could be detected by NMR in the clear rumen fluid. Nonspecific adsorption to microbial surfaces as suggested by Chesson et al. (1982) was not observed, therefore the possibility of metabolism of FA to non-aromatic products must be considered. Bacteria able to use ferulic and sinapic acids under anaerobic conditions as their only carbon and energy source have been described (Healy et al. 1980; Akin 1982). However, it took three days for a culture acclimated to ferulic acid to completely degrade ferulic acid (Healy et al. 1980). Another possibility is the adsorption of phenolic acids or their metabolites to components in the clear rumen fluid (e.g. proteins) (Marvin, unpublished results).
As mechanism of inhibition of rumen microbes by phenolic acids, damaging cell membranes by inactivation of enzymes (Chesson et al. 1982; Borneman et al. 1986) or influencing attachment of fibrolytic microorganisms to fibre particles (Varel and Jung 1986) have been suggested. PCA has also been shown to inhibit the activity of ß-glucanase, endoglucanase and xylanase from rumen bacteria (Martin and Akin 1988).

Fate of phenolic acids from maize stems

Content of PCA in the undigested residue increased during fermentation, whereas FA content decreased. This agrees with findings of Harris et al. (1980) who suggested that these two components are located in different structural or chemical environments within the plant cell wall, where PCA is associated more

with the less digestible plant cell walls than is FA (Buxton and Russell 1988). The absolute amounts of PCA and FA released from the stems during fermentation were different for the five varieties, but their concentrations in the undigested residues were the same. These results suggest that the phenolic acids, extractable with 1.0 M NaOH, could be divided into two pools. One removable by rumen microorganisms during 48 h fermentation, and one that could not be removed. Treatment of grasses with different concentrations of NaOH suggested plant differences in the ways phenolic acids were covalently bound to cell walls (Hartley and Morrison 1991) and difference in cell types (Akin et al. 1992). For bermudagrass it is assumed that 0.1 M NaOH liberates the PCA and FA groups that are ester linked to the arabinoxylans in the hemicellulose. How the other phenolic groups are bound is not known (Hartley and Morrison 1991). In our experiments, percentage of FA, released during fermentation, did not differ fir the samples, whereas the percentage of PCA released was lowest for sample E, which had the highest cell wall content. Probably this sample had also the highest amount of lignified tissue from which phenolic acids (especially PCA) could not be removed. Since all undigested residues had the same concentrations of PCA and FA, the distribution of PCA and FA must be more spreaded throughout the cell walls to explain the differences in DMD. The presence of phenolic acids in the undigested residue might limit further degradation.

Free versus esterified phenolic acids

In general, high levels of PCA (and high PCA/FA ratios) in feed are associated with low forage digestibility (Hartley 1972; Burrit et al. 1984). Our results support these findings, however a causal correlation could not be made, since differences in digestibility might also be caused by differences in cell wall content. When PCA was present in concentrations above 1.0 g/l, the main metabolite was PA. When present in lower concentrations, PCA was metabolized further without detectable phenolics being present. During fermentation of maize inbred lines, the amount of PCA released from the feed could reach 50 mg/l, assuming there is no further metabolism. This concentration is too low to inhibit fermentation. However, it has been suggested that in the microenvironment of bacteria attached to fibers, the phenolic acid concentration may be higher than that of rumen fluid (Chesson et al. 1982; Borneman et al. 1986). In this case an inhibitory concentration might be reached.

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CHAPTER 8

General discussion

MEASURING IN-VITRO GAS PRODUCTION KINETICS

In-vitro methods measuring ruminal digestion kinetics

In-vitro incubation of feedstuffs with rumen fluid (Tilley and Terry 1963) is widely used to estimate digestibility. However, this is an end point method, measuring the amount of material degraded after 48 h.
In high yielding dairy cows, with high feed intakes, not only total digestibility is important, but also the rate of digestion. At high intakes, digestibility is depressed as a result from increased ruminal passage rate. If the rate of ruminal fiber digestion could be increased, an increase in growth of rumen microorganisms as well as a higher dry matter (DM) intake could be established (Hoover 1986). Another advantage of improving the availability of nutrients in feeds is a decrease in the environmental load of waste from animal production.
Rumen degradation rates are now mainly estimated by incubating a feedstuff in porous nylon bags in the rumen and determining disappearance of feed components at various time intervals (Mehrez and Ørskov 1977). However this is a very expensive and laborious method.
An alternative is measuring gas production kinetics of a feedstuff incubated with HCO_3^-/CO_2 buffered rumen fluid. The carbohydrates from the feed will be fermented to volatile fatty acids (VFA) and gas (Menke et al. 1979; Beuvink and Spoelstra 1992). Gas thus produced can be divided in direct gas production (CO_2 released as a fermentation end product) and indirect gas production being the CO_2 released from the buffer by the VFA produced (Chapter 2).
The incubation procedure is similar to that of the in-vitro method described by Tilley and Terry (1963), (omitting the second step, a pepsin-HCl incubation) where disappearance of DM is measured gravimetrically. Both the Tilley and Terry method and the gas production method originally are end-point methods, but they can be extended to determine ruminal digestion kinetics in vitro by measuring at various time-intervals. Both methods have their advantages and disadvantages, which are listed in Table 1.

Table 1. Differences between the in-vitro technique described by Tilley and Terry (1963) and the gas production method.

Tilley and Terry method	Gas production method
Gravimetrical method	Microbiological method
Measured disappearance of OM	Measures gas as a fermentation end product
Different samples needed for each time point	One sample needed for measuring at different time points
Relatively large amount of sample needed for fibre analysis	Small amount of sample can be used (Pell and Schofield 1993)
Difficult to automate	Relatively easy to be automated
No influence of fermentation pattern on disappearance of OM	Fermentation pattern influences the amount of gas produced (Chapter 2). Fermentation of lactic acid yields no gas (Chapter 6)
Role of soluble forage components can not be determined. Not all the disappeared OM is necessarily fermentable (Pearce et al. 1987). Small feed particles are lost in the supernatant.	Gas production reflects the amount of all material fermented, soluble as well as non-soluble, small as well as big particles. Soluble, but not fermentable products do not contribute to gas production.
Second step with pepsin-HCL simulates digestion in the stomach	Only rumen fermentation is simulated

The original aim of this study was to evaluate enzyme treatment of grass silages. Therefore a rapid method was needed to get an impression of the change in ruminal fermentation kinetics upon enzyme treatment of grass silages. It was also known that enzyme treatment of grass silages increased the soluble fraction (van Vuuren et al. 1989). The choice was made for the gas production system, based on the facts that it was a rapid, kinetic method, relatively easy to automate and that it measured fermentability rather than solubility. However, it was not possible to obtain purified enzymes in sufficient amounts to use them for ensiling experiments, so only enzyme cocktails were used (Chapter 6).

Methods to measure gas production kinetics

Gas production kinetics have been measured in various ways, by using calibrated syringes (van der Meer et al. 1990, Krishnamoorty et al. 1991), pressure transducers (Merry et al. 1991) or by using liquid displacement systems (Jouany and Thivend 1986; Beuvink and Spoelstra, 1992). All these methods require frequent periodical readings, making them less suitable as a routine laboratory method. Therefore it was decided to automate the measurements, choosing for the liquid displacement system (Chapter 3; Beuvink et al. 1992). This system proved to be sensible to leakages and it took some time to reach te acquired equilibria between the liquid levels in the displacement bottle and the collection vessel. Even though the gas production was registrated automatically, the preparations before starting the measurements were laborious and complicated. So therefore during the research it was felt that another, less complicated, method was to be preferred. This was found in the use of pressure transducers (Chapter 7). Nevertheless, the liquid displacement produced good reproducible results, so it was used for the measurements described in Chapters 3 to 6. The manual system described in Chapter 2 was used to enable sampling of the incubation mixtures. The principle of all the methods is the same, only the way the gas is measured and registrated differs.

Mathematical evaluation of gas production kinetics

In roughages, plant cell walls, consisting mainly of structural carbohydrates, serve as the major dietary fibre and energy source for ruminants. Because the fibre fraction is never fully degraded in the rumen, a distinction can be made between digestible and non-digestible fibre fractions. This distinction has proven its use in mathematical models to predict intake and fibre digestion in ruminants (Mertens and Ely 1979; Ørskov et al 1988).
The structural polysaccharides found in grass leaves consist of three fractions, a fully digestible fibre fraction consisting of primary cells, a more slowly digested fraction of secondary cell walls and a non-digestible residue, mainly consisting of unfermented secondary cell wall material (Cheng et al. 1980; Chesson et al. 1986). Models describing digestion with a single fixed rate of digestion have the disadvantage that this is an average rate which may not be related to chemical composition (Mertens and Ely 1982). With this in mind, in this thesis gas production was modelled by dividing the gas production into two components. One arising from the rapidly fermentable fraction, and one from the slowly fermentable fraction. This principle has been succesfully used by Robinson et al.

(1986) and Mertens and Ely (1982) to model loss of neutral detergent residue of ryegrass from nylon bags.
Gas production kinetics have been modelled by the exponential model (Xiong et al. 1990; Krishnamoorty et al. 1991; Merry et al. 1991; Blümmel and Ørskov 1993). However, the modified Gompertz model described in Chapter 4, fits gas production data better, especially when a lag phase occurs. The main processes taking place in the lag phase are hydration and colonization of the feed (Cheng et al. 1980). Fermentation of monomeric sugars takes place during the first two hours (van der Meer et al. 1988).
Biological processes have been modelled using sigmoidal equations like the Gompertz and the logistic equation (France and Thornley 1984). These models assume all substrate to be available for fermentation at $t=0$ (Robinson et al. 1986). The modified Gompertz model is distinct from other sigmoidal models at this point, and also in the fact that it does have two fractional degradation constants. The mGom model described gas production kinetics of different feedstuffs better than known sigmoidal models (Chapter 4), thus being a valuable instrument to statistically compare gas production curves.

Physiological references

Gas production after 24 h has been shown to be related to the metabolisable energy content of a feedstuff, via a regression equation with crude protein, crude fat and crude ash as the other parameters (Menke et al 1979; Steingass and Menke 1986). Gas production kinetics of cereal straws have been shown to be related with dry matter intake (DMI), digestible dry matter intake (DDMI) and growth rate (Blümmel and Ørskov 1993). Most of the variance in DMI and DDMI for cereal straws and silages could be explained by differences in the gas production rate (obtained after fitting to an exponential model; Blümmel 1992). Blümmel and Ørskov (1993) also found total gas production to be related to nylon bag degradability at different time intervals, but they did not find a siginifcant relationship between the rate constant for gas production and the rate constant for dry matter loss as obtained by the nylon bag technique (both were obtained after fitting an exponential model). Research in this thesis (Chapter 5) has shown that between the nylon bag method and gas production method correlations only existed between the undegradable fraction (U) and total gas production. No correlations were found between gas production parameters and the potentially digestible fraction (D) and its rate constant (k_d). With the gas production method, the gas production which results of fermentation of the whole sample, is modelled. With the nylon bag technique, only degradation

of D+U is modelled, because it is assumed that the soluble fraction (S) is degraded instantly at an infinite rate. So, the rate parameters of the two methods are not based on the same material and therefore show no relationship with each other.

Rumen fluid as an inoculum

Strained rumen contents do not fully represent the microbial population of the rumen (Senshu et al. 1980). Most of the starch fermenters and part of the cellulose fermenters are extracted from the rumen solids by repeated washing and squeezing. The particle-associated microorganisms can be separated from the material by blending. However, introducing this step means an extra possibility of exposure to oxygen, and leads to the presence of many small feed particles. Presence of feed particles is undesirable because they cause higher blank values. Pell and Schofield (1993) found similar gas production curves for blended and unblended inocula.

For the research described in this thesis, rumen fluid was withdrawn 2 h after feeding, at which time ruminal fermentation activity is maximal. When samples of rumen fluid are taken before morning feeding, there is less fermentation activity, but the composition of the rumen fluid is more stable, and there are less feed particles present. The gas production system should not show limitations of the rumen fluid, but differences in gas production observed should arise from differences in feeds only. That is why in this thesis the procedure described by Menke et al. (1973) was used. This meant the use of a relatively large inoculum (33%) and sampling of rumen fluid 2 h after feeding to ensure maximum fermentation activity. Pell and Schofield (1993) found an inoculum size of 20% sufficient to ensure maximum rate of gas production, whereas lower values were not.

The animals used for the experiments in the present investigation were whether sheep kept on a daily diet of 800 g hay and 200 g concentrates (low in starch). The rumen fluid was thus not adapted to most of the feeds tested in this thesis (grass silage, maize silage, chopped ear corn silage, corn cob mix and maize stems).

APPLICATIONS OF THE GAS PRODUCTION TECHNIQUE

In-vitro fermentation of plant cell walls

Carbohydrates from the feed enter the rumen in three distinct forms:
1. Water-soluble carbohydrates consisting mainly of mono- and oligosaccharides, and water soluble polysaccharides such as fructosans.
2. Starch, an α-glucan storage polysaccharide.
3. Structural polysaccharides contributing to the plant cell wall.

For the isolated carbohydrates, fermentation of these three types can be clearly distinguished. Glucose for example is fermented very rapidly during the first few hours of fermentation, whereas starch is fermented later, and cellulose is fermented at the slowest rate (Chapters 2 and 3). Degradation of the different components cannot be distinguished when whole plant material is fermented because the degradation processes partially coincide.

Within the three carbohydrate fractions mentioned above, differences can also occur. Starch from different sources is fermented at a different rate (McBurney et al. 1990) and processed starch is fermented at a higher rate than raw starch (Xiong et al. 1990). Also different degradation patterns are found for cell walls of different cell types. Mesophyll cells are most rapidly degraded by rumen microorganisms, followed by bundle sheath and sklerenchymal cells, whereas the lignified vascular tissue is unfermentable (Cheng et al. 1980; Chesson et al. 1986).

The composition of plant cell walls varies with age (Jones 1972) and digestibility decreases. For grass silage the effects of ageing on gas production were lower total gas production and a decrease in maximum gas production rate (Chapter 6).

Isolated cell walls (after neutral detergent (ND) treatment) show a higher gas production per gram dry matter than the grass they were isolated from (Chapter 3). Gas produced is an end-product of the fermentation of carbohydrates; fermentation of protein yields negligible amounts of gas (Raab et al. 1983). With the ND treatment, most of the protein is removed, resulting in a higher concentration of fermentable carbohydrates in the cell wall fraction (ND treated material) compared to the original grass. However, the lag phase is prolonged because of the removal of soluble carbohydrates (e.g. fructosans) and monomeric and oligomeric sugars.

Screening the effect of cell wall degrading enzymes in grass silage

Upon enzyme treatment of silages, changes in the cell wall fraction occur (McDonald et al. 1991), which are expressed as a loss of cell wall components, measured as NDF. The question is whether this change in chemical composition also alters the feeding value. It is not exactly known what changes take place in the cell walls upon enzyme treatment; whether components are released that may enhance or disturb fermentation (e.g. phenolic acids). Therefore measuring only the disappearance of dry matter from enzyme treated feeds would not give sufficient information. Next to providing kinetic parameters, the gas production technique is able to detect whether fermentation is inhibited or advanced.
Gas production experiments showed that degradation of cell walls by enzymes resulted in a shortened lag time, probably due to a shift towards more rapidly fermentable material (Chapter 6). No differences in VFA composition were observed (results not shown).
Enzymes used as a silage additive are mainly cocktails of cellulases, hemicellulases and many other unknown activities. These enzyme cocktails are usually from fungal origin (*Aspergillus niger, Trichoderma viride*) (Vanbelle and Bertin 1989). However, the rumen provides a more effective range of enzymes to degrade feed, which is illustrated by the following. A sample of grass (dried and ground over a 1 mm screen) suspended in a 0.1 N HAc/AC^- buffer (pH 4.8) was treated with an enzyme cocktail of cellulases, hemicellulases and pectinases (enzyme preparation C from Chapter 6) until no more reducing sugars were released (about 120 h). Subsequently this sample was incubated with buffered rumen fluid and gas production was followed over time. From this material a substantial amount of gas was produced, be it at a low rate (Figure 1). This illustrates that the enzyme preparation lacked some of the activities that are present in rumen fluid.
To improve the ruminal digestion of feedstuffs by enzyme addition, the enzymes must be either catalyzing a rate limiting step in feed degradation or add a new activity, necessary for feed degradation, to the rumen fluid. Research on enzymes should therefore focus on defining more specific enzymes (to be used alone or in combination with purified cellulases or hemicellulases) instead of undefined cocktails. Such enzymes might include the esterases that break down the ester linkages between carbohydrates and phenolic acids (e.g. p-coumaric acid esterase and ferulic acid esterase, which are produced by rumen fungi (Borneman et al. 1990)) or that remove acetyl substituents from xylose chains in grass hemicellulose (Biely et al. 1986).
When new enzymes are produced or purified, measuring gas production kinetics

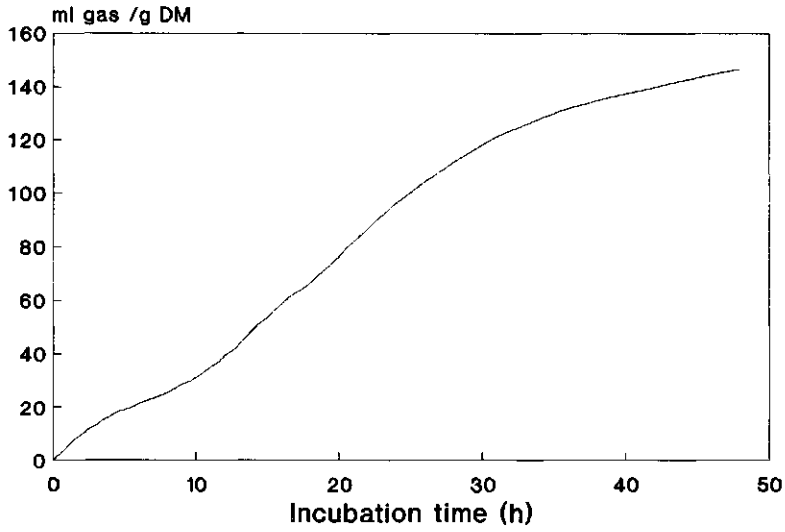

Figure 1. Gas production curve of a grass sample that had been exhaustively treated with cell wall degrading enzymes before incubation in the gas production system.

could give rapid information about the effect of these enzymes on the fermentation of the feed in the rumen. It may serve as a prescreening technique, before conducting feeding trials.

Aid in plant breeding

Improving digestibility of animal feeds can in the long term be acchieved by selecting better digestible varieties of fodder plants. To evaluate their breeding programs, plant breeders need rapid in-vitro methods measuring digestion characteristics. Often only small amounts of plant material are available for assaying which restricts evaluation to in-vitro methods. Measuring gas production kinetics seems to be a suitable method to distinguish between the stems of different maize inbred lines. Ranking could be made according to total gas production or to gas production rate (Chapter 8).

Formation of VFA and gas versus microbial protein

When feeds are fermented, differences in gas production can be caused by 1) differences in amounts of feed fermented, 2) differences in fermentation pattern (Chapter 2) or 3) a shift from the formation of VFA to production of microbial protein.
Naga and Harmeyer (1975) found a negative correlation between microbial growth (measured as the increase in TCA-insoluble nitrogen) and VFA production during 1-h incubation, although there was no constant relationship. Blümmel (1992) found a negative correlation between microbial cell yield and gas production. He estimated microbial growth as the difference between apparently and truly digested dry matter. Apparent digestibility was estimated by ultra centrifugation of the entire feed residue and the estimate of true digestibility was obtained by refluxing the freeze dried pellet with neutral detergent solution. The disadvantage of such chemical methods is that no distinction can be made between microbial and feed protein. Krishnamoorty et al. (1991) used ^{32}P as a marker for synthesis of microbial protein. Net synthesis of microbial protein was positively linearly correlated with gas production. At a higher extent of fermentation more ATP is produced, which should result in a higher biomass production (Hespell and Bryant 1979). The ^{32}P-method is therefore to be preferred to the two chemical methods mentioned above. A chemical method that could differ between microbial and feed protein would be usefull to estimate microbial protein synthesis in the gas production system.
For the experiments described in Chapter 2, for all three substrates (glucose, rice starch and cellulose) 86-90% of the C-atoms from the substrate (assuming 100% fermentation of the substrate) could be recovered in VFA or gas directly produced as a fermentation end product. Recovery was calculated from the VFA contents and composition, using the stoichiometric relations mentioned in Chapter 2. The remaining 10 to 14% had possibly been used for microbial growth or production of reserve material.

Future developments in measuring gas production kinetics

Measuring gas production kinetics can be used for evaluating feed additives and other treatments. It is a usefull technique to determine differences in the way the feed is fermented by rumen microbes and a ranking can be obtained. The precise relationship of the gas production parameters with the animal, needs to be clarified in future experiments.
However, at the moment it is the quickest method to obtain information about

ruminal fermentation kinetics of carbohydrates.

Next to evaluating feeds, the gas production can also be used to determine how the rumen microflora adapts to a new feed, by incubating the same feed sample with different samples of rumen fluid. Feeds for monogastric animals might also be evaluated with the gas production technique, since ruminal fermentation and hind-gut fermentation show a lot of similarities (Demeyer and de Graeve 1991).

More attention has to be paid to the role of protein in gas production. When microbial protein synthesis can be measured, the gas production system can be used to optimize rumen microbial protein syntesis and fibre degradation (van Nevel and Demeyer 1988).

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Angelie Beuvink

Curriculum vitae

Johanna Maria Wilhelmina Beuvink werd op 19 augustus 1964 geboren te Oldenzaal. Zij behaalde in 1982 het VWO diploma aan het Thijcollege te Oldenzaal. In september van datzelfde jaar begon zij met een studie Levensmiddelentechnologie aan de toenmalige Landbouwhogeschool te Wageningen. In september 1988 werd het ingenieursdiploma behaald met als afstudeervakken Levensmiddelenmicrobiologie en Proceskunde. Op 1 augustus 1988 werd zij aangesteld bij het Instituut voor Veevoedingsonderzoek (IVVO-DLO) in Lelystad als tijdelijk onderzoeker op het gebied van biotechnologie in de veevoeding. Het hier uitgevoerde onderzoek resulteerde in dit proefschrift.