



Alternative methodologies – stretching the in vitro box

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Abstract

Current gas-based in vitro evaluation systems are extremely powerful research techniques. However they have the potential to generate a great deal more than simple fermentation dynamics. Details from four experiments are presented in which adaptation, and novel application, of an in vitro system allowed widely differing objectives to be examined. In the first two studies, complement methodologies were utilised. In such assays, an activity or outcome is inferred through the occurrence of a secondary event rather than by direct observation. Using an N-deficient incubation medium, the increase in starch fermentation, when supplemented with individual amino acids (i.e., known level of N) relative to that of urea (i.e., known quantity and N availability), provided an estimate of their microbial utilisation. Due to the low level of response observed with some amino acids (notably methionine and lysine), it was concluded, that they may not need to be offered in a rumen-inert form to escape rumen microbial degradation. In another experiment, the extent to which degradation of plant cell wall components was inhibited by lipid supplementation was evaluated using fermentation gas release profiles of washed hay. The different responses due to lipid source and level of inclusion suggested that the degree of rumen protection required to ameliorate this depression was supplement dependent.

That in vitro inocula differ in their microbial composition is of little interest per se, as long as the outcome is the same (i.e., that similar substrates are degraded at comparable rates and end-product release is equivalent). However where a microbial population is deficient in a particular activity, increasing the level of inoculation will have no benefit. Estimates of hydrolytic activity were obtained by examining fermentation kinetics of specific substrates. A number of studies identified a fundamental

Abbreviations: DM, dry matter; iOMD, in vitro organic matter degradation; IVTDMD, in vitro true dry matter digestion; ME, metabolizable energy; NDF, neutral detergent fibre; NWNNS, non-soluble non-structural carbohydrate; OM, organic matter; RPT, Reading Pressure Technique; VFA, volatile fatty acids; WH, washed hay; WSC, water soluble carbohydrate

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difference between rumen fluid and faecal inocula, with the latter having a lower fibrolytic activity, which could not be completely attributed to microbial numbers. The majority of forage maize is offered as an ensiled feed, however most of the information on which decisions such as choice of variety, crop management and harvesting date are made is based on fresh crop measurements. As such, an attempt was made to estimate ensiled maize quality from an *in vitro* analysis of the fresh crop. Fermentation profiles and chemical analysis confirmed changes in crop composition over the growing season, and loss of labile carbohydrates during ensiling. In addition, examination of degradation residues allowed metabolizable energy (ME) contents to be estimated. Due to difficulties associated with starch analysis, the observation that this parameter could be predicted by difference (together with an assumed degradability), allowed an estimate of ensiled maize ME to be developed from fresh material. In addition, the contribution of the main carbohydrates towards ME showed the importance of delaying harvest until maximum starch content has been achieved.

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1. Introduction

The two-stage methodology of [Tilley and Terry \(1963\)](#), which provides an *in vitro* estimate of *in vivo* digestibility, can be considered one of the great advances in ruminant feedstuff evaluation. Modifications by [Goering and Van Soest \(1970\)](#) led to the IVTDM (in *in vitro* true dry matter (DM) digestibility) assay that is still widely used. However, as the extent of rumen degradation of a feedstuff is the product of ruminal residence time and rate of degradation, these methodologies are deficient in that they provide no kinetic information on degradation. It is, therefore, possible for two feeds to have similar end-point degradation values, but different rates of degradation. This is of fundamental importance, as feeds with higher degradation rates tend to be consumed in greater quantity. In addition, as these assays are generally made after an extended incubation period (usually 48–96 h), it is possible that degradation is over-estimated and subtle differences among similar feeds lost.

While gas production systems (e.g., [Menke et al., 1979](#)) allow fermentation gas release profiles to be used to estimate degradation kinetics, the relationship between these is highly substrate dependent. Thus, use of gas values alone provides insufficient information to assess widely differing feedstuffs. Current systems (e.g., [Pell and Schofield, 1993](#); [Davies et al., 1995](#); [Cone et al., 1996](#); [Mauricio et al., 1999](#)) vary in their complexity, capacity and assay capabilities. However they have the potential to provide a great deal more information than an estimate of end-point degradation. For instance, [Colombatto et al. \(2003\)](#) examined the influence of fibrolytic enzymes on hydrolysis and fermentation of the pure carbohydrate substrates cellulose and xylan by mixed ruminal microorganisms using *in vitro* gas release kinetics. Similarly, [Mlambo et al. \(2002\)](#) assessed the effectiveness of alkalis to inactivate tannins in leguminous tree fruits, and so improve their nutritive value in Zimbabwean smallholder goat systems, by measuring changes in gas release.

The following case studies briefly detail novel applications of the Reading Pressure Technique (RPT; [Mauricio et al., 1999](#)) modified for specific situations.

2. Complement systems

In the first two examples, the Reading Pressure Technique was adapted to a produce complement methodology. In such assays, an activity or outcome is inferred through the occurrence of a secondary event, rather than by direct observation. For instance, while application of an enzyme will hydrolyse a feedstuff, degradation residues or products must be assayed to quantify the reaction. If a suitable microflora was present, the fermentation gas released could provide an estimate of both the quantity, and rate, of substrate degraded by the enzyme.

2.1. Impact of lipid on fibre degradation

Lipids are often incorporated into high yielding dairy cow rations to increase dietary energy density. However, unless included at relatively low levels (<50 g/kg dry matter (DM)), or in a rumen protected or inert form, these supplements will adversely affect ruminal fibre degradation. For instance, Stewart (1977) reported that cellulolytic activity decreased with increasing levels of tallow. The methodology reported here was based on the assumption that the extent to which lipids depress microbial degradative activity in vitro is correlated with their 'rumen-inertness' (i.e., the extent to which their unsaturated fatty acids become saturated in the rumen). As in vitro gas release provides an indication of rumen degradability, the degree to which structural carbohydrate (i.e., fibre) degradation is modified as a result of the type and level of dietary fat inclusion can be estimated from gas release kinetics. Thus this method allows the effectiveness with which a lipid has been protected from the rumen microbial environment to be assessed.

In the study of Mould et al. (2002), three commercial cold-pressed lipid supplements (i.e., fish, linseed and rapeseed oils) were added at levels of 20, 40, 80 and 120 mg/g to grass hay (947 g/kg DM). The RPT was used to estimate rate and extent of gas release and in vitro organic matter (OM) degradation (iOMD) was assessed at 6, 12, 18, 24 and 48 h post-inoculation. Cumulative gas release profiles were generated from head-space gas pressure values obtained at regular intervals over the incubation period. The degradation of hay alone was examined, as was the effect of direct lipid addition to the incubation medium (i.e., no substrate) on gas release. The inoculum was prepared from hand-squeezed rumen samples obtained pre-feeding (i.e., 07.00 h) from a lactating dairy cow offered a grass silage/hay based diet. Cumulative gas release was depressed with lipid supplementation, but the extent varied with lipid. With rapeseed oil, only the highest level depressed gas release, with fish oil all but the lowest level (i.e., 20 mg/g) had a negative effect on gas release (Table 1), while the pattern for linseed oil was similar to rapeseed. The effect of lipid supplementation on degradation was similar to gas release, with little effect of supplementation until higher levels of linseed and rapeseed oils (Fig. 1) were included. In contrast, fish oil significantly depressed digestibility at all levels above 20 mg/g, suggesting that lipids do not all depress fibre degradation to the same extent (with those of plant origin apparently being less deleterious), thereby indicating that the level of rumen protection required to offset ruminal effects is source dependent. Further, gas release kinetics of hay alone identified the fermentation of cell contents, and primary cell wall components, as peaks in gas production at 4 and 12 h post-inoculation, respectively (Fig. 2). Lipid supplemented

Table 1

Cumulative gas release from hay (ml/g OM) as influenced by lipid supplementation and level

Lipid	Level (mg/g)	Hours post-inoculation				
		6	12	24	36	48
Control ^a	0	35.7	75.5	153.7	192.3	209.8
Fish	20	39.1	81.9	155.1	191.4	210.1
	40	39.1	80.2	146.2	176.2	189.3
	80	36.6	75.2	132.7	158.5	168.7
	120	34.5	71.8	123.1	144.8	153.5
Linseed	20	39.4	81.2	154.1	190.7	208.1
	40	37.9	78.6	150.2	187.0	203.9
	80	38.7	77.6	141.9	174.1	189.9
	120	37.0	71.9	127.9	155.9	164.8
Rapeseed	20	37.5	82.9	154.2	189.8	206.2
	40	39.5	84.7	154.6	191.8	208.5
	80	39.0	82.8	149.4	182.9	197.9
	120	35.6	77.4	133.0	155.8	164.6

^a No lipid supplementation.

hays, at 80 mg/g, had similar gas release profiles up to 10 h, but thereafter gas release was reduced and varied with lipid supplemented. The similarity in gas release to 10 h post-inoculation suggests iOMD reduction was associated with depression of cell wall degradation and, in particular, secondary cell wall components, demonstrating that the *in vitro* model was able to detect differences among lipid supplements, with respect to cell wall degradation.

The system also has the potential to assess the efficacy of techniques that render lipid supplements inert in the rumen, or to establish the degree of rumen protection required for modification of the fatty acid composition of ruminant products by using dietary lipid

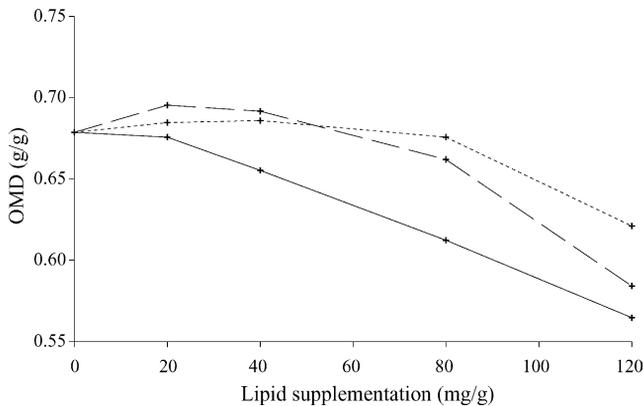


Fig. 1. Effect of lipid supplementation on OM degradation (OMD) of hay: (—) fish oil, (---) rapeseed oil and (· · ·) linseed oil.

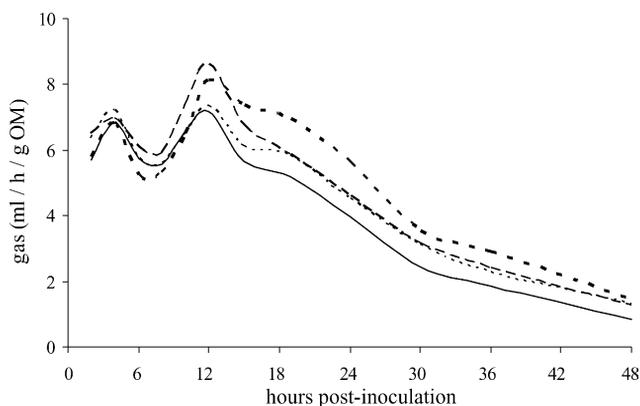


Fig. 2. Influence of lipid supplementation (80 mg/g) on gas release kinetics: (----) hay alone, (—) fish oil, (···) rapeseed oil and (- · -) linseed oil.

supplements. However, this *in vitro* methodology is a batch culture system where neither rumen outflow nor absorption can be simulated. Thus it is possible that the depressing effect of rumen unprotected lipid on fermentation may be overestimated. In contrast, advantages of such *in vitro* systems (i.e., large capacity, rapid and simple assessment, and control of the fermentative environment) not only reduce analytical costs, but offer other possibilities. For instance, sub-samples of the incubation medium can be examined for lipid effects on volatile fatty acids (VFA) and microbial biomass production, or the degree of biohydrogenation.

2.2. Utilisation of amino acids by mixed rumen microorganisms

While the literature regarding ruminal degradation of amino acids appears contradictory, a number of general conclusions can be drawn. Rumen proteolysis is extensive, with a tendency for amylolytic microflora to have a higher level of proteolytic/deaminase activity than one dominated by cellulolytic bacteria. There is an apparent preferential absorption of peptides over single amino acids (Pittman and Bryant, 1964) with slow uptake of the latter tending to limit deamination. The environment in which rumen bacteria have evolved has selected against utilisation (i.e., absorption) of individual amino acids (Bryant and Robinson, 1963). This has been confirmed by *in vitro* studies, which have shown that few rumen microorganisms utilise amino acids when they are included as the sole N source, if energy supply was sufficient (Lewis, 1955; Lewis and Emery, 1962; Hino and Russell, 1985). However, under similar conditions, the majority readily metabolise ammonia, or acid-hydrolysed proteins such as casein. Recent studies have been unable to identify either individual, or combinations, of amino acids that influenced rumen fermentation (Kajikawa et al., 2002; Atasoglu et al., 2003). These findings support the conclusion, first suggested by Cottle and Velle (1988), that if adequate quantities are supplemented, ‘rumen protection’ may not be needed to deliver substantial levels of specific amino acids to the abomasum.

As the utilisation of amino acids by rumen microorganisms cannot be readily determined, except by using radio-isotope labels, an *in vitro* complement assay was developed. The

studies utilised an N-free medium (Morgan et al., 2004) with a rumen fluid inoculum prepared from donor animals in which rumen available N was considered to be marginal to microbial requirements. A pure carbohydrate substrate (i.e., maize starch) was used, as its fermentation is rapidly inhibited when the N supply in the ruminal inoculum is exhausted. The hypothesis on which this model is based assumes that if fermentation continues, in the presence of amino acids, then they are not inert with regards to N availability. Further, the extent to which this fermentation occurs, relative to that of a known N source, provides an estimate of availability.

A dose titration study was conducted to determine effects of supplemental N on maize starch fermentation. On the basis that the standard RPT medium supplied 17 mg N/flask, aliquots of a urea stock solution were added to an N-free medium such that the N content, excluding that supplied by the inoculum, increased from 0 to 17 mg in 10 equal increments. The 11 urea levels, defined as 0–10, respectively, were examined in triplicate with and without inclusion of 1.0 g of maize starch. Initial pH of the rumen fluid inoculum was 6.57, with DM and N contents of 30.3 and 1.2 g/l, respectively. A volume of 10 ml of inoculum and 90 ml of buffer were added/flask. Degradation was not estimated, although headspace gas pressure measurements were made at standard intervals over the 48 h incubation.

Fig. 3 shows that rate of gas release from flasks without supplemental N increased to 6 ml/h/g starch at 6 h post-inoculation and thereafter remained constant. This gas production was assumed to be the basal level of microbial activity that can be supported by the N in rumen fluid inoculum. In contrast, when N was supplemented, rate of gas release increased to 10 h post-inoculation before declining. Gas release increased with each increment of urea, with significant differences in cumulative gas volumes (Table 2). That gas release increased at all levels of N addition suggests that even the highest level of N was insufficient to permit maximal starch fermentation. The highly significant regression ($P > 0.001$, $r^2 = 0.929$) between supplemental N (y , mg) and rate of gas release (x , ml/h) at 10 h post-inoculation of,

$$y = 1.0376x + 0.0008x^2 - 5.062$$

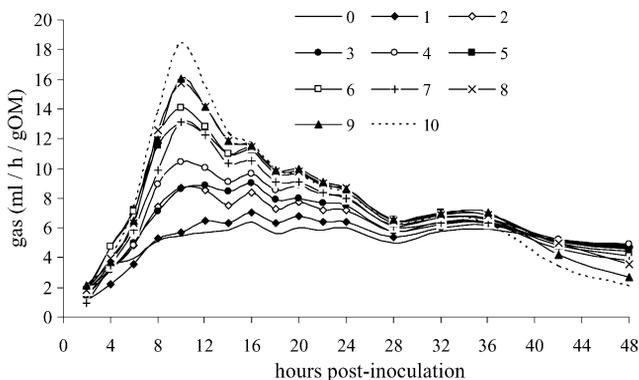


Fig. 3. Gas release kinetics (ml/h/g starch) – influence of supplemental nitrogen (where 0–10 are incremental levels of urea from 0 to 17 mg/g starch).

Table 2

Cumulative gas release from starch (10 h post-inoculation) as influenced by level of N added as urea

Urea level	Nitrogen (mg/flask)	Cumulative gas (ml/g OM)
0	0	39.3 ^{ef}
1	1.7	35.7 ^f
2	3.4	52.0 ^d
3	5.0	50.5 ^{de}
4	6.7	59.7 ^{dc}
5	8.4	76.6 ^b
6	10.1	79.7 ^{ab}
7	11.8	68.9 ^{bc}
8	13.4	80.8 ^{ab}
9	15.1	80.6 ^{ab}
10	16.8	90.7 ^a

Values within columns with common superscripts do not differ (i.e., $P > 0.05$).

confirmed the hypothesis that gas release could be used to determine N availability to the microorganisms. Thus where amino acids are used as supplements, the extent of gas release is a direct function of their utilisation by rumen microorganisms.

This effect was examined in a second study (Mould et al., 2004) in which the experimental procedures were similar to the first except that nine amino acids (i.e., alanine, glutamine, glutamic acid, glycine, histidine, lysine, methionine, phenylalanine and serine) together with urea (positive control) were included at levels equivalent to 8, 12 or 16 mg N/g starch. Gas release was examined at 2 h intervals over a 16 h incubation period. Results, expressed as cumulative gas production (Fig. 4), show that dose responses were obtained with urea and glutamine, while supplementation with alanine, glutamic acid and serine increased gas release, although to a lesser extent. However the absence of any appreciable gas release from glycine, histidine, lysine, methionine and phenylalanine, relative to that of the unsupplemented controls (i.e., 85 ml gas), suggests that these amino acids were poorly utilised by

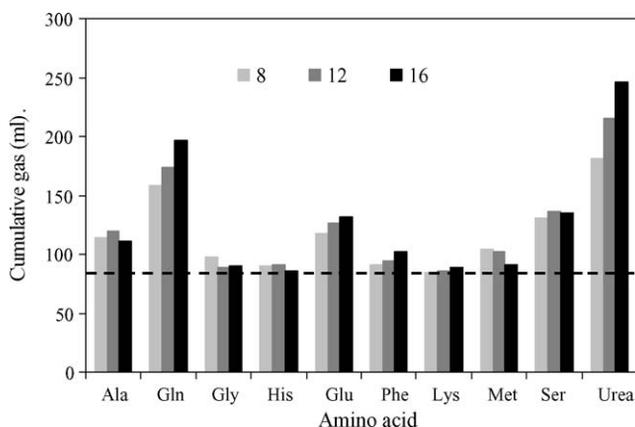


Fig. 4. Influence of amino acid or urea addition at three levels (8, 12 or 16 mg/g starch) on cumulative gas release (ml). '---' indicates level of gas release from controls.

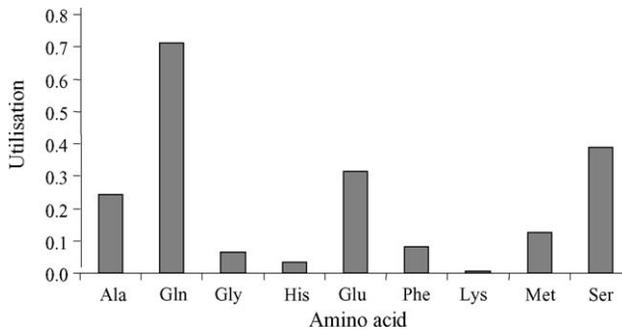


Fig. 5. Amino acid utilisation in vitro relative to urea.

the rumen microbial inoculum, even under N limiting conditions. Utilisation, expressed as a function of gas release corrected for that of the controls relative to urea (Fig. 5), suggests a value of 0.71 for glutamine, while those for serine, glutamic acid and alanine were 0.39, 0.31 and 0.24, respectively, with others varying from 0.13 to only 0.01.

This technique identified utilisation of urea by rumen microorganisms, and showed that a marked difference in utilisation of individual amino acids by a mixed rumen microflora occurred. The results suggest that protection of some supplemental dietary amino acids, e.g., lysine and methionine, from rumen microbial degradation might not be required to deliver substantial quantities of them to the abomasum.

3. Hydrolytic activity – an alternative method of assessing microbial inocula

The function of the inoculum is to generate a fermentative environment representative of the gastro-intestinal tract section being studied. Ideally the inoculum should include all microbial species in the proportions that they are present. However gastro-intestinal microflora exist in three sub-populations, being those associated with the fluid phase, particulate (feed) material and gut epithelial tissues. Problems in sampling these niches may lead to poor estimation of both microbial numbers and the relative importance of their metabolic activity. For example, Stewart (1977) showed that the size of the cellulolytic population did not provide an accurate indication of activity under all conditions. In addition, microorganisms in isolation often metabolise substrate differently than in mixed culture due to nutritional interdependence (Van Gylswyk et al., 1992). Further, where slight variations occur in gas release with different inocula, they are often attributed to microbial concentrations rather than metabolic activity. Thus if a population is deficient in a particular metabolic activity, increasing the level of inoculation will have no benefit on gas release. While this deficiency could be determined by examining a representative microbial sample for specific enzymatic activities, it provides little useful information as enzymic profiles change constantly due to the dynamic nature of the microbial population.

The reason, therefore, for evaluating the ‘hydrolytic activity’ of microflora is to identify the metabolic profile of the inoculum, rather than that of its individual microbial species. That *in vitro* inocula differ in their microbial composition is of little interest per se, as long as the outcome is the same (i.e., that similar substrates are degraded at a comparable rate and end-product release is equivalent).

The metabolic activity of an inoculum is a reflection of its ability to degrade/hydrolyse specific substrates. Thus if these are offered, the resulting fermentation profiles provide a simple method to assess quality/activity of the inocula, rather than species present or microbial population densities. Thus comparison of two inocula or the impact of a dietary manipulation on microbial activity could be readily assessed. The data discussed here, in which the hydrolytic activity of faecal and rumen contents were assessed, are taken in part from Afdal et al. (2002).

Rumen fluid and faecal samples (per rectum) were obtained 1 h before feeding at 07.00 h from two dry cows offered a diet of grass silage and hay, on two consecutive weeks. The faecal material was blended for 30 s with an equal volume of reduced buffer under CO₂ to minimise exposure to air. This was strained through a single muslin cloth twice, while the rumen fluid was strained through a double layer of cloth. A volume of 10 ml of the resulting fluids were used to inoculate 90 ml buffer containing 1.0 g substrate. Saccharolytic, amylolytic and fibrolytic activity of the inocula were assessed using xylose (X), maize starch (S) and cellulose (C, Sigmacell®), respectively (Sigma Chemicals Ltd., St. Louis, USA) over a 48 h incubation period. A fourth substrate, washed hay (WH) was used to estimate cell wall degradation kinetics. This was prepared by washing pre-milled (2 mm) hay with tap water in a modified pipette washer for 16 h, before drying at 40 °C. Its degradation profile was close to that of cellulose, however it had the advantage that it contained a variety of cell wall components.

Based on 48 h cumulative gas production both inocula ranked the substrates in a similar order (i.e., S > X > C > WH), although faecal gas values were lower and broadly equivalent to those of rumen fluid at 24 h (Table 3). Not only were faecal lag times extended but, in contrast to rumen fluid, the least fermentable substrates (i.e., C and WH) could not be differentiated at 48 h. Due to similarities between S fermenta-

Table 3
Effect of inoculum type (F – faeces, R – rumen fluid) on cumulative gas release as affected by substrates

Substrate	Inoculum	Cumulative gas release (ml/g OM)				
		6 h	12 h	19 h	24 h	48 h
Starch	F	34.2 ^b	202.3 ^b	278.5 ^a	298.3 ^a	325.1 ^a
	R	78.1 ^a	230.0 ^a	297.9 ^a	323.5 ^a	356.8 ^a
Cellulose	F	1.2 ^a	3.4 ^a	6.3 ^b	16.1 ^b	114.0 ^b
	R	5.0 ^a	12.4 ^a	44.8 ^a	92.6 ^a	260.7 ^a
Washed hay	F	0.5 ^b	4.0 ^b	7.9 ^b	14.6 ^b	93.1 ^b
	R	2.0 ^a	10.0 ^a	39.0 ^a	70.0 ^a	158.2 ^a
Xylose	F	3.5 ^b	59.4 ^b	161.8 ^b	188.3 ^b	233.3 ^b
	R	24.6 ^a	101.1 ^a	216.0 ^a	255.6 ^a	294.0 ^a

Means within substrate and column with different superscripts differ ($P < 0.05$).

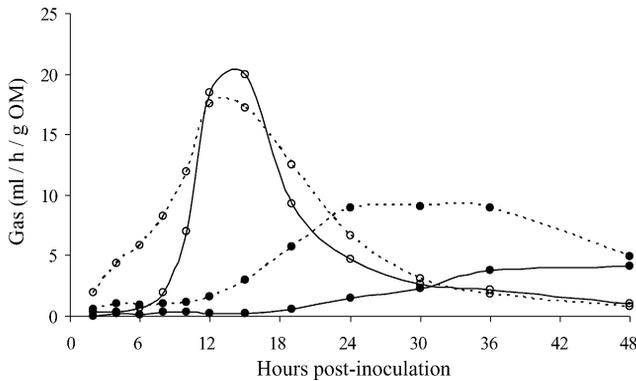


Fig. 6. Effect of inoculum on gas release kinetics: (—) faeces, (---) rumen fluid, (○) xylose and (●) cellulose.

tion profiles, it was concluded that the amylolytic activity of rumen fluid and faeces was, under these conditions, similar. However marked differences in rates of gas release from xylose and cellulose between inocula (Fig. 6) suggested compositional differences between rumen and faecal microflora in terms of species present and/or their hydrolytic activity. The use of faecal inocula resulted in significantly lower values for washed hay degradation versus rumen fluid (Table 4), especially 24 h post-inoculation.

These findings support the idea that faecal microflora have a different hydrolytic profile to rumen microflora, tending to be characterised by fewer, opportunistic (i.e., facultative) microorganisms, with a reduced cellulolytic activity. In contrast, rumen microorganisms are specialised, exhibiting a considerable range of metabolic activity. The results confirm the lower rates of *in vitro* fermentation and degradation associated with the use of faecal inocula. In addition, the hydrolytic approach suggests that metabolic differences, rather than microbial populations (species and/or numbers) were mainly responsible. Parallel studies have shown (Mould et al., 2005) that increasing the quantity of inoculum does not markedly alter degradation. Care should therefore be taken when extrapolating *in vitro* kinetic data obtained with faecal inocula, to *in vivo* situations.

Table 4
Effect of inoculum type on degradation of washed hay (g/kg OM)

Inoculum	Degradation (g/kg OM)				
	6 h	12 h	19 h	24 h	48 h
Faeces	19.5 ^b	19.0 ^b	39.4 ^b	71.8 ^b	363.2 ^b
Rumen fluid	15.7 ^a	36.7 ^a	146.5 ^a	269.9 ^a	495.3 ^a

Means within column with different superscripts differ ($P < 0.05$).

4. Quality prediction of ensiled forage maize

Assessment of feedstuff quality is probably the most common application of *in vitro* methodologies, with tens of thousands of samples assayed annually. With respect to forage maize, the information provided to the producer, on which decisions, such as variety, crop management and harvesting date, and their effect on potential energy and protein yields are made, are based almost entirely on fresh crop measurements. However, essentially all forage maize offered to livestock is ensiled prior to feeding. The data presented here are from a series of studies, including Mould et al. (2000), in which effects of harvesting date on maize silage yields and nutritive quality were investigated. In addition, an attempt was made to estimate ensiled maize quality from an *in vitro* analysis of the fresh crop.

In the starch assay, water-soluble carbohydrates (WSC) were extracted in aqueous ethanol prior to gelatinisation and enzymatic hydrolysis. A separate procedure was used for WSC analysis. Neutral detergent fibre (NDF) and WSC content of the fresh material declined with increasing crop maturity (0.62 to 0.52 and 0.23 to 0.09, 117 d (H1) to 160 d post-sowing (H4), respectively), while starch increased markedly (0.13 to 0.39) and especially from 132 (H2) to 145 d H3 (0.21 to 0.38). In contrast, ensiled material had essentially no WSC, as this had been fermented during the ensiling process. Yields of the various carbohydrate fractions in fresh and ensiled maize are given in Figs. 7 and 8, respectively. Changes in carbohydrate composition and degradability with time (harvest) were readily identifiable from gas release profiles. Early harvested material (H1) had a low level of WSC fermentation (gas peak at 4 h post-inoculation), but by H2 the quantity of WSC had increased, as evidenced by the higher peak (Fig. 9). At H3, the slightly lower peak and its time displacement suggests that WSC are beginning to be stored as more slowly fermentable starch, while material harvested at H4 shows the lowest WSC levels with the slightly lower starch peak indicating that it is becoming more resistant to degradation. Corresponding values for ensiled material show

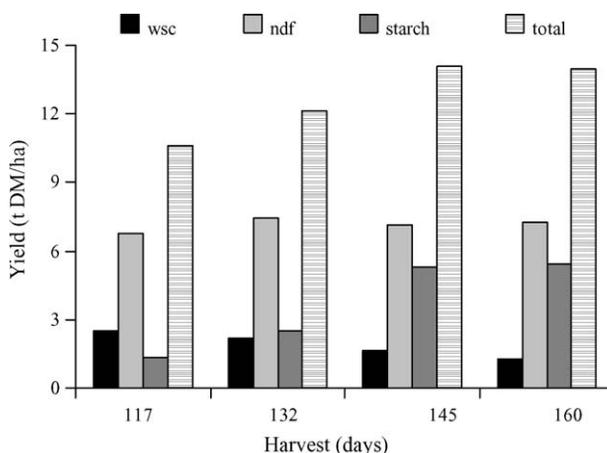


Fig. 7. Carbohydrate yield of fresh forage maize as influenced by harvest date.

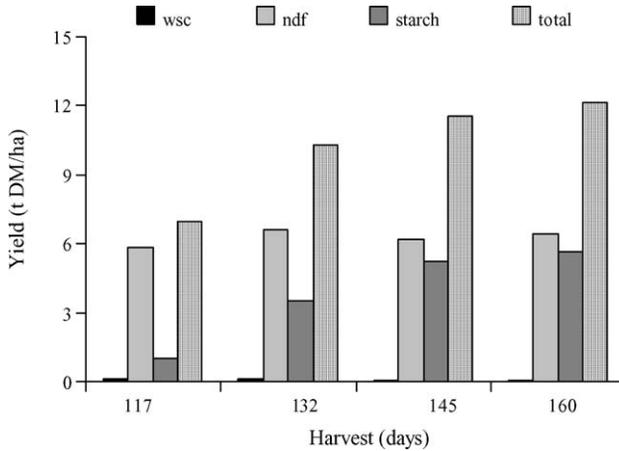


Fig. 8. Carbohydrate yield ensiled forage maize as influenced by harvest date.

that labile WSC is absent at H1 and greatly reduced at H2 (Fig. 10). While the H3 and H4 profiles for ensiled material were not dissimilar to those of fresh maize, the ensiling (i.e., acidification) process appears to have slightly enhanced fermentation. Thus peak gas yields occurred earlier over a shorter time period.

Analysis of fermentation residues allows degradation estimates to be made. By comparing these with crop yields, and correcting for ensiling losses, estimates of both metabolizable energy (ME) harvested and the contribution of various feed components can be estimated. The emphasis on composition relates to the specific substrate requirements of dairy cows to achieve their lactation potential. For instance, while milk yield is related to supply of glucogenic substrates, the quantity of circulating lipogenic VFA directly impacts milk fat secretion. While evidence linking dietary carbohydrate composition and milk component yield is inconsistent, the selection towards later maturing, higher DM yielding, varieties

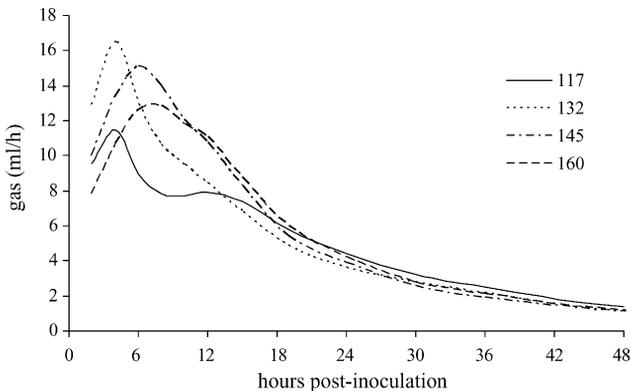


Fig. 9. Fresh forage maize: influence of harvest (days) on gas release kinetics.

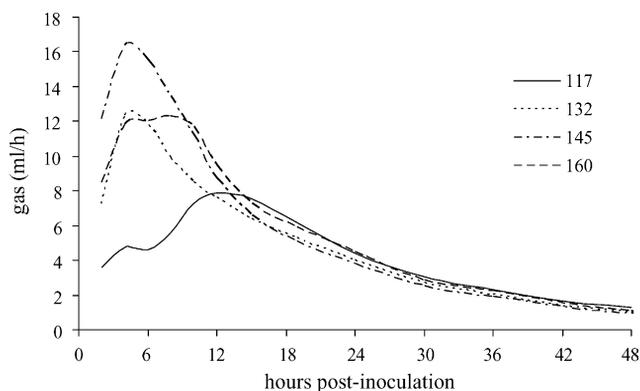


Fig. 10. Ensiled forage maize: influence of harvest (days) on gas release kinetics.

simply on the basis of ME yield, could have severe consequences should these varieties fail to reach maturity (i.e., maximize their starch content). The ME content remains high over the growing season, a consequence of the increase in starch content, while fibre declines along with its degradability. However silage from an immature crop will be low in readily degradable carbohydrate and a depression in fibre degradability will adversely affect animal intake and production. To estimate ME values, an estimate of starch content is required. However, starch analysis is time consuming, relatively expensive and results are often variable (Krystallidou and Mould, 2004). Thus, an alternative estimate is proposed based on the finding that the summation of ash, crude protein, NDF, WSC and starch values, obtained by chemical analysis, comprised 0.94 of total DM. Assuming that the ether extract (EE) comprises a further 0.03 (MAFF, 1990) then a simple estimate of 'starch' or more correctly non-WSC 'non-structural carbohydrates' (NWNS) can be obtained as;

$$\text{NWNS} = 1 - (\text{ash} + \text{CP} + \text{NDF} + \text{WSC} + \text{EE} + e)$$

where e represents the residual analytical error. Regressing NWNS (x) and assayed starch (y) values resulted in a highly predictive relationship ($y = 1.039x + 0.0127$, $r^2 = 0.872$, $P < 0.001$, $n = 34$). Thus by analysing fresh material for NDF, estimating starch content using the difference method, and applying a digestibility coefficient to each (with that for NDF having been obtained *in vitro*) an estimate of the nutritive value of the ensiled crop can be made. In addition, the earliest harvesting date can be predicted by using gas release kinetics to identify when the transformation of WSC to starch (i.e., its translocation to the cob), is complete.

5. Conclusions

In vitro gas-based feed evaluation systems are extremely powerful research tools and capable of a great deal more than routine analysis of feedstuffs. The ability of complement systems to provide simple solutions to relatively complex situations was demonstrated. The differences identified in the lipid study suggest that the degree of rumen protection

required to prevent ruminal fibre degradation being depressed varies with lipid type. The methodology will allow such treatments to be refined and examined under various rumen conditions prior to animal studies.

A similar conclusion can be drawn from the amino acid studies, however these indicate that the current level of rumen protection may not be required. This offers the possibility of incorporating free amino acids, such as methionine and lysine, directly into the diet, rather than as N-free precursors such as methionine hydroxy analogue, while obtaining substantial ruminal outflow.

Use of specific substrates allows the metabolic activity of an inoculum to be examined directly, thus the suitability of alternative inocula or impact of dietary manipulations on a known microflora can be readily assessed. Finally, use of *in vitro* techniques to identify optimum harvest periods, possibly linked with near infrared reflectance techniques, to predict the nutritional value of feedstuffs post-ensiling from an examination of fresh material has wide ranging possibilities, not just for the producer but also in disciplines such as plant breeding where fresh crop DM yield is an inadequate forage selection criteria.

Unlike other techniques, gas production systems provide detailed kinetic data on substrate degradation and end-product release together with information on microbial population dynamics. The ability to strictly control the fermentative environment, coupled with the high capacity of many current systems allows several factors to be examined simultaneously. However every system has limitations and ‘over-interpretation’ of data must be avoided. Nevertheless, with ingenuity and thinking ‘outside the box’, a great deal remains to be achieved with this methodology.

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