

The use of cumulative gas and volatile fatty acid production to predict *in vitro* fermentation kinetics of Italian ryegrass leaf cell walls and contents at various time intervals

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Differences between the fermentation characteristics of cell contents (CC) and protease-treated cell walls (CW) of young leaves of Italian ryegrass (*Lolium multiflorum* Lam.) cultivar Multimo (tetraploid), were studied *in vitro*. Gas and volatile fatty acid (VFA) production rates were measured at regular intervals, as was the degradability of organic matter (OM) of CW. The measured VFA were used to predict the gas production and fermentable OM using stoichiometric calculations. For CW the volume and kinetics of measured gas production were the same as those predicted from the VFA formed. In contrast, the measured gas production for CC was consistently less than predicted, indicating that the stoichiometric equations were not valid for rapidly fermenting substrates. For both CC and CW, the relative rate of acetic acid production levelled off more slowly than for other VFA, resulting in an increasing gas yield (in ml/g fermentable OM) after 12 (CW)–24 (CC) h incubation. Consequently, the fermentation of OM was not linearly related to gas production kinetics. For CW, the kinetics of decline of degradable OM and fermentable OM were the same, after correction for a constant 'lost fraction' of degradable OM of 205 g/kg OM. This work indicates the value of detailed studies of fermentation processes to evaluate herbage quality. In particular, the role of CC and the difference between degradation and fermentation require further attention.

Fermentation: Grass: Volatile fatty acids

Forages are usually evaluated using *in vitro* and *in situ* methods (e.g. Erwin & Elliston, 1959; Tilley & Terry, 1963; Goering & Van Soest, 1970; Ørskov *et al.* 1980). However, the degradation of feedstuffs in the rumen requires sequential processes of solubilization and fermentation. Both processes depend on the chemical composition and structure of the substrate (Salvador *et al.* 1993; Merchen & Bourquin, 1994). Solubilization of cell walls requires hydration, colonization by rumen micro-organisms and then the release of enzymes from these micro-organisms, to digest the complex cell-wall structures (McAllister *et al.* 1994). These processes result in degradation of cell walls. The *in vitro* and *in situ* techniques now in use have the disadvantage that the contribution of the soluble components of the original feed cannot be assessed, since they

rely mainly on the measurement of particle loss. Fermentation, on the other hand, is the anaerobic decomposition of substrates by micro-organisms and can be assessed according to the production of end-products, of which volatile fatty acids (VFA) and gas are the most important (Lin *et al.* 1985). At present, cumulative gas production is increasingly being used to measure the fermentation kinetics of ruminant feeds (Beuvinck & Spoelstra, 1992; Pell & Schofield, 1993; Theodorou *et al.* 1994).

The complex interactions within a mixed rumen microbial population leading to the conversion of plant components to gas and VFA, have been summarized in the form of stoichiometric reaction equations (Wolin, 1975, 1979; Russell & Wallace, 1988; Van Soest, 1994). All of the equations show the relationship between the hexose-

Abbreviations: CC, cell contents; CW, protease-treated cell walls; NDF, neutral-detergent fibre; OM, organic matter; VFA, volatile fatty acids.

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equivalents fermented, and the proportions of fermentation products produced. Such equations have been used to estimate the extent of digestion of hexose-equivalents on the basis of the fermentation products formed (e.g. Blümmel & Ørskov, 1993).

The aim of the present work was to investigate the dynamics of degradation and fermentation of the protease-treated cell walls (CW) and cell contents (CC) of the leaves of Italian ryegrass (*Lolium multiflorum*). Gas and VFA production rates were measured at regular intervals throughout incubation. The VFA data obtained during fermentation were used in stoichiometric equations to predict both organic matter (OM) disappearance and formation of gas. The predicted values were then compared with those measured, to determine whether cumulative gas production reflected changes in other fermentation variables, including VFA and the disappearance of OM.

Materials and methods

Substrates and cumulative gas production

Italian Ryegrass (*Lolium multiflorum* Lam., tetraploid cultivar Multimo) was sown on 28 October 1993. The seeding rate was nine plants per pot (0.0169 m² surface area) containing a mixture of sand and peat (1:1, v/v). The plants were grown in a heated glasshouse, at day and night temperatures of 18 and 13° respectively. Day temperatures were maintained between 08.00 and 20.00 hours. Day length was natural, and the relative humidity was approximately 70%. Additional light was supplied by Philips 400W SONT lamps (Philips, Eindhoven, The Netherlands) for 14 h/d. The pots were placed in large trays, which were flushed with nutrient solution (Steiner, 1984). The pots were placed in two replicate blocks. Plants were harvested after 113 d of growth, when they had formed eight leaves on the main stem (18 February 1994). The last fully expanded leaf was dissected from the main stem of each plant and frozen separately before analysis.

Cell wall and contents fractionation, and cumulative gas production

Fresh leaves were first chopped into 10–20 mm pieces using a paper guillotine and then further chopped in a Knifetec Sample Mill (Tecator, Sweden). This material was pressed (HAPA hydraulic press, IKA, Germany; 250 ml) through a double nylon cloth (Nybolt PA 40/30, Zurich, Switzerland) under a pressure of 10 100 kPa. The residue was mixed thoroughly with distilled water, and the process repeated three times. The resultant filtrate was cooled with solid CO₂, while the residue was washed. Both components were freeze-dried and ground through a 1 mm sieve (Retsch mill, Haan, Germany). The residue was then treated with neutral detergent (Goering & Van Soest, 1970) and protease (Sigma Protease Type XXIII P-4032; Sigma, St Louis, MO, USA) resulting in the CW fraction. The protease treatment lasted for 1 h in 60 ml phosphate buffer containing 0.25 ml protease/g DM.

Two replicate samples were incubated for cumulative gas production measurements during various incubation

periods, according to the method of Theodorou *et al.* (1994). Bottles containing CC were removed at 3, 6, 12, 24, 48 and 120 h after inoculation, while the CW removal times were 6, 12, 24, 48, 72 and 120 h. The fermentation was stopped by plunging the bottles into iced water for 20 min. A liquid sample was then collected for VFA analysis and the residual CW estimated by filtration through sintered glass crucibles (Schott Duran, porosity no. 2, Mainz, Germany) and rinsing with hot water. Blank bottles containing only medium and inoculum, but no substrate, were incubated for 120 h.

Chemical and volatile fatty acid analysis

Neutral-detergent fibre (NDF; Goering & Van Soest, 1970), crude protein and ash were determined according to standard procedures. VFA were analysed using GLC (Packard 419, (CE Instruments, Milan, Italy), glass column filled with Chromasorb 101, carrier gas N₂ saturated with methanoic acid, 190° with isocaproic acid as the internal standard).

Stoichiometric calculations

The theoretical stoichiometric calculations used to obtain an approximate indication of OM fermentation, were based on the VFA production measured at each time interval (Van Soest, 1994). OM fermentation was expressed in glucose equivalents (g). It was assumed that the glucose equivalents were fermented to form the end-products: acetic (HAc), propionic (HPr), and butyric acids (HBu), and the gases CO₂ and CH₄, as well as being incorporated into microbial biomass.

To calculate the amount of glucose required for microbial growth, the amount of ATP (mmol) synthesized can be estimated from the VFA production. VFA production results in direct gas production (1 mmol from acetic acid and 2 mmol from butyric acid; Hungate, 1966; Beuvink & Spoelstra, 1992). To estimate ATP production for microbial growth, the molar proportions of CO₂ and CH₄ in the gas produced also need to be derived. From stoichiometric equations it can be calculated that:

$$\text{CO}_2 = \frac{\text{HAc}}{2} + \frac{\text{HPr}}{4} + \frac{3 \text{HBu}}{2}, \quad (1)$$

$$\text{CH}_4 = \text{HAc} + 2 \text{HBu} - \text{CO}_2. \quad (2)$$

The production of microbial biomass was estimated from the total ATP production (2, 3 and 3 mol/mol acetic, propionic and butyric acids respectively) and the yield of microbial biomass per mol ATP (Y_{ATP} , assumed to be constant at 10 mg/mmol ATP; Hespell, 1979). It was assumed that 80% of bacterial structures were synthesized from glucose skeletons. The glucose consumption for production of biomass ($\text{Glucose}_{\text{biomass}}$) and for production of products ($\text{Glucose}_{\text{products}}$) were calculated as:

$$\text{Glucose}_{\text{biomass}} = 0.8 Y_{\text{ATP}}(2 \text{HAc} + 3 \text{HPr} + 3 \text{HBu} + \text{CH}_4), \quad (3)$$

Glucose_{products}

$$= 162 \frac{2 \text{ HAc} + 3 \text{ HPr} + 4 \text{ HBU} + \text{CO}_2 + \text{CH}_4}{6} \quad (4)$$

Total fermentation of hexose-equivalents was calculated as Glucose_{products} + Glucose_{biomass}. Theoretically, one mmol CO₂ should be released from the bicarbonate buffer in the medium per mmol VFA produced. This was used to calculate an 'indirect' measure of gas production, which was added to the calculated 'direct' gas, to predict the total gas production. This predicted gas production was then compared with the measured values.

Statistical analysis

Profiles of cumulative gas (ml/g OM) and VFA (mmol/g OM) production were calculated, as were the degradation and fermentation of OM (g/kg OM). These were fitted iteratively to the model described by Groot *et al.* (1996) (equation 5). In this equation, G (ml/g OM; mmol/g OM; g/kg OM) denotes the amount at time *t* after incubation. A_{*i*} (ml/g OM; mmol/g OM; g/kg OM) represents the asymptote. B_{*i*} (h) is the time after incubation at which half of the asymptotic amount has been formed, and C_{*i*} is a constant determining the sharpness of the switching characteristic of the profile. The value of *i* indicates the number of phases in the profile (*i* = 1, *n*). In this experiment, the monophasic curve (*i* = 1) was used, since only six observations were available per profile.

$$G = \sum_{i=1}^n \frac{A_i}{1 + \frac{B_i^{C_i}}{t^{C_i}}} \quad (5)$$

Significance of the differences of the parameters was determined by ANOVA. Changes in the proportions of

acetic, propionic and butyric acids, and the gas yield during the incubation were analysed by linear regression. Genstat 5.3 (Genstat 5 Committee, 1993) was used for all statistical analyses.

Results

The crude protein (258 (SE 18.2) g/kg OM) and ash (213 (SE 6.1) g/kg OM) contents were higher for CC than for CW, which contained 120 (SE 7.0) g crude protein/kg OM and 18 (SE 0.3) g ash/kg OM. NDF content in CW was 771 (SE 14.7) g/kg OM, while this was negligible for CC and so is not reported here.

Gas and volatile fatty acid production

The measured cumulative gas production and gas production rates are presented in Fig. 1, while the fitted curve parameters for both measured and predicted gas production are shown in Table 1. Although the final measured gas volumes per g OM were not significantly different between CC and CW (Fig. 1(a); Table 1), the maximum gas production rate was higher for CC than for CW (Fig. 1(b)). The maximum gas production rate was reached more rapidly for CC than for CW. As a consequence, the half-time of the fitted curve (B parameter) occurred later for CW than for CC.

For the gas production predicted from VFA, the kinetic parameters B and C and, thus, the relative gas production rate, were the same as for the measured curves. However, the measured gas volume for CC was considerably lower than predicted from VFA produced, due to a lower absolute gas production rate, which was most evident during the first 24 h of the incubation (Fig. 1(b)). For CW the predicted volume of gas produced corresponded closely with the measured value (Table 1).

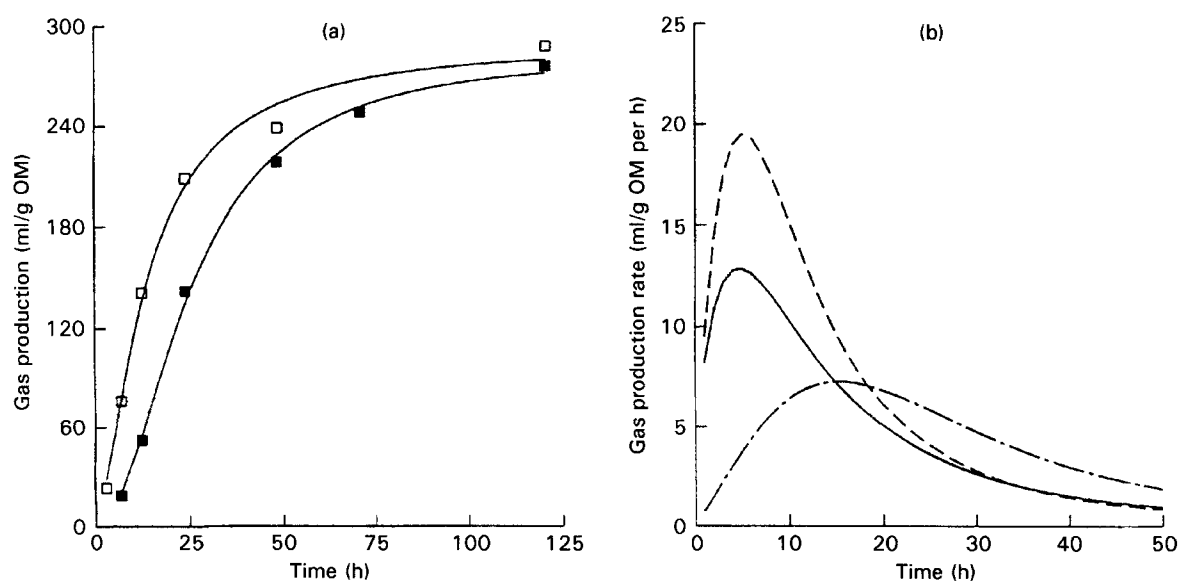


Fig. 1. (a) Measured gas production from cell contents (CC, □) and protease-treated cell walls (CW, ■) of Italian ryegrass during 120 h fermentation, and the fitted monophasic curves. (b) Gas production rate for CC measured (—) and predicted from volatile fatty acids (— · —), and for CW measured (---) during the first 50 h of fermentation. Rates were calculated from fitted curves. OM, organic matter.

Table 1. Mean values* at 120 h incubation and fitted parameter A for measured and predicted gas production (ml/g organic matter (OM)), volatile fatty acid production (mmol/g OM) and OM fermentability and degradability (g/kg OM) for cell contents (CC) and protease-treated cell-wall (CW) fractions of the leaves of Italian ryegrass; fitted half-time (B, h) and the shape parameter (C) are also presented

Variate	Fraction	120 h	A	B	C
Measured gas	CC	287	291	13.8	1.50
	CW	275	284	25.6	2.06
Predicted gas	CC	350	362	11.4	1.70
	CW	274	290	26.5	2.11
LSD		21.3	29.4	3.93	0.322
Measured HAC	CC	3.76	3.94	10.2	1.48
	CW	3.95	4.20	28.2	2.02
Measured HPr	CC	3.23	3.32	9.6	2.35
	CW	1.25	1.35	18.6	2.94
Measured HBu	CC	0.71	0.72	13.0	2.76
	CW	0.32	0.37	19.7	2.31
LSD		0.256	0.305	2.65	0.632
Predicted FOM	CC	847	869	10.2	1.92
	CW	597	621	23.7	2.23
Measured DOM†	CW	808	928	19.5	1.18
	CW corrected‡	603	631	23.8	2.05
LSD		94.6	101.1	3.53	0.342

HAC, acetic acid; HPr, propionic acid; HBu, butyric acid; LSD, least significant difference according to the Tukey test ($\alpha = 0.05$); FOM, fermentable organic matter; DOM, degradable organic matter.

* All values were corrected for the blank.

† Not determined for the CC fraction.

‡ CW degradation corrected for the calculated 'lost fraction' of 205 g/kg OM (see p. 524).

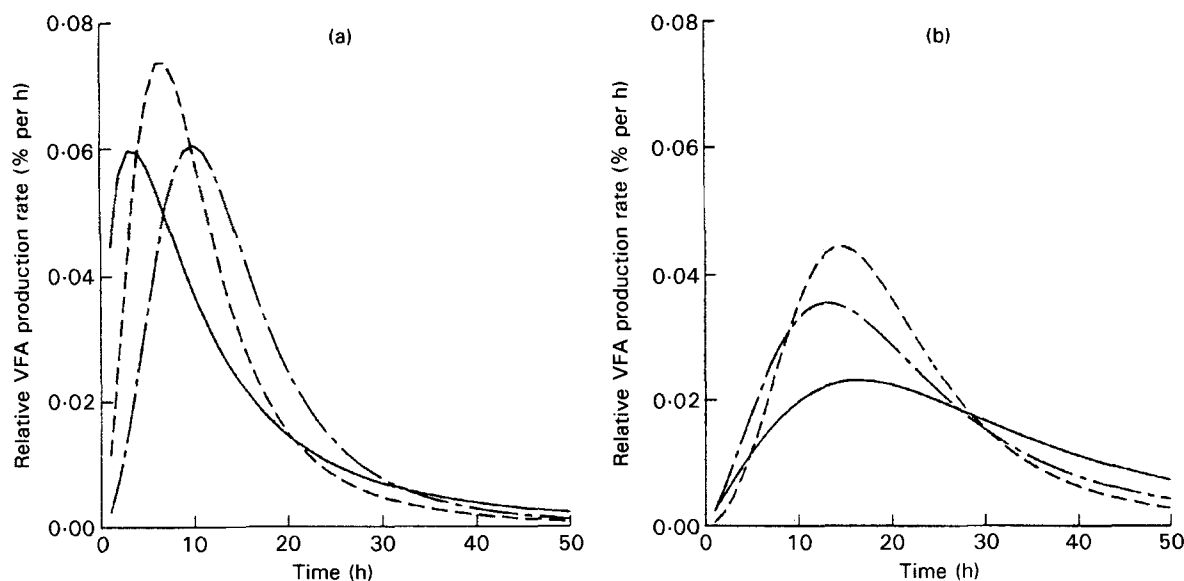


Fig. 2. Relative production rates of acetic acid (—), propionic acid (- - -) and butyric acid (- · -) from (a) cell contents and (b) protease-treated cell walls of Italian ryegrass during the first 50 h of incubation. Relative rates were calculated from fitted curves. VFA, volatile fatty acids.

After 120 h of incubation, 11.0 (SE 0.53) and 8.2 (SE 0.29) mmol VFA/g OM had been produced from CC and CW respectively. For both fractions, 89% of the total VFA consisted of acetic, propionic and butyric acids. The amounts and kinetics of VFA production are presented in Table 1 and Fig. 2 and molar percentages of these acids are shown in Fig. 3.

Two phases could be distinguished in the changes of the proportions of VFA for CC. Until 24 h of incubation, the

proportion of acetic acid declined and propionic and later butyric acid proportions increased (Fig. 3(a)). After 24 h of incubation, there was a linear increase in the proportion of acetic acid and a decrease in the proportion of propionic acid (both $P < 0.001$). These trends could be related to the changes in the relative rates of VFA production (Fig. 2(a)). A succession of maxima in the relative production rates of acetic, propionic and butyric acids occurred, after 3, 7 and 10 h of incubation respectively. After the maxima had been

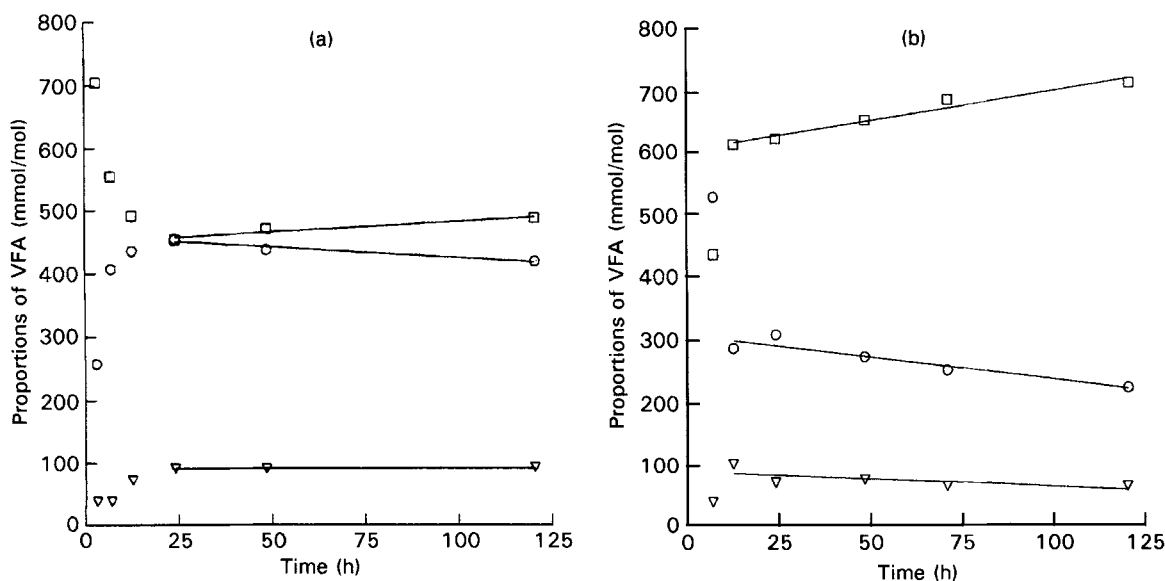


Fig. 3. Proportions of the total volatile fatty acids (VFA) occurring as acetic acid (□), propionic acid (○) and butyric acid (▽) during 120 h fermentation of (a) cell contents and (b) protease-treated cell walls of Italian ryegrass. The lines indicate the significant linear relationship determined by regression analysis after 24 h and 12 h incubation for cell contents and cell walls respectively.

reached, the relative production rates declined rapidly for all VFA, though more so for propionic and butyric acid than for acetic acid.

For CW, rapid changes in the proportions of VFA occurred between 6 and 12 h of incubation (Fig. 3(b)). After 12 h of incubation, changes were more gradual, with an increase in the proportion of acetic acid, and declining propionic and butyric acid proportions ($P < 0.001$). The propionic acid production was lower than with CC (Table 1). As a result the proportions of acetic and butyric acid in total VFA were higher. The maxima in the relative

production rates of VFA occurred almost simultaneously, but the relative rate of acetic acid production levelled off more slowly than for propionic and butyric acids (Fig. 2(b)).

Degradation, fermentation, and gas yield

Stoichiometric equations were also used to predict how much OM should have been fermented to produce the amounts of VFA measured from CC and CW (Fig. 4(a)).

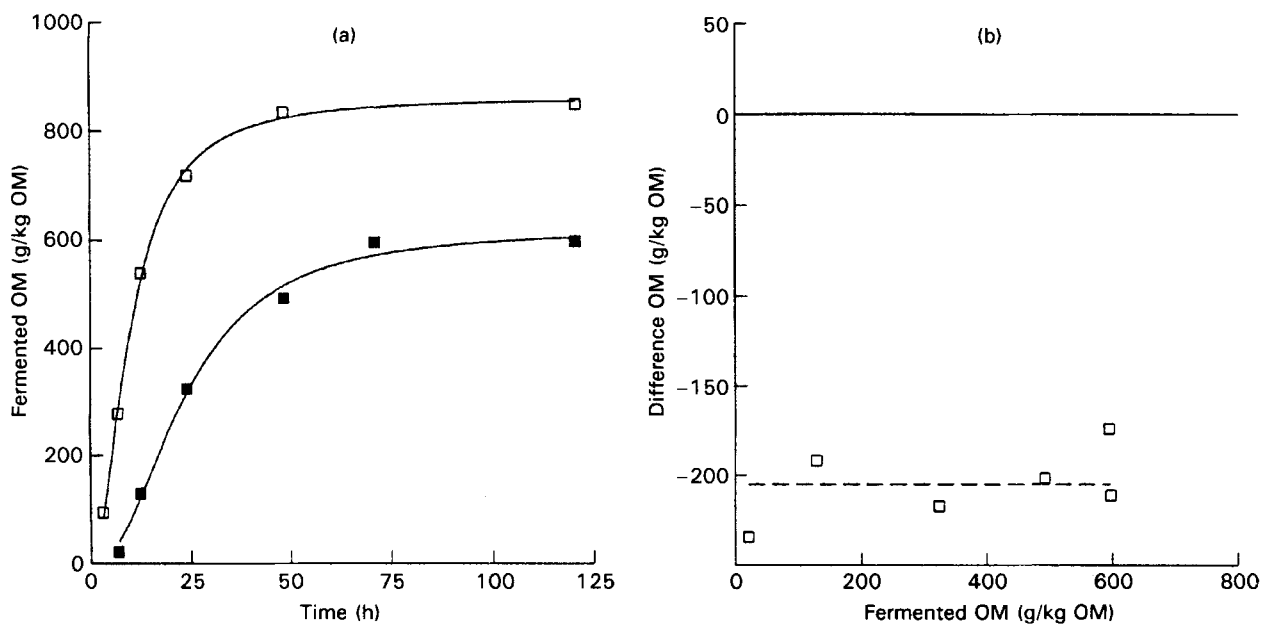


Fig. 4. (a) Stoichiometrically calculated organic matter (OM) fermentability for cell contents (□) and protease-treated cell walls (■) from Italian ryegrass, and the fitted monophasic curves. (b) The difference between predicted OM fermentability and measured OM degradability v. predicted OM fermentability. (---), Average value.

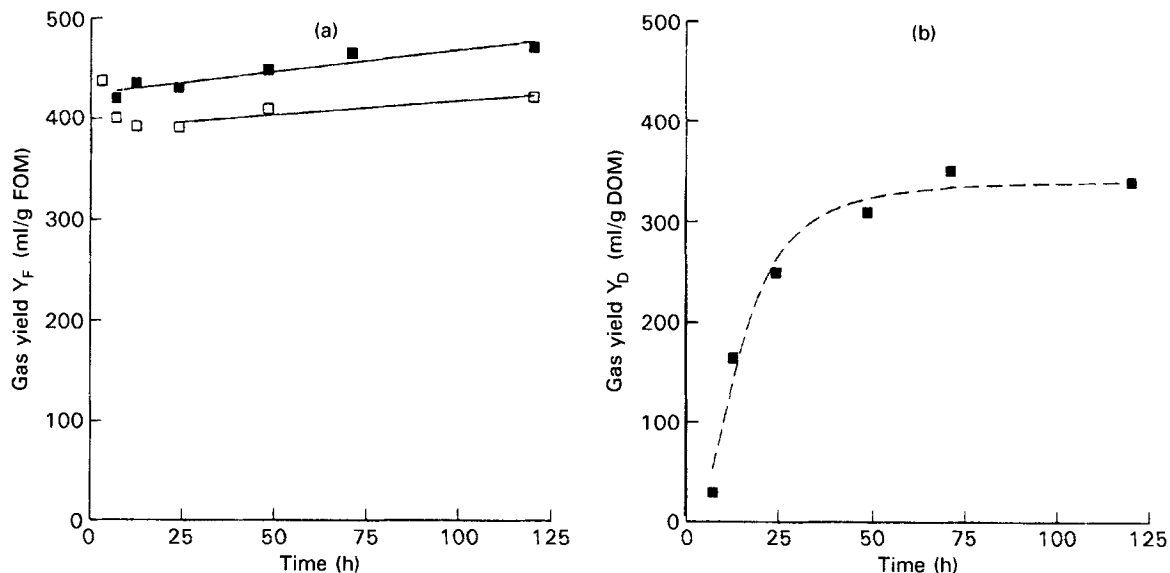


Fig. 5. (a) Changes in predicted fermentative yield of gas (Y_F ; ml/g predicted fermentable organic matter (FOM)) for cell contents (□) and protease-treated cell walls (■) of Italian ryegrass. (b) Changes in measured degradative yield of gas (Y_D ; ml/g measured degradable organic matter (DOM)) for protease-treated cell walls (■).

The predicted final fermentability of both CC and CW was considerably lower than 100% (Table 1).

Fig. 4(b) shows that for CW the predicted OM fermentability was 205 (SE 21.0) g/kg OM lower than the measured degradability. This difference was considered to be a 'lost fraction'. Moreover, the asymptotic gas volume A was higher and the kinetic parameters B and C were lower than for the measured degradation. However, when the degradability was corrected for the 'lost fraction' of 205 g/kg OM at every point of observation, the fitted parameters of degradability of CW corresponded with those of fermentability (Table 1).

The yields of gas per g fermented OM (Y_F) and per g degraded OM (Y_D) are shown in Fig. 5(a) and 5(b). For CC, Y_F declined until 24 h of incubation and increased thereafter ($P < 0.001$). The Y_F from CW increased linearly throughout the incubation. Y_D for CW (Fig 5(b)) increased throughout the incubation period to reach a maximum of 340 ml gas/g degradable OM. Y_D was consistently lower than Y_F .

Discussion

The results suggest that gas production profiles are not necessarily linearly related to degradation or fermentation of substrate. The higher predicted compared with measured gas production for CC indicated that the stoichiometric relations (Van Soest, 1994) are probably not valid for rapidly fermenting substrates.

For CC, the approximately 25% higher gas production predicted by stoichiometric calculations than measured was similar to overestimations of gas production reported for cellulose (by 20%) and glucose (by 10%) by Beuvinck & Spoelstra (1992). Typically, these overestimations occur for substrates producing high proportions of propionic acid. The use of pure substrates by Beuvinck & Spoelstra (1992) seems to rule out the possible mechanism of reduction of

gas production by bonding of CO_2 to NH_3 . A second process which could reduce the gas produced per mol VFA, would be the conversion of CO_2 and H_2 to acetate instead of CH_4 (Miller, 1995), which would result in the net consumption of one mol CO_2 . This process mainly occurs when low roughage diets containing high proportions of sugars and protein are fed (Leedle & Greening, 1988). The possible role of this process could not be estimated for the present experiment as the gases were not analysed. A third possibility could relate to the large crude protein fraction of CC. Little work has been done in the area of the stoichiometric relations for the fermentation of protein, and it is likely that these differ from those for hexose equivalents. This suggests that care must be taken with stoichiometric balances for feedstuffs which contain large amounts of protein.

Relatively large amounts of propionic and butyric acid from CC are more frequently reported when easily digestible substrates are supplied to rumen microbes (Van Houtert, 1993). When the rate of gas production was high, the proportions of propionic and butyric acid increased and consequently the calculated gas yield (Y_F) was low. After 24 h of incubation, the gas production rate was lower, and mainly acetic acid was produced which led to an increase in Y_F . This corresponds with the more frequently observed two-phasic gas production (e.g. Pell & Schofield, 1993; Groot *et al.* 1996). However, multiphasic analysis of the gas production profiles was not possible, due to the limited number of observations per curve.

For CW, the value of Y_F increased throughout the incubation, due to the higher production of acetic acid compared with other VFA. This resulted in a higher half-time (B parameter) for gas production (26.5 h) than for fermentation of OM (23.7 h). The value of Y_D increased even more rapidly, and could probably be attributed to the loss of some material when the residual CW was

determined by use of neutral detergent (Goering & Van Soest, 1970). This 'lost' CW fraction may have been a component such as soluble lignin-carbohydrate complexes (Chesson, 1993), which are not fermentable. Such a loss of CW was probably also the cause of the overestimation of gas production by Blümmel & Ørskov (1993), which they attributed to substrate trapping by microbes.

In conclusion, the fermentation studies resulting in gas and VFA production data can give more insight into the processes occurring in *in vitro* forage evaluation systems. The results also indicate that caution is required in the interpretation of gas production profiles particularly for forages of heterogeneous composition. For forage evaluation, it is important to distinguish between degradation and fermentation, as not all degraded CW material is necessarily fermentable.

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