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Methodological considerations in using gas production techniques for estimating ruminal microbial efficiencies for silage-based diets☆

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Abstract

The relationship between in vivo and in vitro estimates of efficiency of microbial production (EMP) was tested using four silage-based diets. In vivo estimates were based on renal allantoin excretion in steers and in vitro estimates were based on a combination of gas volume and true substrate degradability (TSD_{OM}) measures conducted at substrate-specific times. After an initial test to evaluate 96 h gas production profiles of diets incubated in both N-low and N-rich media, the time to half maximal gas production ($t_{1/2}$) was calculated and a second incubation conducted in both media with fermentation stopped at $t_{1/2}$ for each substrate. True substrate degradability was measured from incubation residues

Abbreviations: ATP, adenosine triphosphate; *B*, asymptotic gas volume; CP, crude protein; DM, dry matter; EMP, efficiency of microbial biomass production; FA, fermentation acids; MBP, microbial biomass production; MCP, microbial crude protein; ME, metabolizable energy; MN, microbial N; ND, neutral detergent; NDFN, N content of truly undegraded residue; RAE, renal allantoin excretion; SBM, soybean meal; SCFA, short chain fatty acids; SF, stoichiometric factor; SIL, silage; TSD, true substrate degradability; $t_{1/2}$, half time of maximal gas production

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and combined with gas volumes to estimate EMP. Additionally, microbial N (MN) production estimates were made using two equations from information on dietary N, N content of residues and NH_3–N content of media before and after incubation. In vitro estimates of EMP were related to in vivo EMP in both N-low ($R^2 = 0.94$, P=0.03) and N-rich ($R^2 = 0.91$, P=0.04) incubation media. No relationships occurred between in vitro efficiency of microbial N production and in vivo EMP when microbial N production was calculated from direct N determinations using N content of the apparently degraded residue after centrifugation and NDFN of the incubation residue. When in vitro microbial N production was estimated indirectly by accounting for dietary N, changes in NH_3–N concentrations in the incubation medium and NDFN in the incubation residue, efficiency of microbial N production relative to 100 mg TSD_{OM} tended ($R^2 = 0.83$, P=0.09) to be positively related to in vivo EMP. Silage fermentation acids appeared to interfere with relationships between gas production and in vitro substrate degradability measures because they were removed from the incubation residue without having contributed to fermentation. Therefore, silage acids should be accounted for when using combined in vitro gas production and true substrate degradability estimates to evaluate EMP of silage-based diets. N supplementation of incubation medium can influence substrate use for microbial growth, and attempts should be made to simulate in vivo N availability. It may be possible to rank silage-based diets for microbial efficiency using combined gas volume and true substrate degradability measures conducted at substrate-specific times ($t_{1/2}$).
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1. Introduction

Microbial biomass is the major source of protein for the ruminant host animal and prediction of efficiency of microbial production (EMP) can be crucial in ruminant nutrition (Beever, 1993; Leng, 1993). Several ruminant feeding systems (NRC, 1996, 2001; Offer et al., 2002) include EMP to predict animal performance. However, lack of a practical laboratory technique to detect feed-specific differences in EMP makes this a problem. It has been proposed that variations in EMP can be detected by a combination of in vitro gas volume measurements (Menke et al., 1979) with a concomitant measure of true substrate degradability (TSD; Goering and Van Soest, 1970; see Blümmel, 2000). The degradability measurement accounts for feed conversion into all products of microbial degradation and synthesis, essentially microbial biomass, short chain fatty acids (SCFA) and gases, whereas the gas volume measurement reflects feed conversion into SCFA and gases. The difference between the measures is assumed to be microbial biomass.

Production of SCFA and gas are stoichiometrically related (Wolin, 1960; Beuvink and Spoelstra, 1992; Blümmel and Ørskov, 1993) and in vitro gas production from forage-based diets can be multiplied by a stoichiometric factor (SF) of 2.20 to estimate substrate conversion into the SCFA–gas complex (2.34 in case of high (>40%) proportional propionate production; Blümmel, 2000). This allows in vitro microbial biomass production (MBP) and EMP to be estimated from concomitant gas volume and TSD measurements (Blümmel, 2000) as:

$$\text{MBP} = \text{TSD} - (\text{gas volume} \times \text{SF}),$$

and

$$\text{EMP} = \frac{\text{TSD} - (\text{gas volume} \times \text{SF})}{\text{TSD}}.$$

Blümmel et al. (1999b) examined the in vitro EMP of four silage-based diets after 24 h of incubation and reported acceptable agreement between this measurement and in vivo estimates of EMP based on renal allantoin excretion in steers. They suggested that these relationships might be improved by conducting gas volume and substrate degradability measurements at substrate-specific incubation times, rather than uniformly after 24 h, to better account for differences in microbial growth patterns among feeds.

In vitro and in vivo estimates of EMP could differ due to lack of product flow in and out of the system. This may be particularly true in feeds that lack synchronisation of ruminal N and carbohydrate degradation, such as silages (Beever, 1993). N recycling can also influence in vivo digestion, and comparisons can be further confounded by the N content of the in vitro buffer (Blümmel and Lebzien, 2001).

The present work had three objectives: (1) to examine the relationship between in vivo and in vitro estimates of EMP for silage-based diets when the in vitro EMP is analysed at substrate-specific incubation times; (2) to compare in vitro estimates of EMP with in vitro determinations of microbial N production; (3) to examine the effect of in vitro N supplementation on these relationships.

2. Materials and methods

2.1. Diets used and in vivo experimentation

Details of the experimental work have been reported by Philipczyk et al. (1996) and Blümmel et al. (1999b). Briefly, four silages (SIL) were prepared from perennial ryegrass grown at the Federal Dairy Research Centre near Kiel (Germany) and harvested at four stages of maturity, being late shooting (SIL 1), early to mid heading (SIL 2), late heading (SIL 3) and early flowering (SIL 4). Four diets (D1–D4) were formulated from the silages using a premix containing barley, a mineral/vitamin premix and the digestibility marker titanium(IV)-oxide. Diets D2–D4 were supplemented with soybean meal to provide approximately 130 g/kg crude protein (CP) in the dry matter (DM; Table 1).

Table 1
Date of grass harvest and concentration (g/kg dry matter) of dietary ingredients of four diets (D1–D4) fed to steers

Diet ingredients	Harvest date	D1	D2	D3	D4
Silage 1	8 May	944			
Silage 2	22 May		908		
Silage 3	7 June			840	
Silage 4	19 June				799
Soybean meal			45	107	140
Premix[a]		56	47	53	61

[a] Premix contains barley, a mineral–vitamin mix, and titanium oxide in the ratio of 76:19:5.

The diets were fed to four ruminally cannulated Angler Rotvieh steers in a 4 × 4 Latin square design. Silages, soybean meal and premix were weighed separately according to the proportions in Table 1, and then completely mixed by hand prior to feeding. Steers were fed in two equal meals at 07:00 and 19:00 h. Steers were offered ad libitum access to diets for a 14 days adaptation period, and the diets were offered at a rate of 85% of the intake observed in the preceding 14 days, and faeces and urine were collected for 14 days. During this period, faecal samples (200 g) were collected twice a day and urine was collected quantitatively into collection bags over two consecutive 24 h periods.

Silages and supplements were sampled and analysed separately. Thawed silage fresh matter was used for determination of fermentation products in silages. Silages (50 g) were mixed with 0.2 l of 0.1 M H_2SO_4; lactic acid, short chain fatty acids and ethanol were estimated by HPLC according to the methods of Siegfried et al. (1984). The DM of the silages was estimated by freeze-drying and subsequent oven-drying at 105 °C overnight, and corrected for losses of volatiles during drying (Weissbach and Berg, 1977). Freeze-dried silages were used for all other analyses. Silages were successively ground in mills with 3 and 1 mm screens. Ash was determined by ignition in a muffle furnace at 550 °C for 16 h. The N was determined using a standard Kjeldahl procedure using Cu^{2+} as a catalyst according to “Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten” (method 4.1.1; VDLUFA; Bassler, 1988). The NDF and ADF analyses were conducted according to Van Soest et al. (1991). Detergent fibre analyses were performed without the use of decalin. Sodium sulfite was omitted and triethylene glycol was used instead of 2-ethoxyethanol in the NDF procedure, and NDF on barley grain was determined with α-amylase (aNDF). The NDF and ADF values are expressed inclusive of residual ash.

Allantoin excretion in urine was analysed by reverse-phase HPLC according to Rosskopf et al. (1991) and microbial crude protein (MCP) was calculated from renal allantoin excretion (RAE) using the relationship of Ehrentreich (1992) and the equation:

$$\text{MCP (g/day)} = 131.7 \times \text{renal allantoin-N (g/day)}$$

where RAE (mmol/day × 56) = renal allantoin-N (mg/day).

In vivo EMP was expressed as g MCP/kg digestible organic matter intake (DOMI), where DOMI was a measured value in steers based on use of titanium dioxide as a digestibility marker. It was also expressed as g MCP/MJ MEI with metabolizable energy (ME) calculated from in vitro gas production for both N-low and N-rich incubation media using the equation of Close and Menke (1986), with lipid content ignored, where:

$$\text{ME} = 2.20 + (0.136 \times \text{gas volume produced}) + (0.0057 \times \text{CP content})$$

2.2. *In vitro gas production measurements*

In vitro studies were conducted at the Fort Keogh LARRL in Miles City, Montana. Nitrogen-low and N-rich incubation media were prepared as described by Blümmel and Lebzien (2001), but incubation procedures were modified in that sample weight and incubation medium were reduced from 500 mg and 40 ml to 250 mg and 20 ml, respectively. Prior to in vitro incubations, ingredients were mixed in the proportions that reflected the actual diet composition of the in vivo trial. Ground substrate (about 250 mg) was weighed

into 100 ml glass syringes, the syringe pistons were lubricated with petroleum jelly and inserted into the syringe.

Rumen inoculum was collected from two cross-bred cows offered ad libitum access to sorghum-sudangrass hay and supplemented every other day with 1.50 kg of lucerne hay. Ruminal fluid and particulate matter in the approximate proportion 60:40 were collected before feeding on the day after lucerne hay supplementation into a pre-warmed, CO_2-filled thermos bottle. Ruminal fluid and particulate matter were homogenised in a blender, strained through nylon material of 40 μm pore size, and filtered through glass wool. The filtrate was then mixed with carbonate buffer (containing ammonium bicarbonate at 4 g/l) and sodium bicarbonate (35 g/l in N-rich incubation medium and only sodium bicarbonate at 39.25 g/l in N-low medium), macromineral solution (5.7 g anhydrous Na_2HPO_4, 6.2 g anhydrous KH_2PO_4 and 0.6 g $MgSO_4 \cdot 7H_2O$ per liter), and deionized water in a ratio of 1:1:0.5:1.5 and 0.1 ml micromineral solution (13.2 g $CaCl_2 \cdot 2H_2O$, 10.0 g $MnCl_2 \cdot 4H_2O$, 1 g $CoCl_2 \cdot 6H_2O$ and 8.0 g $FeCl_3 \cdot 6H_2O$ per 100 ml) was added per liter. The medium was then reduced by addition of 41.7 ml reducing agent (40 ml deionized water, 1 ml 1N NaOH and 1 g $Na_2S \cdot 9H_2O$) per liter of medium. Twenty milliliters of medium were dispensed into the 100 ml glass syringes through tubing at the tip and placed upright in a 39 °C waterbath. Blank samples (i.e., medium only, no substrate) were placed throughout the water bath and used to measure any gas production from the medium alone. All handling of ruminal inoculum was under a constant stream of CO_2 and all containers used were pre-warmed and filled with CO_2. All incubations were conducted in both N-low and N-rich incubation media and ruminal inoculum was the same for both media.

Cumulative gas volume measurements of silages and diets were read manually for incubations in N-low and N-rich media from four replicates each after 2, 4, 6, 8, 10, 12, 14, 16, 24, 30, 36, 48, 54, 60, 72 and 96 h of incubation. After subtraction of gas production from blank syringes, data were fitted to exponential models without (Ørskov and McDonald, 1979) and with a lag phase as:

$$y = B \times (1 - \exp^{-ct}),$$

and

$$y = B \times (1 - \exp^{-c \times [t - \text{lag}]}).$$

where ‘y’ is the cumulative volume of gas produced at time ‘t’ (h), ‘B’ the asymptotic gas volume, ‘c’ the rate constant and ‘lag’ is the time (h) between inoculation and commencement of gas production.

After fitting both models to the data from each substrate, the model with the best fit for that substrate was used for further calculations. Models were compared and if the fit differed significantly ($P<0.05$), the model with the highest R^2 was used. When models did not differ, the model with the fewest parameters (i.e., no lag phase) was chosen.

Halftime of gas production ($t_{1/2}$) [i.e., the time (h) when half of the asymptotic gas volume (B; ml) was produced] was calculated as:

$$t_{1/2} = \frac{\ln 2}{c}; \quad \text{for model } y = B \times (1 - \exp^{-ct}),$$

and

$$t_{1/2} = \left(\frac{\ln 2}{c}\right) + \text{lag}; \quad \text{for model } y = B \times (1 - \exp^{-c\times[t-\text{lag}]}),$$

respectively.

2.3. In vitro apparent and true substrate degradability measurements

After the initial 96 h gas run, $t_{1/2}$ was calculated and a second incubation with the diet as substrate was conducted to obtain degradability measures at substrate-specific times (i.e., $t_{1/2}$ for each substrate). Collection and handling of ruminal fluid was the same as that described for the 96 h incubations. Four syringes were prepared for each substrate, providing two syringes for apparent and two for true degradability measures. The incubations were terminated at $t_{1/2}$ and the volume of gas was recorded. True substrate degradability of diets (Goering and Van Soest, 1970) at $t_{1/2}$ was measured by refluxing the incubation residue with neutral detergent (ND) solution (prepared without sodium sulfite) for 1 h with subsequent recovery of the truly undegraded substrate in sintered glass crucibles of porosity '*C*' (i.e., 40–60 μm). Apparent substrate degradability was determined and calculated at $t_{1/2}$ by high-speed centrifugation (20,000 × *g*) of incubation residue at 20 °C (Blümmel and Lebzien, 2001) following placement into an iced waterbath (about 14 °C) to stop fermentation. Four 20 ml aliquots of the N-low and N-rich media (i.e., 0 h blanks) were collected, as were four blanks without substrate (20 ml) for each $t_{1/2}$. All blank samples were centrifuged (20,000 × *g*) and supernatant was siphoned off, frozen and stored. Residue was weighed and used to correct apparent substrate degradability determinations for residue from the ruminal inoculum.

2.4. Microbial N determinations, chemical analysis and stoichiometric calculations

The N determination on solid material used a Technicon Auto Analyzer (Technicon Industrial Systems, 1977), and NH_3–N was analysed by the method of Broderick and Kang (1980). Microbial N production of diets at $t_{1/2}$ was estimated by two techniques. The first (microbial N production I) estimates microbial N production indirectly by differentially quantifying all N sources, except microbial N, and includes use of information on the N content of diets, truly undegraded N (NDFN) in diets at $t_{1/2}$, and the change in NH_3–N levels in the incubation medium between 0 h of incubation and $t_{1/2}$ using the equations:

$$\text{microbial N production I at } t_{1/2} = \text{diet N} + \Delta NH_3\text{–N} - \text{NDFN at } t_{1/2} \quad \text{(I)}$$

where ΔNH_3–N = NH_3–N in 0 h blanks − NH_3–N in diet incubations at $t_{1/2}$.

The second method (microbial N production II) estimates microbial N production directly by using the N content of the apparently degraded residue remaining after centrifugation (pellet N) and NDFN in diets at $t_{1/2}$, using the equation:

$$\text{microbial N production II at } t_{1/2} = \text{pellet N at } t_{1/2} - \text{blank pellet N at 0 h incubation} - \text{NDFN at } t_{1/2}. \quad \text{(II)}$$

In the remaining text, equation (I) is referred to as "microbial N determination by N balance" and equation (II) is referred to as "direct microbial N determination".

The short chain fatty acids acetate, propionate, butyrate, valerate, isobutyrate and isovalerate were analysed in supernatant of $t_{1/2}$ and 0 h incubations of diets and blanks by gas chromatography. One milliliter of 25% meta-phosphoric acid was added to 5 ml of supernatant, allowed to stand for 30 min, and then centrifuged at $551 \times g$ for 10 min. No internal standard was used. The gas chromatograph was a Hewlett Packard Series II Model 5899 with a 10% SP 1200/1% H_3PO_4 column on Chromosorb WAW packing. Oven temperature was a constant 118 °C and injector temperature 195 °C. Net SCFA production (mmol) was calculated by subtracting SCFA recovery at 0 h incubation from SCFA production at $t_{1/2}$. Stoichiometric relationships between SCFA and gas production and carbon (C), hydrogen (H) and oxygen (O) requirements for production of SCFA and fermentative CO_2, CH_4 and H_2O (SCFA–gas complex) were calculated as described in detail by Blümmel et al. (1997). Measured gas volumes were multiplied by 0.92 to correct the altitude at the laboratory (723 m) to altitude and air pressure at sea level for stoichiometric calculations according to the formulae suggested by Blümmel et al. (1999a).

2.5. *In vitro incubation of silages in autoclaved rumen inoculum for determination of gas production from silage fermentation acids*

This experiment was prompted by some of the earlier results. Because the amount of gas produced per unit of substrate degraded was less than in previous work with non-silage forages, and did not agree as well with stoichiometric calculations, it was thought that silage fermentation acids may be altering relationships between gas production and substrate degradability. Therefore, a test was conducted to determine the contribution of silage acids to gas production, and MBP and EMP calculations. Rumen inoculum prepared for some of the incubations described above was autoclaved (Market Forge Sterilmatic Autoclaver) for 20 min. Triplicate samples of 1 g of freeze-dried silage were weighed into incubation syringes and, after fitting the syringes with plungers, 5 ml of autoclaved rumen inoculum were dispensed into each syringe. After removing all gas space from a syringe, and recording the volume occupied by the sample and the rumen inoculum, exactly 15 ml of reduced N-low incubation medium were dispensed into the syringe. The syringes were shaken and gas volume was recorded immediately and after 1 h of incubation.

2.6. *Statistical analysis*

Differences in in vivo measurements were examined by analysis of variance using the SAS (1992) general linear model procedure considering treatment (i.e., harvest date of perennial ryegrass), animal and period as fixed effects in a Latin Square design. Differences between diets for in vitro results were tested by analysis of variance and differences between N-low and N-rich incubation media were analysed by a paired *t*-test using GraphPad InStat (1998). Linear regressions and non-linear regressions for curve fitting procedures of gas production profiles were done using GraphPad Prism (1999).

3. Results

3.1. Diet composition, energy intake and microbial protein production of the silage-based diets fed to steers

The N content of D1 was higher than D2–D4, which were adjusted by soybean meal (SBM) inclusion to similar N contents (Table 2). Fibre constituents in diets increased with maturity of the ryegrass ensiled. Lactic acid accounted for approximately 70% of total fermentation acid in D1 and D2, but decreased to approximately 50% in D3 and D4.

Allantoin excretion was highest in D1 and D2. In vivo efficiency of microbial protein production declined with maturity of silages (Table 3).

Table 2
Nitrogen content, fibre constituents, total fermentation acid and lactic acid content, and metabolizable energy (ME) concentrations of diets

Diet composition	Diet 1	Diet 2	Diet 3	Diet 4
Organic matter (g/kg)	897	915	921	926
Nitrogen (g/kg)	25.4	21.4	21.3	22.2
NDF (g/kg)	347	427	489	532
ADF (g/kg)	218	252	286	312
Fermentation acids (g/kg)	197	176	119	130
Lactic acid (g/kg)	136	118	62	67
$ME_{N\text{-low}}$ (MJ/kg)[a]	9.9	9.9	9.2	8.4
$ME_{N\text{-rich}}$ (MJ/kg)	10.2	10.2	9.6	8.9

Data are expressed on a dry matter basis.

[a] ME in N-low and N-rich incubation medium was estimated according to Close and Menke (1986) as based on in vitro gas production as follows: ME = (2.20 + (0.136 × gas volume) + (0.0057 × crude protein)) but lipid content was ignored.

Table 3
Metabolizable energy intake (MEI; MJ/day), total renal allantoin excretion (RAE, mmol/day), microbial crude protein production (MCP)[a] in relation to digestible organic matter intake (DOMI) and microbial crude protein production in relation to MEI (g MCP/MJ MEI) of four silage-based diets fed to steers

Diet	MEI[b] (MJ/day)		RAE[c] (mmol/day)	MCP/DOMI[c] (g/kg)	MCP/MEI (g/MJ)	
	N-low	N-rich			N-low	N-rich
D1	87.7 ab[d]	90.3 ab	148.5 a	173.3 a	12.5 a	12.2 a
D2	105.1 b	108.4 b	154.8 a	151.9 ab	10.9 ab	10.5 ab
D3	86.3 ab	89.9 ab	105.2 b	126.9 b	9.0 ab	8.6 ab
D4	68.0 a	72.2 a	80.1 b	115.8 b	8. 7b	8.2 b
S.E.	5.8	6.0	8.5	11.3	0.6	0.6

[a] Calculated as MCP (g/day) = [131.7 × renal allantoin-N (g/day)] (Ehrentreich, 1992), where [RAE (mmol/day) × 56] = renal allantoin-N (mg/day).

[b] Calculated from organic matter intakes (OMI; averages are based on four OMI values per diet) reported by Blümmel et al. (1999b) and in vitro ME estimate reported in Table 2 and adjusted to an OM basis. Incubations to determine ME were conducted in both N-low and N-rich incubation media.

[c] Renal allantoin excretion and MCP relative to DOMI values were calculated from Blümmel et al. (1999b).

[d] Numbers within columns followed by different letters (a and b) differ (P<0.05).

3.2. In vitro gas production profiles and gas volumes and apparent and true substrate degradability at $t_{1/2}$ of gas production

Gas production profiles of the diets incubated in N-low and N-rich media are in Fig. 1. Gas production profiles for N-low incubations were adequately described by the exponential model without lag, but the exponential model with lag fitted the gas profiles of D1–D3 incubated in N-rich medium better ($P<0.05$) than the model without lag.

There were differences among the diets in asymptotic (B) gas production in both N-low ($P<0.0001$) and N-rich ($P=0.002$) incubation media. Similarly, $t_{1/2}$ of asymptotic gas production differed ($P<0.0001$) among diets within N-low and N-rich incubation media. The N level did not affect asymptotic gas production, but $t_{1/2}$ were shorter in N-rich incubations ($P=0.006$). Asymptotic gas volumes from silages were similar to those reported for diets in Fig. 1, but $t_{1/2}$ were longer being 10.2, 12.2, 15.7, 16.7 h and 8.5, 9.2, 10.8 and 15.4 h for SIL 1, SIL 2, SIL 3 and SIL 4 incubated in N-low and N-rich incubation medium, respectively.

Gas volumes, and apparent and true degradabilities, for substrate-specific $t_{1/2}$ are in Table 4. At both N levels, differences among diets occurred for gas volumes and for substrate true dry and organic matter degradabilities. Apparent degradability differed among diets in N-low but not N-rich incubations. True substrate degradabilities, and apparent degradability in N-low medium, declined from D1 to D4, whereas gas volumes were highest in D3 (Table 4). The N supplementation of the incubation medium did not alter mean degradability measurements, but gas volumes were higher ($P<0.05$) in N-low compared to N-rich incubation medium.

3.3. In vitro ratios of degraded substrate to gas volumes produced, estimates of microbial production by these ratios and direct microbial N determinations at $t_{1/2}$

Ratios of apparently and truly degraded substrate to gas volumes produced varied among diets (Table 5). The mean ratios (mg/ml) of substrate apparently degraded to gas volume produced, were substantially higher than 2.2 in both N-low and N-rich incubation me-

Table 4
Gas volumes and apparent (ASD) and true dry (TSD_{DM}) and organic matter degradability (TSD_{OM}) of four silage-based diets incubated in N-low and N-rich incubation medium

Diet	Gas volume (ml)		ASD (mg)		TSD_{DM} (mg)		TSD_{OM} (mg)	
	N-low	N-rich	N-low	N-rich	N-low	N-rich	N-low	N-rich
D1	38.5 a[a]	35.2 a	131.8 a	131.0	189.0 a	186.7 a	159.9 a	157.7 a
D2	41.8 b	39.4 b	126.9 a	116.9	174.3 ab	172.0 ab	149.7 a	147.5 ab
D3	42.5 c	40.1 c	114.2 b	116.2	161.7 b	157.5 bc	140.5 a	136.7 bc
D4	36.8 a	36.1 d	99.1 c	121.4	144.8 c	149.5 c	122.2 b	126.5 c
Mean	39.9*	37.7	117.8	121.4	167.5	166.4	143.1	142.1
S.E.	0.6	0.6	3.0	2.5	4.1	3.5	3.6	2.9

Data relate to the incubation of 250 mg of dry substrate and were obtained by terminating the incubation at $t_{1/2}$.
[a] Numbers within columns followed by different letters (a–d) differ ($P<0.05$).
* Indicates differences ($P<0.05$) between N levels tested by paired t-test.

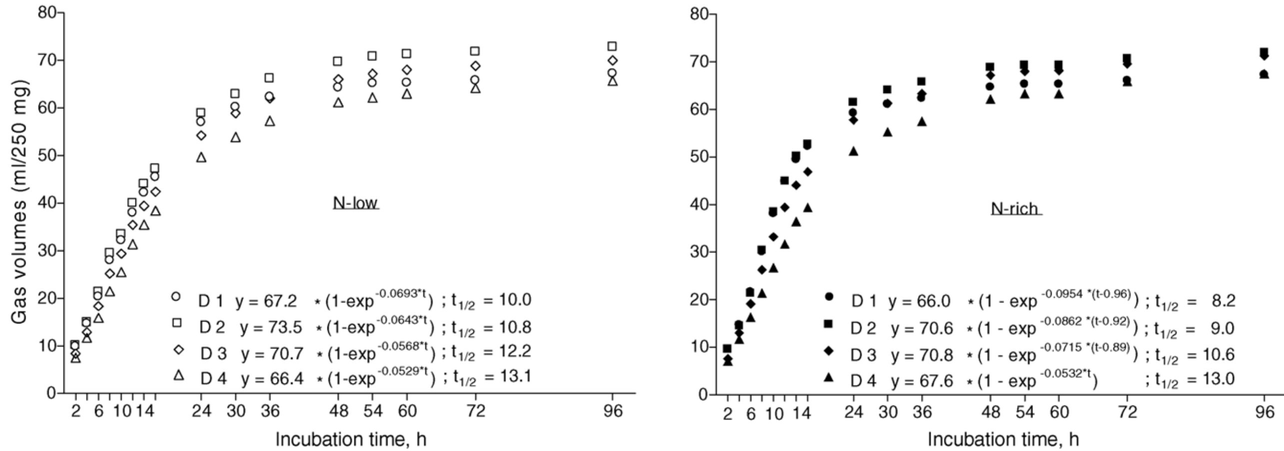

Fig. 1. In vitro gas production profiles of four silage-based diets (D1–D4) incubated in N-low and N-rich incubation media. Gas production profiles have been fit to curves using the equations indicated in the figure with time to half maximal gas production ($t_{1/2}$) expressed in hours. Choice of equation was based on best fit at $P<0.05$. Average rate of gas production differed between N-rich and N-low incubations resulting in shorter $t_{1/2}$ for N-rich incubations ($P<0.01$) than for those for N-low incubations. Standard errors for maximal gas production and rate of gas production, respectively, for N-low medium were 0.63, 0.0019; 0.49, 0.0013; 0.55, 0.0012; 0.67, 0.0015 for D1–D4 and for N-rich medium were 0.85, 0.0042; 0.86, 0.0035; 0.69, 0.0023; 0.76, 0.0016 for D1–D4.

Table 5
Ratios of apparently degraded dry (ASD) and truly degraded organic matter (TSD_{OM}) to gas volumes produced, microbial biomass production[a] (MBP) and efficiency of microbial biomass production[b] (EMP)

Diet	ASD:gas (mg/ml)		TSD_{OM}:gas (mg/ml)		MBP (mg)		EMP (mg/mg)	
	N-low	N-rich	N-low	N-rich	N-low	N-rich	N-low	N-rich
D1	3.42 a[c]	3.71 a	4.11 a	4.40 a	75.2 a	80.3 a	0.47 a	0.51 a
D2	3.02 b	2.97 b	3.57 b	3.77 b	57.7 b	60.8 b	0.39 b	0.41 b
D3	2.68 c	2.91 b	3.31 b	3.43 b	47.0 c	48.5 c	0.34 c	0.36 c
D4	2.69 c	3.36 ab	3.45 b	3.52 b	41.2 d	47.1 c	0.34 c	0.37 c
Mean	2.95	3.24	3.61*	3.78	55.3*	59.2	0.38*	0.41
S.E.	0.08	0.10	0.10	0.12	3.4	3.5	0.015	0.016

In vitro variables were obtained at $t_{1/2}$ (see Table 4) and relate to the incubation of 250 mg of dry substrate.
[a] MBP was estimated as TSD_{OM} − [gas volume × 2.20].
[b] EMP was estimated as [TSD_{OM} − (gas volume × 2.20)]/TSD_{OM}.
[c] Numbers within columns followed by different letters (a–d) differ (P<0.05).
* Indicates differences (P<0.05) between the two N levels as tested by paired t-test.

dia. Ratios of substrate OM truly degraded (TSD_{OM}) to gas volume were higher in D1 compared to the other three diets with both media. Microbial biomass production, estimated as MBP = TSD_{OM} − (gas volume × 2.2), was highest in D1, and declined with maturity of silages, but differences between D3 and D4 depended on N supplementation. The in vitro estimates of MBP were related to in vivo EMP (microbial CP per unit ME intake) in N-low (R^2 = 0.98, P=0.01) and N-rich (R^2 = 0.97, P=0.01) incubations. In vitro efficiency of microbial production, estimated as EMP = [TSD_{OM} − (gas volume × 2.2)]/TSD_{OM}, was higher in D1 and D2 than in D3 and D4 but there was no difference between D3 and D4 (Table 5). These in vitro estimates of EMP in N-low (R^2 = 0.94, P=0.03) and N-rich (R^2 = 0.91, P=0.04) incubations were also related to in vivo EMP.

In vitro N partitioning of diets, total microbial N and efficiency of microbial N production at $t_{1/2}$ are in Table 6. In N-low incubations, NH_3–N recovery was higher at $t_{1/2}$ than at 0 h in all diets, whereas in N-rich incubations NH_3–N net uptake occurred in D1–D3. Estimates of in vitro microbial N production were substantially higher when calculated from N balance than from direct N determination. Substrate rankings for microbial N production were not the same for the two methods, with microbial N calculated by N balance being more discriminatory among diets than estimates from direct N determinations. Efficiency of microbial N production was estimated by relating microbial N to either 100 mg of TSD_{OM} or gas volume. No relationships occurred between in vitro efficiency of microbial N production and in vivo EMP when microbial N production was calculated from direct N determinations in both N-low and N-rich incubations. When microbial N production was estimated indirectly by N balance, efficiency of microbial N production relative to 100 mg TSD_{OM} tended to be positively related (R^2 = 0.83, P=0.09) to in vivo EMP. There was a relationship when microbial N production was related to the amount of gas produced (R^2 = 0.92, P=0.04).

Table 6
In vitro feed nitrogen (N) partitioning, microbial N (MN) balances, and efficiency of microbial N production (EMNP) of four silage-based diets (D1–D4) incubated in N-low and N-rich incubation media

Treatment	Feed-N (mg)	NDFN (mg)	ΔNH_3–N[a] (mg)	MN I (mg)	MN II (mg)	EMNP I_{TSD} (mg N/100 mg TSD)	EMNP II_{TSD} (mg N/100 mg TSD)	EMNP I_{GAS} (mg N/ml GAS)	EMNP II_{GAS} (mg N/ml GAS)
N-low									
D1	6.35	0.53	−0.20 ac[b]	5.62 a	3.57	3.52 a	2.34 ab	0.146 a	0.093 a
D2	5.35	0.53	−0.16 a	4.66 b	2.91	3.11 b	1.94 b	0.112 b	0.070 b
D3	5.33	0.50	−0.33 c	4.50 b	2.92	3.27 c	2.08 ab	0.108 c	0.069 b
D4	5.55	0.56	−0.63 d	4.36 c	3.15	3.58 da	2.58 a	0.119 d	0.086 ab
S.E.		0.015	0.044	0.12	0.11	0.049	0.085	0.004	0.003
Mean	5.65	0.53	−0.33	4.79	3.14	3.37	2.49	0.121	0.079
N-rich									
D1	6.35	0.41	0.55 a	6.49 a	3.46 a	4.25 a	2.17 a	0.186 a	0.098 a
D2	5.35	0.47	0.48 a	5.36 b	3.95 a	3.64 b	2.68 b	0.137 b	0.100 a
D3	5.33	0.51	0.13 a	4.95 b	3.50 a	3.61 b	2.56 ab	0.123 bc	0.087 a
D4	5.55	0.49	−0.99 b	4.07 c	2.43 b	3.18 c	2.84 b	0.113 c	0.067 b
S.E.		0.018	0.17	0.23	0.16	0.11	0.080	0.070	0.004
Mean	5.65	0.47	0.04	5.21	3.34	3.67	2.57	0.140	0.088

Data were obtained by terminating the incubation at $t_{1/2}$. There were no differences (P>0.05) due to N level of the incubation media.

[a] ΔNH_3–N = NH_3–N at 0 h incubation minus NH_3–N measured in the respective diet at $t_{1/2}$. Zero hour NH_3–N recovery in 20 ml of incubation medium was 0.32 and 2.56 mg in N-low and N-rich media, respectively. MN I = diet N + ΔNH_3–N − NDFN at $t_{1/2}$; "microbial N determined by N balance". MN II = pellet N at $t_{1/2}$ − blank pellet N at 0 h incubation − NDFN at $t_{1/2}$; "direct microbial N determination".

[b] Numbers within columns followed by different letters (a–d) differ (P<0.05).

3.4. Short chain fatty acid production, SCFA proportions and stoichiometric relationships at $t_{1/2}$

Net SCFA production, proportions of acetate, propionate, butyrate (butyrate + isobutyrate) and valerate (valerate + isovalerate), stoichiometrically calculated gas volumes, and C, H and O requirement for the SCFA–gas complex for diets at $t_{1/2}$ are in Table 7. In N-low incubations, SCFA production increased with maturity of ryegrass. This was similar for N-rich incubations, except that D4 produced less SCFA than D3. Stoichiometrically calculated mean values of gas volumes were 36.3 and 33.7 ml in N-low and N-rich incubations, respectively, which is close to measured and altitude-corrected mean volumes of 36.7 and 34.7 ml in N-low and N-rich incubations, respectively.

3.5. In vitro incubation of silages in autoclaved rumen inoculum for determination of gas production from silage fermentations acids

Immediate gas production was observed when silages were incubated in autoclaved ruminal fluid (Table 8). These gas volumes corresponded to total fermentation acid (FA) and lactic acid concentrations in silage-based diets by the regression equations:

$$\text{FA} = 21.6 + (5.2 \times \text{gas}), \quad R^2 = 0.96, \quad P = 0.02,$$

and

$$\text{lactic acid} = 7.0 + (5.1 \times \text{gas}), \quad R^2 = 0.93, \quad P = 0.04.$$

A comparison of the two regression equations showed that the intercepts differed ($P<0.001$), but the slopes were similar, with a pooled slope of 5.1.

4. Discussion

4.1. In vivo microbial protein synthesis in diets

In vivo EMP for the silage-based diets varied from about 8 to 12 g of microbial CP/MJ of ME intake (Table 3) and was highest in D1 and smallest in D4. The higher N content of D1 (Table 2) may have contributed to the higher EMP for this diet, but dietary N content cannot completely account for the steady decline in EMP from D2 to D4. Efficiency of microbial production is affected by animal factors, such as level of feed intake and ruminal outflow rate (AFRC, 1993; GfE, 2001), and by feed characteristics such as N forms (Hespell and Bryant, 1979). Well-synchronized ruminal N and carbohydrate metabolism is an important determinant of EMP (Sinclair et al., 1993) and Beever (1993) noted that carbohydrate and N degradabilities are poorly matched in silages.

Overall nutritive value of the silage-based diets decreased with maturity of the ryegrass (Table 2) used for ensiling, while CP contributed by SBM increased from D2 to D4 (Table 1). Time to half maximal gas production of incubated silages also increased from 10.2 to 16.7 h and from 8.5 to 15.4 h from SIL 1 to SIL 4 in N-low and N-rich incubations, respectively. This indicates slower rates of energy release with increasing grass maturity, which may

Table 7
Short chain fatty acid (SCFA) production and proportions, gas volumes stoichiometrically calculated from SCFA's, and carbon (C), hydrogen (H) and oxygen (O) requirement for SCFA and fermentative CO_2, CH_4 and H_2O production of four silage-based diets incubated in N-low and N-rich incubation media

Diet	SCFA (mmol)		Acetate (% of total SCFA)		Propionate (% of total SCFA)		Butyrate[a] (% of total SCFA)		Valerate[a] (% of total SCFA)		Gas volume (ml)		C + H + O (mg)	
	N-low	N-rich	N-low	N-rich	N-low	N-rich	N-low	N-rich	N-low	N-rich	N-low	N-rich	N-low	N-rich
D1	0.74 a[b]	0.64 a	61.7	63.2 a	24.9 a	24.4 a	10.8 a	10.3^{a}	2.3^{a}	2.1^{a}	33.9^{a}	28.5^{a}	78.2^{a}	65.2^{a}
D2	0.78 ab	0.75 b	61.6	63.9 bc	25.0 a	24.7 b	11.0 bd	10.6^{b}	2.3^{a}	0.8^{b}	34.9^{ab}	34.4^{b}	80.7^{ab}	77.7^{b}
D3	0.81 bc	0.82 c	62.9	64.1 c	23.9 b	23.6 c	10.7 c	10.1^{a}	2.6^{b}	2.2^{c}	37.3^{cb}	38.4^{c}	85.2^{bc}	87.5^{c}
D4	0.86 c	0.73 b	61.8	63.1 a	24.3 c	23.7 c	10.9 ad	10.5^{b}	3.0^{c}	2.7^{d}	38.9^{c}	33.5^{b}	89.9^{c}	76.8^{b}
Mean	0.80	0.74	62.0*	63.5	24.5*	24.1	10.9*	10.4	2.6*	2.0	36.3	33.7	83.5	76.8
S.E.	0.03	0.02	0.13	0.12	0.12	0.13	0.04	0.055	0.07	0.18	0.65	0.92	1.49	2.16

Data relate to the incubation of 250 mg of dry substrate.
[a] Iso-butyrate and iso-valerate were added to butyrate and valerate values, respectively.
[b] Numbers within columns followed by different letters (a–d) differ ($P<0.05$).
* Indicates differences ($P<0.05$) between N levels tested by paired *t*-test.

Table 8
Gas production (GP, ml) of four silages buffered in N-low incubation medium containing autoclaved rumen inoculum

Silage	Immediate GP	GP after 1 h	Immediate GP_{SIL}	GP_{SIL} after 1 h
SIL 1	5.9 a[a]	6.0 a	5.6 a (14.5%)[b]	5.7 a (14.8%)
SIL 2	4.1 b	4.2 b	3.8 b (9.1%)	3.9 b (9.3%)
SIL 3	1.7 c	2.0 c	1.6 c (3.8%)	1.9 c (4.5%)
SIL 4	2.5 d	2.6 c	2.4 d (6.5%)	2.4 c (6.5%)
S.E.	0.48	0.47	0.56	0.44

Values are expressed per 250 mg of dry substrate and gas production of silage in a diet (GP_{SIL}) was calculated from the proportion of silage in the diet.

[a] Numbers within columns followed by different letters (a–d) differ (P<0.05).

[b] Values in parentheses are percentage of gas production in autoclaved rumen inoculum in relation to measured gas production of diets at $t_{1/2}$ in N-low incubation medium (Table 3).

have resulted in unsynchronised ruminal energy and protein availability, thereby reducing EMP.

4.2. Detection of differences in in vivo EMP by concomitant in vitro gas volume and substrate degradability measurements

In previous research, when the silage-based diets were examined for in vitro MBP and EMP after 24 h of incubation in N-rich incubation medium, MBP and EMP were 52.5, 42.8, 34.1 and 37.6 mg and 0.30, 0.25, 0.22 and 0.26 mg MBP/mg TSD_{OM} for D1, D2, D3 and D4, respectively (Blümmel et al., 1999b). These estimates were in reasonable agreement with renal allantoin excretion relative to digestible OM intake (as in Table 3), except that D4 had a higher in vitro EMP than D3, which did not agree with in vivo findings. This disagreement led to the suggestion to analyse EMP at substrate-specific incubation times in incubation media varying in N level (Blümmel et al., 1999b). As hypothesized, D3 and D4 did not differ in EMP when analysed at substrate-specific times. These results are consistent with in vivo values, suggesting that use of substrate-specific $t_{1/2}$ may be preferred over 24 h measures, but this should be evaluated under a broader range of dietary conditions.

Microbial biomass production and EMP estimated at substrate-specific $t_{1/2}$ was higher than when estimated after 24 h. Estimates of MBP obtained at $t_{1/2}$ were well related to microbial CP production per MJ of ME intake in vivo when ME was calculated using gas production values from both N-low ($R^2 = 0.98$, P=0.01) and N-rich ($R^2 = 0.97$, P=0.01) incubations. Interestingly, the relationship between the EMP estimates at $t_{1/2}$ and in vivo EMP was less close (N-low: $R^2 = 0.94$, P=0.03 and N-rich: $R^2 = 0.91$, P=0.04). Similar findings were reported by Blümmel and Lebzien (2001), who observed that in vitro MBP estimates were more closely related to in vivo EMP, estimated by ^{15}N infusion, than in vitro EMP estimates for dairy cow diets.

There are indications that concomitant in vitro gas volume and substrate degradability measurements might be too simple a concept for silage-based diets. For example, in previous research, apparent substrate degradability determined by high-speed centrifugation always agreed with the amount of C, H and O recovered in SCFA and fermentative CO_2, CH_4 and

H_2O (i.e., requirements for the SCFA–gas complex) in a wide array of forages and mixed diets (Blümmel, 2000). In the present work, C, H and O recovery for SCFA–gas complex was substantially less than apparent substrate degradability (i.e., compare Tables 4 and 7) and on average 2.92 mg (N-low) and 3.24 mg (N-rich) substrate were apparently degraded for production of 1 ml of gas. In Blümmel (2000), 1 ml of gas was associated with an average of 2.26 mg of substrate apparently degraded, which agrees well with C, H and O requirements for the SCFA–gas complex in production of 1 ml of gas, which varies between 2.20 mg (high acetate) and 2.34 mg (high propionate). The reason for the deviation of the silage-based diets from this relationship is probably due to the content of FA produced during ensiling. In the present study, these accounted for up to 20% of silage DM (Table 2), although they do not present fermentable matter to rumen microbes (AFRC, 1993). However, they do count as apparently and truly degraded substrate as they do not appear to be recovered in incubation residue. This confounds interpretation of concomitant substrate degradability and gas volume measurements.

The presence of FA in silages may also affect gas volume measurements in bicarbonate-buffered in vitro measures, where about half of the gas volume is accounted for by CO_2 released upon buffering SCFA (Blümmel and Ørskov, 1993). In the current study, immediate gas production occurred when silages were incubated in autoclaved rumen fluid. Intercepts of the regression equations for FA and lactic acid from gas production can be interpreted as an indication of the amount of FA buffered during ensiling or an indication of the amount of FA lost during the freeze-drying of silages. Accepting the latter assumption, TSD_{OM} could be corrected for FA as $TSD_{CORR} = TSD_{OM} - SFA$, where SFA = immediate gas volume in autoclaved rumen fluid × 5.1. In vitro estimates of MBP and EMP could be corrected for FA as $MBP_{CORR} = (TSD_{OM} - SFA) - (\text{gas volume} \times 2.2)$ and $EMP_{CORR} \times [(TSD_{OM} - SFA) - (\text{gas volume} \times 2.2)]/(TSD_{OM} - SFA)$. Microbial biomass production at $t_{1/2}$ after these corrections was 47.2, 38.3, 38.7 and 28.8 mg and 52.3, 41.4, 40.2 and 30.3 mg for D1, D2, D3 and D4 incubated in N-low and N-rich incubation media, respectively. The efficiency of microbial production thus estimated was 0.36, 0.28, 0.29 and 0.26 and 0.40, 0.32, 0.31 and 0.30 for D1, D2, D3 and D4 incubated in N-low and N-rich incubation media, respectively. However, while these estimates consistently ranked D1 higher than the other diets, none of these estimates was related (N-low: $R^2 = 0.72$, $P = 0.15$ and N-rich: $R^2 = 0.84$, $P = 0.08$) to in vivo EMP. This contrasts with the FA uncorrected estimates, and more research is required to accurately quantify FA in the substrate, thereby elucidating their fate during incubation. The effect of FA on gas volume interpretation is probably of less importance than when degradability is being considered. Gas evolution in autoclaved ruminal fluid was immediate, and this gas may be expelled when adjusting the plunger to the volume of the incubation medium after injection of the complete incubation medium.

In vitro estimates of MBP and EMP were higher ($P < 0.05$) in N-rich versus N-low incubations (Table 5), resulting from a decrease in gas production upon N supplementation, while TSD_{OM} remained unaffected (Table 4). The 6% decrease in gas production was approximately matched by the 8% decrease in SCFA recovery (Table 7). A likely explanation for this is a shift in partitioning of TSD_{OM} from the SCFA–gas complex to MBP with N supplementation. That the relationships between in vitro MBP and EMP estimates and in vivo EMP were closer for N-low than for N-rich incubations suggests that in vitro N supplemen-

tation impacts the relationship between these measures. In the current research, 2.56 mg of N were introduced into the N-rich incubation by rumen inoculum and buffer, which might have resulted in unrealistically high N availability in vitro (i.e., 0.13 mg N/ml for 250 mg substrate). It appears worthwhile to investigate reduced levels of N supplementation to simulate in vivo conditions.

4.3. Effects of N supplementation on microbial N recovery and detection of differences in in vivo EMP by in vitro microbial N determinations

In all diets, more NH_3–N was recovered at $t_{1/2}$ in N-low incubations than was introduced into the system, while the reverse occurred for N-rich incubations, except for D4 (Table 6). The change in NH_3–N in N-low incubations could have originated from microbial lysis, as well as from degraded feed N not used for MBP. The latter assumption would suggest a lack of energy (ATP) or precursors required by microbes for MBP. However, this assumption is apparently inconclusive since net uptake of NH_3–N was prevalent in N-rich incubations, even though less SCFA and, by implication, ATP (Table 7) was produced in these than in N-low incubations.

That less gas and SCFA, and more microbial N, were recovered in N-rich versus N-low incubations, together with the findings that TSD_{OM} were unaffected by N level, supports the hypothesis of a shift in partitioning of substrate use from SCFA–gas complex to MBP. Three factors could influence this shift, being N limitation in N-low incubations, a higher microbial efficiency under greater surplus of NH_3–N and/or N supplementation increased the rate of fermentation (Fig. 1) and positively affected the ratio of ATP available for microbial growth in relation to requirements for microbial maintenance (Russell et al., 1992).

In vitro estimates of microbial N and efficiency of microbial N production were higher ($P<0.0001$) when calculated from N balance than when calculated from direct microbial N determination, findings that agree with Blümmel and Lebzien (2001) who suggested that some feed or microbial amino acids and peptides are not completely recovered in the residual pellet after centrifugation. In estimates of microbial N by in vitro N balance, these N sources were accounted for, whereas they would be attributed to microbial N in in vitro direct microbial N determinations.

5. Conclusions

Efficiency of microbial biomass production estimated from true substrate degradability and gas production measures at substrate-specific times was well related to in vivo measures of microbial efficiency. Diet rankings of microbial biomass production were dependent on N supplementation of the incubation medium with results from N-low incubations being more closely related to in vivo EMP than N-rich incubations. In vitro estimates of microbial N production were better related to in vivo EMP when estimated using N balance rather than by direct N determinations. Further research is warranted on the impact of N content of incubation media and impacts of silage fermentation acids on microbial efficiency estimates from gas production and concomitant substrate degradability measures.

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