

Influence of diet on growth yields of rumen micro-organisms *in vitro* and *in vivo*: influence on growth yield of variable carbon fluxes to fermentation products

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The efficiency of rumen microbial production (EMP) *in vitro* and *in vivo* was examined for three roughages (lucerne (*Medicago sativa* L.) hay, oat (*Avena sativa* L.)–berseem clover (*Trifolium alexandrinum* cultivar BigBee) hay and maize (*Zea mays* L.) crop residue (MCR)) and for five isonitrogenous (106 g crude protein (N × 6.25)/kg) diets formulated from lucerne hay, oat–berseem clover hay, MCR, soya-bean meal and maize grain to provide degradable intake protein for the production of 130 g microbial protein/kg total digestible nutrients. EMP *in vivo* was determined by intestinal purine recovery in sheep and ranged from 240 to 360 g microbial biomass/kg organic matter truly degraded in MCR and in one of the diets respectively ($P < 0.05$). EMP *in vitro* was estimated by the substrate degraded:gas volume produced thereby (termed partitioning factor, PF (mg/ml)) at times of estimated peak microbial production and after 16.0 and 24.0 h of incubation. For the diets, PF values were significantly related to EMP *in vivo* at peak microbial production ($P = 0.04$), but not after 16.0 ($P = 0.08$) and 24.0 h ($P = 0.66$). For roughages, PF values were significantly related to EMP *in vivo* only when measured after 16.0 h ($P = 0.04$). For MCR and diets, a close non-linear relationship was found between PF values at peak microbial production and EMP *in vivo* ($R^2 = 0.99$, $P < 0.0001$) suggesting a maximum EMP *in vivo* of 0.39. Low gas production per unit substrate degraded (high PF) was associated with high EMP *in vivo*. The *in vitro* study of the products of fermentation, short-chain fatty acids, gases and microbial biomass (by purine analysis) after 16.0 h of incubation showed very strong relationships ($R^2 \geq 0.89$, $P < 0.0001$) between short-chain fatty acids, gases and gravimetrically measured apparent degradability. Except for maize grain, the true degradability of organic matter estimated by neutral-detergent solution treatment agreed with the sum of the products of fermentation ($R^2 = 0.81$, $P = 0.0004$). After 16.0 h of incubation, the synergistic effects of diet ingredient on diets were greater for microbial biomass (18 %) than for short-chain fatty acids and gas production (7 %). It is concluded that measurement of gas production only gives incomplete information about fodder quality; complementation of gas measurements by true degradability measurements is recommended.

Fermentation products: *In vitro* gas production: Microbial efficiency

Proportionally high conversion of rumen-degraded feed into microbial biomass, i.e. a high efficiency of microbial production (EMP), is desired in ruminant animal nutrition because it leads to efficient feed N and C utilization (Beever, 1993; Leng, 1993; Van Soest, 1994). It was, for example, demonstrated that the nature and fermentation characteristics of feed protein (N) and carbohydrates can affect EMP (Brown & Pittman, 1991; Clark *et al.* 1992; Sinclair *et al.* 1995), thus rejecting the assumption of a constant EMP still prevalent in some feeding systems (Ausschuss für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie der Haustiere, 1986; Agriculture and Food Research Committee, 1993). As an exception, the Cornell net carbohydrate and protein system (Fox

et al. 1992; Russell *et al.* 1992; Sniffen *et al.* 1992) considers feed- or diet-specific differences in EMP, suggesting their prediction by the analysis of numerous feed carbohydrate and N fractions with associated degradation rates (National Research Council, 1996).

A simpler *in vitro* technique for the estimation of EMP of roughages was suggested by Blümmel *et al.* (1997) and consists of a combination of two *in vitro* measurements in one 24.0 h incubation using rumen inoculum. Gas volume produced during the incubation is recorded as described by Menke *et al.* (1979) and the substrate truly degraded is gravimetrically quantified by the modification of the technique of Tilley & Terry (1963) suggested by Goering & Van Soest (1970). The degradability measurement

Abbreviations: EMP, efficiency of microbial production; LH, lucerne hay; MCR, maize crop residue; MG, maize grain; OBH, oat–berseem clover hay; OM, organic matter; PF, partitioning factor; SBM, soyabean meal; SCFA, short-chain fatty acid; $t_{1/2}$, time of half asymptotic gas production.

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reflects how much substrate was used for the formation of all products of fermentation, namely short-chain fatty acids (SCFA), gases and microbial biomass. The gas measurement indicates how much substrate was used for the formation of SCFA and gases, since SCFA and gas production are very closely associated stoichiometrically (Wolin, 1960; Beuvink & Spoelstra, 1992; Blümmel & Ørskov, 1993; Blümmel *et al.* 1999a). The substrate truly degraded (mg) : gas thereby produced (ml) was termed the partitioning factor (PF) and, proportional to the amount of substrate degraded, low gas production (high PF) was indicative of a high EMP *in vitro* (Blümmel *et al.* 1997). For roughages, PF values from 2.75 to 4.45 mg/ml approximately reflected Y_{ATP} ranges from 10 to 32 (for review, see Blümmel *et al.* 1997).

Microbial growth, however, has several stages, notably lag, growth, stationary and decline phases (Van Soest, 1994). Microbial growth phases relative to incubation time will vary between substrates and comparisons of EMP in batch systems at different microbial growth phases might result in false conclusions. This might be the case with PF analysis fixedly conducted after 24.0 h incubations without regard for substrate-specific differences in microbial growth kinetics.

In the present study, eight roughages and mixed diets were investigated with two objectives in mind. The first was to examine if PF comparisons at similar microbial growth phases would result in greater agreement between *in vitro* and *in vivo* estimates of EMP. Second, by studying the products of microbial degradation, the study addresses some fundamental considerations about *in vitro* techniques, namely the comparative advantages and disadvantages of measuring substrate disappearance (Goering & Van Soest, 1970) or appearance of fermentation products such as gases (Menke *et al.* 1979; Steingass & Menke, 1986).

Materials and methods

Feeds and feeding

The forages used for *in vivo* experimentation have previously been described in detail by Karsli (1998). In brief, a first harvest oat (*Avena sativa* L.)–berseem clover (*Trifolium alexandrinum* cultivar BigBee) was mowed at late vegetative stage and third harvest lucerne (*Medicago sativa* L.) was mowed at first flowering stage. Maize (*Zea mays* L.) crop residues (MCR) were obtained from maize fields and all three roughages were round-baled and ground through a 50 mm screen of a tub-grinder shortly before the start of the feeding trial. The three roughages were offered in restricted amounts and *ad libitum* (about 115% of intake; only results on *ad libitum* intake are reported in the present paper) to six rumen- and duodenum-cannulated wether sheep in a 6 × 6 Latin square design. Five diets designed from the three roughages were adjusted to approximately isonitrogenous levels by maize grain (MG) and soyabean meal (SBM) incorporation and were offered in a consecutive experiment to five sheep in a 5 × 5 Latin square design. The diets had degradable intake protein levels to provide 130 g microbial protein/kg total digestible nutrients, consumed according to level 1 of

the Nutrient Requirements of Beef Cattle computer program (National Research Council, 1996). In the calculation of dietary crude protein, degradable protein intake and total tract digestible organic matter (OM; see later) of MCR, oat–berseem hay (OBH) and lucerne hay (LH) determined in a previous experiment (Karsli, 1998) were used. Each period lasted for 22 d, consisting of a 12 d adaptation and a 10 d collection period. The animals were housed in individual pens under controlled (25°C) temperature conditions and had free access to water and a trace minerals salt block.

Digesta flow, digestibility measurements and estimates of *in vivo* microbial biomass production

Cr-mordanted fibre (1.5 g) containing approximately 20 g Cr/kg was added to the rumen daily at 08.00 and 20.00 hours to determine digesta flow as described by Rojas-Bourillon *et al.* (1987). Feed and ort samples were collected on days 12 to 16 of each period and composited. On days 14, 15 and 16 of each period, 200 ml duodenal digesta and 15 g fresh faecal matter were collected four times per d. Sampling time was advanced 2.0 h per d so that the samples collected over the 3 d represented each 2.0 h of a 24.0 h cycle. After collection, samples were frozen and stored. Approximately 1500 ml rumen contents were collected from the ventral sac of the rumen for the isolation of rumen microbes by differential centrifugation according to Adamu *et al.* (1989).

DM concentrations of feeds, Orts and faeces were determined by drying in a forced-air oven at 60°C for 48.0 h. Duodenal digesta samples and the bacterial pellets were freeze-dried. Dried feed, duodenal digesta and faecal samples were ground through a 1 mm screen. Dry duodenal digesta and faecal samples were composited on an equal dry weight basis for each animal in each period. OM concentrations of dried feeds, Orts, duodenal digesta and faecal samples were determined as the weight loss during combustion at 600°C for 2.0 h in a muffle furnace. Concentrations of neutral-detergent fibre and acid-detergent fibre in dry feeds, Orts, duodenal digesta and faecal samples were determined by the sequential procedure of Van Soest & Robertson (1985) and N concentrations were analysed by the Kjeldahl method. Purine concentrations of duodenal digesta and rumen microbial pellet samples were determined by the procedure of Zinn & Owens (1986). The amounts of N and truly degraded OM in the rumen were calculated by correcting the amounts of apparently degraded N and OM for microbial N and OM as determined by the N:purines and N:OM ratios in the rumen bacterial pellet. Similarly, microbial biomass produced was calculated by dividing duodenal purine flow by the concentration of purines in the DM of the bacterial pellet.

In vitro incubation procedures

Substrates were incubated in 100 ml calibrated glass syringes commonly used for the Hohenheim gas-production test (Menke *et al.* 1979; Steingass & Menke, 1986), but the incubation protocol followed was that of Blümmel & Becker (1997). Briefly, four replicates of 500 mg air-dry substrate were weighed into the syringes and these were

incubated with 40 ml mixed suspension of rumen digesta. This suspension consisted of 10 ml rumen contents, 10 ml bicarbonate buffer, 5 ml macro- and micro-minerals solutions (0.002 ml of which was micro-mineral solution) and 15 ml distilled water. As a modification to the method of Blümmel & Becker (1997), no NH_4HCO_3 was used in the preparation of the bicarbonate buffer, which consisted entirely of NaHCO_3 (0.467 M). Rumen inoculum was collected from a ruminally fistulated German Hinterwalder cow kept on a medium- to good-quality grass hay diet before morning feeding. The rumen inoculum consisted of about 600 ml rumen contents and 400 ml rumen particulate matter/l. For details of inoculum handling and preparation, see Blümmel & Becker (1997).

In vitro gas volume and apparent DM and true organic matter degradability measurements

Five different incubations were conducted. The rate and extent of *in vitro* gas production from roughages, concentrates and diets were obtained during a 96.0 h incubation with gas volume recordings after 2, 4, 6, 8, 10, 12, 24, 30, 36, 48, 54, 60, 72 and 96.0 h of incubation. The rate and extent of gas production were calculated using an exponential model $y = V(1 - e^{-ct})$, in which y is the gas volume at time t , V is the asymptotic value of gas production and c is the fractional rate of gas production.

In vitro true OM degradability was determined after recording the gas volume and terminating the incubation at the desired time by transferring the entire syringe contents through the outlet of the syringe into 600 ml spoutless Berzelius beakers. The syringes were rinsed twice with neutral-detergent solution (prepared without sodium sulfite and amylase) by dispensing 25 ml neutral-detergent solution through the outlet into the syringe each time. The syringe was shaken each time to remove residual particles, after which the contents were added to the respective Berzelius beaker. The procedure of Goering & Van Soest (1970) for the determination of true digestibility was then applied by refluxing the incubation residue for 1.0 h and recovering the undigested matter on filter crucibles (porosity 2). The PF value at a given time of incubation was calculated as OM truly degraded (mg):gas volume produced thereby (ml). The PF values were determined after 24.0 and 16.0 h of incubation as well as at the time at which half-maximum gas production was achieved ($t_{1/2}$). Preliminary data about kinetics of *in vitro* gas production and microbial biomass yield had suggested that $t_{1/2}$ could serve as a common time denominator to facilitate across-substrate comparisons of PF values (Blümmel *et al.* 1999b). $t_{1/2}$ in the model $y = V(1 - e^{-ct})$ was calculated as:

$$t_{1/2} = \ln 2/c.$$

In vitro apparent DM degradability was determined after 16.0 h of incubation by high-speed centrifugation. The entire syringe content was transferred into centrifuge tubes and centrifuged at 20 000 g for 30 min at 4°C. The supernatant fraction was carefully removed with a Pasteur pipette and stored for SCFA and NH_3 analysis. The syringes were washed three times with NaCl solution (4 g/l), each time

dispensing 15 ml of solution through the spike into the syringe. The syringe was shaken each time to remove residual particles and the contents were added to the respective centrifuge tube. After completing rinsing of the syringes, centrifugation was repeated once and the supernatant fraction was discarded. The pellets were lyophilized overnight and residual moisture was removed by drying in a forced-air oven at 105°C for 3.0 h. *In vitro* apparent DM degradability equals DM substrate incubated (mg) – (DM pellet (mg) – DM blank pellet (mg)), where blank pellet weight was determined by centrifugation of 4×40 ml rumen suspension sampled at the 0 h of incubation.

In vitro short-chain fatty acid production, ammonia concentration, stoichiometrical calculations and purine analysis after 16.0 h of incubation

SCFA in the supernatant fraction were analysed by GC (Hewlett Packard 5880 A (Hewlett Packard, Palo Alto, CA, USA) with flame ionization detection) as described by Aiple (1993). Gas production was calculated from SCFA according to Blümmel *et al.* (1999a) and measured gas volumes were corrected for pressure as described in the same paper. The amounts of C, H and O required for the production of SCFA (including the isoacids) and associated fermentative CO_2 , CH_4 and H_2O were summed according to Blümmel *et al.* (1997). ATP production from the acids was calculated using standard values from the literature as described by Blümmel *et al.* (1997). $\text{NH}_3\text{-N}$ in the supernatant fraction was determined by the Kjeldahl method using direct steam distillation. Purines (adenine and guanine) were analysed by HPLC as described by Makkar & Becker (1999). Purines were analysed after ball-milling the pellets obtained by high-speed centrifugation in the determination of *in vitro* apparent DM degradability and net purine production (μmol) was calculated based on pellet weights and purine analysis. The purine concentration in the microbial biomass was estimated by purine analysis of the lyophilized blank microbial pellet.

Statistical analysis

Statistical differences in the variables were analysed using ANOVA procedure (1988; Statistical Analysis Systems, Cary, NC, USA) and mean values were separated by applying least squares difference procedures with the probability level set to $P=0.05$. Significance statements refer to this probability level unless otherwise stated. The computer program GraphPad Prism (1994; GraphPad Inc., San Diego, CA, USA) was used to calculate the rate and extent of gas production by non-linear regression and also to calculate the simple linear regression relationships used in the present study.

Results

Roughages, concentrates, diets and in vivo experimentation

The chemical compositions of LH, OBH, MCR, SBM, MG and diets (diets 1 to 5) are summarized in Table 1.

Table 1. DM, organic matter, crude protein (N × 6.25), neutral-detergent fibre and acid-detergent fibre in soyabean meal, maize (*Zea mays* L.) grain, lucerne (*Medicago sativa* L.) hay, oat (*Avena sativa* L.)–berseem clover and (*Trifolium alexandrinum* cultivar BigBee) hay and maize crop residue and of diets made from these components*

	SBM	MG	LH	OBH	MCR	Diet 1†	Diet 2‡	Diet 3§	Diet 4	Diet 5¶
DM (g/kg)	944	914	930	931	933	934	933	930	932	928
OM (g/kg DM)	932	987	918	892	936	938	935	928	939	940
CP (g/kg DM)	436	79	181	139	41	107	106	109	103	104
NDF (g/kg DM)	206	169	420	622	757	659	645	610	628	554
ADF (g/kg DM)	136	30	284	374	433	369	359	339	356	315

SBM, soyabean meal; MG, maize grain; LH, lucerne hay; OBH, oat–berseem clover hay; MCR, maize crop residue; OM, organic matter; CP, crude protein; NDF, neutral-detergent fibre; ADF, acid-detergent fibre.

* Composition of diets.

† Diet 1: 0.790 (MCR)+0.05 (MG)+0.16 SBM.

‡ Diet 2: 0.690 MCR+0.103 OBH+0.083 MG+0.124 SBM.

§ Diet 3: 0.450 MCR+0.35 OBH+0.15 MG+0.05 SBM.

|| Diet 4: 0.691 MCR+0.103 LH+0.103 MG+0.103 SBM.

¶ Diet 5: 0.450 MCR+0.350 LH+0.20 MG.

The mean crude protein (N × 6.25) content of diets was 106 (range 103–109) g/kg with little variation between the diets. For roughages, the crude protein content was lowest in MCR and highest in LH, while the reverse was true for fibre constituents. All diets included two roughages and at least one concentrate. MCR and MG were included in all diets.

Daily intakes of roughages and diets, OM truly degraded in the rumen and EMP are summarized in Table 2. DM and OM, crude protein and fibre intakes were substantially higher in LH and OBH than in MCR and diets. For roughages, OM truly degraded in the rumen was substantially higher for LH than OBH and MCR, but only small differences in OM truly degraded in the rumen were observed for the diets. The EMP *in vivo*, expressed as g microbial biomass production/kg OM truly degraded in the rumen, was lowest for MCR and highest for diet 5. The diets had higher EMP than roughages, but the difference was small for LH and OBH.

In vitro gas volume, substrate degradability measurements and partitioning factor values: their relationships with the efficiency of microbial production *in vivo*

Asymptote, *c* and $t_{1/2}$ of *in vitro* gas production, *in vitro* true OM degradability and PF of concentrates, roughages and diets are summarized in Table 3. There were significant differences in *V*, *c* and $t_{1/2}$ between components and

between diets. It can be calculated that the mean *V* value of the diets was slightly (1.5%), but significantly, higher ($P < 0.05$) than expected from the summation of the respective values of the feed components according to their proportion in the diets. The rate of gas production of the diets was about 13% higher than calculated from the incubations of the dietary components and their respective proportions in the diets. Similarly, $t_{1/2}$ was about 13% less in the diets than calculated from the dietary components.

Gas volumes, *in vitro* true OM degradability and PF measured at $t_{1/2}$ and after 16.0 h of incubation differed significantly between components and diets. $t_{1/2}$ varied widely from 7.6 and 8.5 h for SBM and LH to 21.5 and 22.7 h for MG and MCR respectively. The difference between the shortest and longest $t_{1/2}$ in diets was about 2.0 h. All $t_{1/2}$ were < 24 h and gas volumes and *in vitro* true OM degradability after 24.0 h were consequently higher than their respective $t_{1/2}$ and 16.0 h values (Table 3), but the reverse was true for PF values after 24.0 h (results not shown). Gas volumes measured at $t_{1/2}$ were not exactly 50% of the asymptotic volumes, reflecting some deviation in the repeatability of gas production kinetics in different incubations.

PF at $t_{1/2}$ was significantly related to EMP *in vivo* in the diets, but not in the roughages (Fig. 1). *In vivo* EMP across diets and roughages were not significantly related to gas volumes but were related to the *in vitro* true OM

Table 2. Daily intakes (g/kg live weight) of DM, organic matter and crude protein (N × 6.25), organic matter truly degraded in the rumen and efficiency of microbial production*

(Mean values for six sheep)

	LH	OBH	MCR	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
DM intake	45.1 ^a	31.1 ^b	16.9 ^c	17.1 ^c	17.7 ^c	22.0 ^c	18.8 ^c	21.7 ^c
OM intake	41.4 ^a	28.6 ^b	15.9 ^c	16.0 ^c	16.5 ^c	20.5 ^c	18.3 ^c	20.4 ^c
CP intake	8.4 ^a	4.4 ^b	0.86 ^d	2.0 ^c	2.1 ^c	2.5 ^c	2.2 ^c	2.3 ^c
OM truly degraded (g/kg)	704 ^a	612 ^c	582 ^c	655 ^{ab}	647 ^b	670 ^a	645 ^b	657 ^{ab}
EMP (g microbial biomass/kg OM truly degraded)	295 ^c	303 ^c	241 ^d	312 ^{bc}	333 ^b	344 ^{ab}	326 ^b	364 ^a

LH, lucerne (*Medicago sativa* L.) hay; OBH, Oat (*Avena sativa* L.)–berseem clover (*Trifolium alexandrinum* cultivar BigBee) hay; MCR, maize (*Zea mays* L.) crop residue; OM, organic matter; CP, crude protein; EMP, efficiency of microbial production.

^{a,b,c}Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* For details of diets and procedures, see Table 1 and p. 626.

Table 3. Gas volumes, substrate degradabilities and partitioning factors (PF) of soyabean meal, maize (*Zea mays* L.) grain, lucerne (*Medicago sativa* L.) hay, oat (*Avena sativa* L.)–berseem clover (*Trifolium alexandrinum* cultivar BigBee) hay, maize crop residue and of diets (diets 1 to 5) measured at time of half asymptotic gas production ($t_{1/2}$) and after 16.0 h of incubation*†

Variable	SBM	MG	LH	OBH	MCR	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Gas asymptote (V, ml)	149.6 ^{ef}	234.0 ^g	123.6 ^b	112.9 ^a	138.4 ^c	140.9 ^c	146.3 ^{de}	144.2 ^d	144.2 ^d	151.2 ^f
Gas rate (c, % per h)	9.01 ^g	3.23 ^b	8.16 ^f	4.61 ^d	3.05 ^a	4.22 ^c	4.19 ^c	4.25 ^c	4.49 ^d	4.85 ^e
Half-time ($t_{1/2}$, h)	7.69 ^a	21.5 ^g	8.50 ^b	15.1 ^d	22.7 ^h	16.4 ^f	16.5 ^f	16.3 ^f	15.4 ^e	14.3 ^c
Gas at $t_{1/2}$ (ml)	71.9 ^f	106.8 ^g	55.2 ^a	53.5 ^a	63.3 ^b	66.8 ^c	71.1 ^{ef}	67.8 ^{cd}	70.8 ^e	69.5 ^{de}
<i>iv</i> TDOM at $t_{1/2}$ (mg)	374.5 ^e	425.9 ^f	251.1 ^d	202.9 ^b	167.6 ^a	216.6 ^c	226.8 ^c	228.0 ^c	230.1 ^c	263.4 ^d
PF at $t_{1/2}$ (mg/ml)	5.21 ^f	3.99 ^d	4.55 ^e	3.79 ^c	2.64 ^a	3.10 ^b	3.19 ^b	3.36 ^b	3.25 ^b	3.70 ^c
Gas 16 h (ml)	108.6 ^h	90.5 ^g	85.5 ^f	59.5 ^b	53.1 ^a	72.6 ^c	72.1 ^c	70.6 ^c	76.0 ^d	80.0 ^e
<i>iv</i> TDOM 16 h (mg)	414.7 ^g	445.3 ^h	292.5 ^f	205.3 ^b	158.9 ^a	217.8 ^c	220.3 ^c	235.1 ^d	235.6 ^d	262.4 ^e
PF 16 h (mg/ml)	3.81 ^d	4.92 ^e	3.42 ^{bc}	3.45 ^c	2.99 ^a	3.00 ^a	3.05 ^a	3.33 ^{bc}	3.09 ^a	3.28 ^b

SBM, soyabean meal; MG, maize grain; LH, lucerne hay; OBH, oat–berseem clover hay; MCR, maize crop residue; *iv*TDOM, *in vitro* truly degraded organic matter.

a,b,c,d,e,f,g,h Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* For details of diets and procedures, see Tables 1 and 2 and p. 626.

† Variables are related to the incubation of 500 mg dry substrate.

degradabilities (R^2 0.60, $P=0.02$). Within diets, this relationship was again insignificant for gas volumes, but was significant for *in vitro* true OM degradabilities (R^2 0.78, $P=0.049$). Within roughages, gas volumes and EMP *in vivo* were inversely related (R^2 0.99, $P=0.016$), but this relationship was insignificant for *in vitro* true OM degradabilities. The PF and EMP *in vivo* of the five diets and MCR could be fitted onto the same regression line, which was better described by a curvilinear than a linear function (Fig. 1). The curvilinear function projected an asymptote of 386 g microbial biomass/kg OM truly degraded in the rumen. Combined gas volumes of the diets and MCR were linearly related to EMP *in vivo* (R^2 0.65, $P < 0.05$). This relationship was closer between *in vitro* true OM degradabilities and EMP *in vivo* (R^2 0.94, $P < 0.001$). No curvilinear regression of the kind reported in Fig. 1 could be fitted to these two latter relationships.

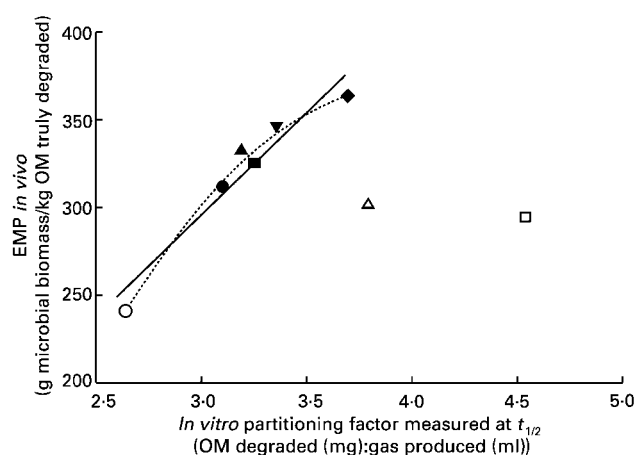


Fig. 1. Relationship between *in vitro* efficiency of microbial production (EMP) estimated by combined substrate degradability and gas volume measurements at time of half asymptotic gas production ($t_{1/2}$) and EMP measured in sheep. OM, organic matter. O, Maize (*Zea mays* L.) crop residue; Δ , oat (*Avena sativa* L.)–berseem clover (*Trifolium alexandrinum* cultivar BigBee) hay; \square , lucerne (*Medicago sativa* L.) hay ($P=0.33$ for roughages); \bullet , diet 1; \blacktriangle , diet 2; \blacktriangledown , diet 3; \blacksquare , diet 4; \blacklozenge , diet 5 ($P=0.04$ for diets 1 to 5); \dots , curvilinear (R^2 0.99); $—$, linear (R^2 0.93). For details of diets and procedures, see Tables 1 and 2 and p. 626.

The relationship between the PF after 16.0 h of incubation and the EMP *in vivo* was significant for the roughages ($P=0.04$), but insignificant for the diets (Fig. 2). The PF after 16.0 h and EMP *in vivo* of the five diets and MCR could not be fitted onto the same regression line. The relationship between the PF after 24.0 h and EMP *in vivo* tended to be significant for roughages ($P=0.07$), but was insignificant for diets ($P=0.66$; results not shown).

In vitro fermentation products and their relationships

SCFA production, NH_3 concentration, total and proportional purine recovery and *in vitro* apparent DM degradabilities were measured after 16.0 h of incubation (Table 4). Acetate proportion was lowest in the concentrates and highest in the roughages. Isoacids and valerate were formed from SBM and were generally

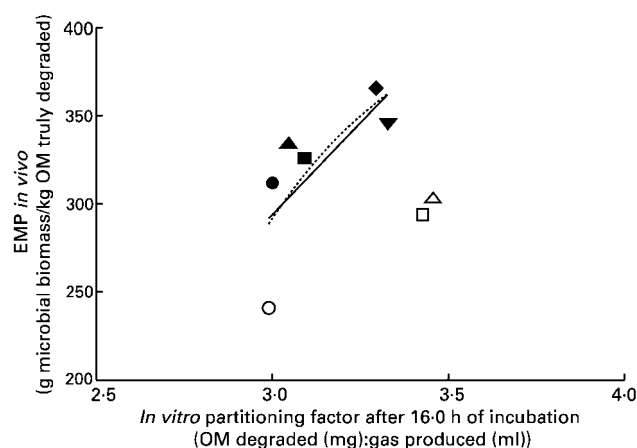


Fig. 2. Relationship between *in vitro* efficiency of microbial production (EMP) estimated by combined substrate degradability and gas volume measurements after 16.0 h of incubation and EMP measured in sheep. OM, organic matter. O, Maize (*Zea mays* L.) crop residue; Δ , oat (*Avena sativa* L.)–berseem clover (*Trifolium alexandrinum* cultivar BigBee) hay; \square , lucerne (*Medicago sativa* L.) hay (roughages $P=0.04$); \bullet , diet 1; \blacktriangle , diet 2; \blacktriangledown , diet 3; \blacksquare , diet 4; \blacklozenge , diet 5 ($P=0.08$ for diets 1 to 5); \dots , curvilinear (R^2 0.56); $—$, linear (R^2 0.51). For details of diets and procedures, see Table 1 and p. 626.

Table 4. Short-chain fatty acid (SCFA) production, ammonia-nitrogen, total purine bases (PB), PB relative to ATP and *in vitro* apparent degradabilities of DM obtained from the incubation of diet components and of diets*†

Variable	SBM	MG	LH	OBH	MCR	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
SCFA (mmol)	2.70 ⁱ	1.47 ^g	1.69 ^h	1.12 ^b	1.02 ^a	1.38 ^{ef}	1.33 ^{cd}	1.31 ^c	1.42 ^{fg}	1.46 ^g
Acetate (mmol/100 mmol total fatty acids)	57.5 ^a	58.3 ^b	69.7 ^e	71.4 ^f	69.3 ^e	66.1 ^c	66.3 ^c	67.2 ^d	66.4 ^c	67.4 ^d
Propionate (mmol/100 mmol total fatty acids)	25.2 ^h	23.5 ^g	21.1 ^c	20.1 ^a	23.8 ^g	22.9 ^f	22.3 ^e	20.5 ^b	21.8 ^d	19.8 ^a
Butyrate (mmol/100 mmol total fatty acids)	9.2 ^c	15.6 ^h	6.0 ^a	6.8 ^b	6.8 ^b	9.0 ^c	9.7 ^d	10.8 ^f	9.9 ^e	11.2 ^g
Isobutyrate (mmol/100 mmol total fatty acids)	1.4 ^e	0.4 ^c	0.5 ^d	0.4 ^c	0 ^a	0.5 ^d	0.4 ^c	0.3 ^b	0.4 ^c	0.3 ^b
Valerate (mmol/100 mmol total fatty acids)	3.0 ^g	1.0 ^e	2.0 ^f	1.0 ^e	0.5 ^a	0.8 ^{cd}	0.7 ^c	0.6 ^b	0.8 ^{cd}	1.0 ^e
Isovalerate (mmol/100 mmol total fatty acids)	3.7 ^f	1.2 ^e	0.7 ^c	0.3 ^a	–§	0.7 ^c	0.5 ^b	0.3 ^a	0.5 ^b	0.3 ^a
NH ₃ (mg)	15.2 ^a	0.03 ^g	3.0 ^b	2.2 ^c	0.6 ^f	2.5 ^c	1.9 ^d	1.2 ^e	1.7 ^d	0.8 ^f
PB (μmol)	8.48 ^e	9.99 ^f	6.81 ^b	4.94 ^a	5.02 ^a	7.14 ^c	6.73 ^b	6.81 ^b	7.32 ^c	7.82 ^d
Adenine (μmol)	4.57 ^e	4.73 ^e	3.03 ^c	2.36 ^a	2.48 ^b	3.49 ^{cd}	3.24 ^c	3.29 ^c	3.51 ^{cd}	3.76 ^d
Guanine (μmol)	3.91 ^b	5.26 ^c	3.78 ^b	2.58 ^a	2.54 ^a	3.65 ^b	3.49 ^b	3.52 ^b	3.81 ^b	4.06 ^b
PB/ATP (μmol/mmol)	1.28 ^a	2.56 ^g	1.59 ^b	1.72 ^c	1.87 ^d	2.00 ^{ef}	1.94 ^{de}	1.98 ^{ef}	1.98 ^{ef}	2.05 ^f
<i>iv</i> ADDM (mg)	311.5 ^f	178.2 ^d	197.4 ^e	149.4 ^b	125.1 ^a	155.1 ^{bc}	158.1 ^c	150.6 ^b	159.1 ^c	171.4 ^d

SBM, soyabean meal; MG, maize (*Zea mays* L.) grain; LH, lucerne (*Medicago sativa* L.) hay; OBH, oat (*Avena sativa* L.)–berseem clover (*Trifolium alexandrinum* cultivar BigBee) hay; MCR, maize crop residue; *iv*ADDM, *in vitro* apparently degraded DM.

a,b,c,d,e,f,g,h,i Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* For details of diets and procedures, see Tables 1 and 2 and p. 626.

† Variables are related to the incubation of 500 mg dry substrate.

§ Negative value calculated.

< 2 mmol/100 mmol total fatty acids in the other substrates. In the diet components, NH₃ concentration was lowest in MG and highest in SBM. Significant differences in NH₃ concentrations were also observed in the diets, and it can be calculated that their NH₃ concentrations and EMP *in vivo* were strongly inversely related (R^2 0.93, $P = 0.007$). The highest purine concentration was found with MG and lowest with OBH. Except for SBM, guanine concentration was higher than adenine concentration. It should be noted here that the purine contents in the non-incubated substrates were 5.7, 5.7, 6.2, 6.3 and 6.1 μmol/500 mg in SBM, MG, LH, OBH and MCR respectively. Purine yield per unit ATP produced (μmol/mmol) from the incubation of 500 mg substrate ranged from 1.28 in soyabean meal to 2.56 in MG. There was no significant relationship between purine yield per unit ATP produced and EMP *in vivo* either across diets and roughages or within diets and roughages. The relationship tended to be inverse for the roughages ($P = 0.22$).

For diets and roughages, a strong relationship was found between stoichiometrically calculated and actually measured gas volumes (Fig. 3). In MG, measured gas volume was about 20% higher than the calculated value, while for SBM measured gas volume was less than the stoichiometrically calculated value by approximately 16%. The relationship was described by the regression equation $y = 24.8 + 0.68x$ (R^2 0.89, $P < 0.0001$).

In vitro apparently degraded substrate was calculated by summation of the mass of C, H and O recovered in SCFA and the mass of C, O and H stoichiometrically calculated for CO₂, CH₄ and H₂O produced. There was a very good agreement between *in vitro* apparent DM degradability as determined by high-speed centrifugation and *in vitro* apparent DM degradability calculated by C, O and H balance

(Fig. 4). The relationship was described by the regression equation $y = -2.0 + 1.0x$ (R^2 0.98, $P < 0.0001$).

In vitro truly degraded substrate was calculated as the sum of C, O and H recovered in SCFA and fermentative CO₂, CH₄ and H₂O plus the amount of microbial biomass produced (the latter calculated from purine production). The relationship between *in vitro* true OM degradability as measured by neutral-detergent solution treatment and calculated true degradability was strong, except for MG, where the measured degradability was about 130 mg

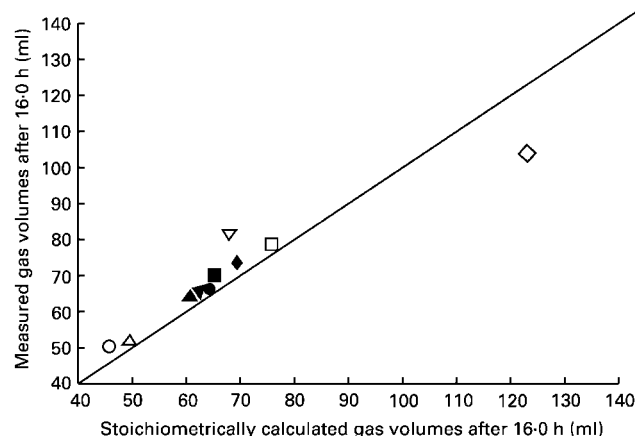


Fig. 3. Relationship between *in vitro* gas volumes stoichiometrically calculated from short-chain fatty acid recovery after 16.0 h of incubation and measured gas volumes. O, Maize (*Zea mays* L.) crop residue; Δ, oat (*Avena sativa* L.)–berseem clover (*Trifolium alexandrinum* cultivar BigBee) hay; □, lucerne (*Medicago sativa* L.) hay; ▽, maize grain; ◇, soyabean meal; ●, diet 1; ▲, diet 2; ▼, diet 3; ◆, diet 4; ◆, diet 5; —, $y = x$. For details of diets and procedures, see Tables 1 and 2 and p. 626.

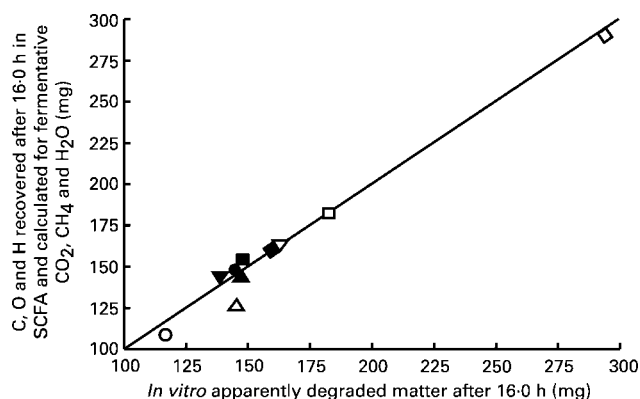


Fig. 4. Relationship between *in vitro* apparent degradability measured by high-speed centrifugation after 16.0-h of incubation and recovery of C, H and O in short-chain fatty acids, (SCFA) and gases. O, Maize (*Zea mays* L.) crop residue; Δ , oat (*Avena sativa* L.)-berseem clover (*Trifolium alexandrinum* cultivar BigBee) hay; \square , lucerne (*Medicago sativa* L.) hay; ∇ , maize grain; \diamond , soyabean meal; \bullet , diet 1; \blacktriangle , diet 2; \blacktriangledown , diet 3; \blacksquare , diet 4; \blacklozenge , diet 5; —, $y = x$. For details of diets and procedures, see Tables 1 and 2 and p. 626.

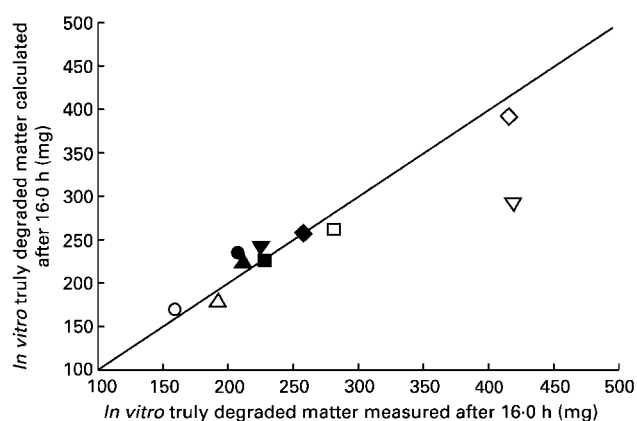


Fig. 5. Relationship between *in vitro* true organic matter degradability estimated by neutral detergent solution after 16.0-h of incubation and recovery of short-chain fatty acids, gases and microbial biomass. O, Maize (*Zea mays* L.) crop residue; Δ , oat (*Avena sativa* L.)-berseem clover (*Trifolium alexandrinum* cultivar BigBee) hay; \square , lucerne (*Medicago sativa* L.) hay; ∇ , maize grain; \diamond , soyabean meal; \bullet , diet 1; \blacktriangle , diet 2; \blacktriangledown , diet 3; \blacksquare , diet 4; \blacklozenge , diet 5. For details of diets and procedures, see Tables 1 and 2 and p. 626.

higher than the calculated value (Fig. 5). The relationship was described by the regression equation $y = 84.6 + 0.62x$ (R^2 0.81, $P = 0.0004$).

Discussion

Relationships between efficiency of microbial production *in vitro* and *in vivo*

As a high conversion of degraded feed into microbial biomass, i.e. high EMP, decreases the need for supplementing rumen undegradable feed protein and decreases the feed C flow into fermentative CO_2 and CH_4 , high EMP is preferred over high SCFA production (Beever, 1993; Leng, 1993; Van Soest, 1994). The prediction of possible

feed-related differences in EMP is, therefore, of considerable interest in feed analysis. In the present work, significant differences in EMP *in vivo* were found for both roughages and diets, even though the latter were designed by Karsli (1998) to provide uniformly the degradable intake of protein required for the synthesis of 130 g microbial protein/kg total digestible nutrients consumed (National Research Council, 1996; level 1). These findings support the concept of varying EMP implicit in the Cornell net carbohydrate and protein system (National Research Council, 1996; level 2). Unfortunately, the analytical tools suggested by the Cornell net carbohydrate and protein system for the prediction of EMP are quite laborious and simpler techniques are required.

The results presented in Fig. 1 suggest that combined true degradability of substrate and gas volume measurements *in vitro*, i.e. PF analysis (Blümmel *et al.* 1997), is a promising technique with regards to the detection of variations in EMP. For diets, there was a significant relationship between PF values measured at $t_{1/2}$ and EMP *in vivo*, which accounted for 94% (non-linear relationship) of the variation in EMP. However, despite the relative time proximity between PF measurements at $t_{1/2}$ and after 16.0 h of incubation (Table 3), this relationship was only close to significant ($P = 0.08$) when measured after 16.0 h and was insignificant ($P = 0.66$) when determined after 24.0 h. The 24.0 h findings can be explained by a distortion of PF measurements through secondary fermentation of lysed microbial cells into SCFA and, consequently, of the gases after microbial peak yield (Blümmel & Ørskov, 1993; Cone *et al.* 1997). Still, the PF value in diet 5, for example, decreased by about 11%, from 3.70 (PF at $t_{1/2}$) to 3.28 (PF after 16.0 h) mg/ml within the relative short incubation period from 14.3 to 16.0 h (Table 3). These findings suggest that for the kind of diets designed by Karsli (1998) detection of variations in EMP might require more laboratory input than PF analysis at only one fixed incubation time. The additional laboratory input consists of the incubation required for estimating asymptote and rate of gas production for the calculation of $t_{1/2}$.

For the roughages, time-uniform PF analysis after 16.0 h (and 24.0 h) of incubation was better related to EMP *in vivo* than PF analysis at $t_{1/2}$. This was caused by the high PF value of LH (4.55 mg/ml) when determined at $t_{1/2}$, which was only 8.5 h. It is possible that this PF value of 4.55 mg/ml, which according to Blümmel *et al.* (1997) would indicate a Y_{ATP} of approximately 32, presents an artifact caused by removal of still unfermented cellular components of LH soluble by neutral-detergent solution treatment, resulting in an overestimation of *in vitro* true OM degradability and consequently the PF value. This problem will be addressed in greater detail later on. Despite these reservations, theoretical PF values for diets calculated from PF values of dietary components at $t_{1/2}$ and the proportion of each component in a diet were strongly related to the *in vivo* EMP of diets (R^2 0.93). These findings suggest that the *in vivo* EMP may be predicted from PF values of possible feed components.

Interestingly, the regression of the EMP *in vivo* on PF of diets and MCR at $t_{1/2}$ could be fitted onto one regression

line, which was better (R^2 0.99 v. 0.93) described by a non-linear than a linear function (Fig. 1). Both curvilinearity, as well as projected asymptote of EMP (386 g microbial biomass/kg OM truly degraded in the rumen), agree well with the findings on maximum EMP (in the presence of protozoa) reported by Russell *et al.* (1992) and Van Soest (1994). For LH and OBH, estimates of EMP *in vitro* by PF analysis were consistently higher than the respective EMP *in vivo* (Figs 1 and 2). Crude protein content was higher in these two roughages than in diets and MCR, and this crude protein might have been used more efficiently for microbial synthesis *in vitro*, where no absorption and passage of NH_3 occurs, than *in vivo*. It can be calculated from *in situ* results on N and OM degradation of the LH and OBH (Karsli, 1998) that rumen N availability in LH was much higher than the OM availability, which might have resulted in a less than optimal EMP *in vivo* (Sinclair *et al.* 1995; Witt *et al.* 1999). Similarly, Blümmel *et al.* (2001) calculated the synchronization indices of N:OM fermentation of the five diets according to Sinclair *et al.* (1995) and Witt *et al.* (1999) and concluded that both overall N and synchronization of N:OM fermentation was insufficient for maximum EMP. However, in the current *in vitro* work, NH_3 was still present in the diet incubation (Table 4), which does not support the assumption of N as the primarily limiting factor for EMP. Interestingly, the NH_3 concentration of the diets *in vitro* was highly negatively related to the EMP *in vivo* (R^2 0.93) and differences in the EMP are therefore probably less due to lack of total N and more to microbial inefficiencies in utilizing it.

In the roughage incubations, the PF analysis after 16.0 and 24.0 h ranked the roughages in the same order as the EMP in *in vivo* determinations, the relationship being significant for the PF after 16.0 h (Fig. 2), but not for the PF after 24.0 h ($P=0.07$). The 24.0 h incubation time was apparently too long for LH, considering the short $t_{1/2}$ (8.5 h) of this roughage, resulting in a low PF value after 24.0 h because of microbial lysis. However, only three roughages have been investigated in the present work and caution is, therefore, required to not over-interpret these results.

Relationships between *in vitro* fermentation products

The concept of PF analysis demands a close stoichiometrical relationship between SCFA and gas production and a reliable determination of true degradability of the substrate. In the present work, gas volumes of diets and roughages were well predicted by SCFA analysis and the application of the stoichiometrical relationships outlined by Wolin (1960). In MG, however, more gas was measured than was stoichiometrically calculated, while the reverse was true for SBM (Fig. 3). The latter findings agree with results reported by Blümmel *et al.* (1999a) obtained with SBM of a different provenance. These authors concluded that the stoichiometrical relationship between SCFA and gas production is not good for feeds with a crude protein content >400 g/kg.

A possible explanation for this observation resides with the high production of NH_3 in these feeds, ultimately

impairing the bicarbonate buffering system in the *in vitro* gas test (Cone 1998; Blümmel *et al.* 1999a). Thus, even though SCFA are produced, the buffering effect does not necessarily allow the expulsion of CO_2 into the gas phase. In the bicarbonate-buffered gas test with an intact buffering mechanism, approximately 50% of the total gas volume consists of CO_2 originating from buffering the SCFA produced (Blümmel & Ørskov, 1993). More gas was measured in the MG incubation than was accounted for by SCFA analysis and stoichiometrical calculations. Lactate was not analysed in the present work because several authors (Steingass & Menke, 1986; Getachew *et al.* 1998) have shown the buffering capacity of the employed *in vitro* test to be adequate for maintaining $\text{pH} > 6$ in concentrate and starch incubations. These pH conditions do not favour lactate production.

Nevertheless, buffering of lactate would lead to gas production, but, besides the unfavourable pH conditions mentioned earlier, it appears unlikely that lactate was produced to any significant extent for another reason. There was a very good agreement between the amount of substrate apparently degraded *in vitro*, as determined by high-speed centrifugation and the amount of C, O and H recovered in SCFA, and calculated for fermentative CO_2 , CH_4 and H_2O (Fig. 4). Were lactate in the MG incubation responsible for the stoichiometrical underestimation of gas production, more substrate should have been apparently degraded than recovered in SCFA and fermentative CO_2 , CH_4 and H_2O , but this was not the case. More work is required to understand why more gas was recovered in MG than was accounted for by SCFA, and it appears worthwhile investigating if the complete uptake of NH_3 (very little NH_3 was recovered after terminating the MG; Table 4) was responsible, possibly due to some mechanism converse to the one observed in SBM (excess NH_3). However, lactate analysis should be included in this work.

Gravimetrically determined true degradability and recovery of fermentation products

As mentioned earlier, reliable determination of true substrate degradability is a second condition for meaningful PF analysis. The relationship between *in vitro* true OM degradability, as determined by neutral-detergent solution treatment, and true OM degradability calculated as the sum of C, H and O recovered as SCFA, CO_2 , CH_4 and H_2O plus the microbial biomass produced, is presented in Fig. 5. There was a good agreement between measured and calculated true degradability for the diets and most of the diet components, confirming that neutral-detergent solution treatment achieves an effective separation of microbes and undegraded feed, thereby yielding a reliable estimate of true feed degradability (Van Soest, 1994). However, neutral-detergent solution-derived *in vitro* degradability in MG was about 130 mg higher than was accounted for by the fermentation products (Fig. 5). The low rate of gas production from MG (3.23% per h, Table 3) strongly suggests that not all the starch was fermented after 16.0 h of incubation. Starch can be soluble in neutral-detergent solution (Van Soest, 1994) and

removal of unfermented starch is probably responsible for the high discrepancy between the measured and calculated true degradability evident in MG. It appears that determinations of *in vitro* true degradability of pure, high-starch concentrates by neutral-detergent solution treatment are potentially prone to substantial analytical errors, resulting in an overestimation of degradability.

In the balance of fermentation products, microbial biomass was estimated by purine analysis. It should be mentioned here that the relationship between 16.0 h purine production per unit ATP and EMP *in vivo* was insignificant for diets and roughages, the relationship tending to be inverse for the roughages. The purine contents of non-incubated diet components was on average 6.00 $\mu\text{mol}/500\text{ mg}$, which appears high when compared with the purine yield after incubating the five substrates for 16.0 h (mean value 7.05 $\mu\text{mol}/500\text{ mg}$; see Table 4). Even though McAllan & Smith (1973*a,b*) suggested that feed purines are completely degradable in the rumen, Djouvinov *et al.* (1998) have recently shown the effective feed purine degradability in the rumen to be dependent on incubation time. This could affect *in vitro* measurements where the entire apparently undegraded pellet was analysed for purines after a short (16.0 h) incubation period relative to rumen retention times. Studies of the kind presented in Fig. 5 should therefore be confirmed using different microbial markers.

Complementarity of measurements of substrate disappearance and of fermentation products

Gravimetric methods measuring substrate disappearance have frequently been criticized on the grounds that substrate may be lost, for example by solubilization or filtration, without having actually been fermented (Menke *et al.* 1979; Blümmel & Ørskov, 1993; Groot *et al.* 1998; Pell *et al.* 1998) and these concerns can be justified (see Fig. 5). These reservations provided a strong rationale for the development of feed evaluation systems based on *in vitro* gas production (Menke *et al.* 1979). Blümmel *et al.* (1997), on the other hand, have argued that measurement of only one fermentation product, particularly of gases that are waste products, is questionable unless the proportionality of fermentation products, including SCFA, gases and microbial biomass, is constant. If this relationship is not constant, gravimetric measurement of true substrate degradability (Goering & Van Soest, 1970) provides a more convincing concept.

Yet more information about the fermentation can be obtained by combining true degradability and gas volume measurements. The results presented in the present study support this hypothesis, despite the occasional problems encountered with neutral-detergent solution treatment in the *in vitro* determination of true OM degradability discussed earlier. For example, *in vitro* true OM degradability of diets measured at $t_{1/2}$ was significantly related to their EMP *in vivo*, while gas volume at $t_{1/2}$ was not. *In vitro* OM degradability measured at $t_{1/2}$ is probably a good indicator of the rate of substrate degradation. As high substrate degradability rates decrease microbial maintenance requirements, EMP is potentially increased (Pirt, 1982;

Russell *et al.* 1992). The problem associated with measuring *in vitro* gas production only is also manifest in the roughage incubation, where gas volumes at $t_{1/2}$ and EMP *in vivo* were inversely related ($P=0.02$). MCR had a higher ($P<0.05$) gas volume at $t_{1/2}$ than LH and OBH, reflecting a proportionally higher conversion of degraded substrate into SCFA and gases. In a related context, after 16.0 h of incubation the mean purine production from 500 mg of the mixed diets was 7.16 μmol . It can be calculated from the purine content of components and their proportion in the diet that only 6.06 μmol purines were to be expected, assuming additive effects, i.e. the associative effect of supplementation on purine yield was about 18%. On the other hand, mean SCFA yield from the incubation of 500 mg mixed diets was 1.38 mmol, while 1.29 mmol SCFA were to be expected from additive effects of SCFA production from the incubation of the dietary components, i.e. the associative effect on SCFA was only about 7% (calculated from Table 4).

In conclusion, nutritionally significant variations were found in the proportions of rumen fermentation products, including microbial biomass yield. *In vitro* degradability measurements were related to EMP *in vivo* in a more meaningful manner than gas volume measurements, but more comprehensive information about substrate degradation was obtained by combining both measurements. Therefore, to achieve a high EMP it appears sensible to suggest the selection of feeds with a high true degradability and low gas production in proportion to the amount of substrate degraded.

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