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The modification of an *in vitro* gas production test to detect roughage related differences in *in vivo* microbial protein synthesis as estimated by the excretion of purine derivatives

M. BLÜMMEL[1]*, R. MGOMEZULU[2], X. B. CHEN[3], H. P. S. MAKKAR[1], K. BECKER[1] AND E. R. ØRSKOV[3]

[1] *Institute for Animal Production in the Tropics and Subtropics, Fruwirthstraße 12, 70599 Stuttgart*
[2] *Bunda College of Agriculture, P.O. Box 219, Lilongwe, Malawi*
[3] *The Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB2 9SB, Scotland*

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SUMMARY

The relationship between variations in *in vitro* microbial biomass production and microbial protein supply to the host animal was examined in 13 Malawian goats fed on stover leaves from two varieties of maize at Bunda College, Malawi, in 1995. The *in vitro* parameters were analysed based on the concept of the partitioning factor (PF) which is obtained by the combination of gas volume measurements with determinations of the amount of substrate truly degraded. The PF reflects substrate-dependent variation in the *in vitro* partitioning of degraded substrate between short chain fatty acids (SCFA), gases and microbial biomass. The *in vivo* microbial protein synthesis was estimated by the urinary excretion of the purine derivatives (PD) allantoin, uric acid and xanthine + hypoxanthine. For the two types of stover leaves examined, the higher microbial efficiency of one variety *in vitro* was reflected by different PD excretions on practically identical digestible dry matter intakes *in vivo*. It is concluded that substrate-dependent variations in microbial efficiencies as detected by the PF are also evident and relevant for *in vivo* microbial protein synthesis.

INTRODUCTION

Microbial biomass and short-chain fatty acids (SCFA) are the useful products and gases (mainly CO_2 and CH_4) are the waste products of ruminal degradation. Conventional *in vitro* systems measure either the amount of substrate degraded (Tilley & Terry 1963) or one of the products generated after a given time of incubation in a mixed rumen suspension. The *in vitro* gas method (Menke *et al*. 1979) records the accumulating gas volume and is either empirically justified by the good relationship between *in vitro* gas production and *in vivo* digestibilities or by the stoichiometrical association between SCFA generation and gas volumes (Beuvink & Spoelstra 1992; Aiple 1993; Blümmel & Ørskov 1993; Opatpatanakit *et al*. 1994; Blümmel *et al*. 1999).

* To whom correspondence should be addressed at: USDA-ARS, Fort Keogh Livestock and Range Research Lab, Miles City, MT 59301, USA. Email: blummel@larrl.ars.usda.gov

However, the yield of SCFA and microbial biomass from a unit of substrate degraded is not a constant; an inverse relationship can exist between them (Beever 1993; Leng 1993). This relationship also exists between *in vitro* gas volumes or SCFA production and microbial biomass (for review see Blümmel *et al*. 1997*a*). These authors suggested a combination of *in vitro* gas volume measurements with residue determinations (Goering & Van Soest 1970) after 24 h of incubation to calculate the amount of substrate truly degraded. The residue determination reveals how much substrate was used in the fermentation and the gas measurement indicates how much of this substrate was converted into SCFA and gases. Substrates with a high true degradability but, proportionally to the amount of substrate degraded, low gas production should be selected to obtain feeds with high microbial efficiencies. This concept was validated by ^{15}N studies (Blümmel *et al*. 1997*a*).

The ratio of substrate degraded to gas volume produced by it was termed partitioning factor (PF) and was found to be valuable in feed intake (DMI)

predictions. The inclusion of PF into stepwise multiple regressions with parameters of rate and extent of *in vitro* gas production and/or *in sacco* degradability improved the prediction of DMI considerably. Roughages with high substrate degradability but comparatively low gas production (higher PF), had higher DMI (Blümmel & Bullerdieck 1997; Blümmel *et al.* 1997*b*). The positive association between PF and DMI may be due to the stimulating effect of enhanced microbial protein supply to the small intestine.

The objective of this work was to investigate the direct relationship between the PF value *in vitro* and the microbial protein supply to the host animal *in vivo*. The latter was assessed by the determination of the purine derivatives in urine (PD) according to Chen *et al.* (1992). The excretion of PD in urine has been used extensively as an index of the intestinal flow of microbial protein (Mayes *et al.* 1995). A higher PD excretion indicates a greater flow of microbial protein. Two roughages were chosen with similar *in vitro* true degradabilities but different gas production and, consequently, different PF values.

MATERIALS AND METHODS

In vitro *studies*

Manually-fractionated stover leaves from a hybrid (HSL) and a local (LSL) maize variety, previously used (unfractionated) in intake and selection trials (Chikagua & Blümmel 1996), were chosen because they had very similar *in vitro* true degradabilities but different *in vitro* gas volumes. This resulted in significantly different ($P < 0{\cdot}05$) PF values of 3·3 and 3·1 mg/ml in LSL and HSL, respectively, and the experiment was planned based on the hypothesis that higher *in vivo* microbial efficiencies were to be expected from feeding LSL. The stover leaves had similar neutral detergent fibre (NDF) contents (HSL 74 and LSL 76%) and similar low nitrogen (N) contents (HSL 0·8 and LSL 0·6%). *In vitro* studies were conducted at Bunda College, Malawi, and rumen liquor and particulate matter were collected from two Malawian Zebu cattle fitted with large rumen cannulas (Bar Diamond, USA) and fed on a hay diet of medium quality, with access to a mineral lick. The partitioning factor (PF) was determined in triplicate samples according to the procedure of Blümmel *et al.* (1997*b*). This approach, in brief, combines the Menke *et al.* (1979) method with that of Tilley & Terry (1963) as modified by Goering & Van Soest (1970). The incubations were terminated after 24 h, the gas volumes recorded and the incubation residues refluxed with neutral detergent solution (NDS) to completely separate microbial biomass from the undegraded feed residues. The PF was calculated as the ratio of mg substrate truly degraded to ml gas produced by it. The analysis of PF value was also repeated at Hohenheim University to detect possible differences in the activity of rumen liquor between the cattle from Malawi and Germany. The observed differences in the PF values were small and insignificant.

Further *in vitro* studies were conducted at Hohenheim University since high-speed centrifugation facilities were not available in Malawi. Rumen liquor was collected from a Hinderwälder cow (German landrace) fed a medium quality hay. The *in vitro* studies were similar to those described above but the 24 h incubation residue was transferred into 90 ml centrifuge tubes and centrifuged at 20000 ***g*** for 30 min to determine the apparent degradability and microbial biomass production as described in detail by Blümmel *et al.* (1997*b*). Biomass production was determined as the weight difference of the centrifuged and freeze-dried pellet before and after treatment with NDS.

Experimental animals and feeding

Thirteen Malawian male goats of mean weight 20·6 (S.D. 3·0) kg were treated against external and internal parasites and accustomed to metabolism cages for 4 weeks. The stover leaves were hammer-milled to *c.* 0·5–3 cm particle size to minimize selective feeding and were supplemented with soybean meal (SBM, with 7% N and 20% fat) to provide 1·25% of N in the feed offered. The feeds were offered at a rate of 2·5% of the body weight of the goats and SBM accounted for *c.* 7·5 (HSL) and 10% (LSL) of the total feed on offer. SBM was offered from 08.00 to 10.00 h and was always completely consumed. The milled stover leaves were offered afterwards and refusals were collected the next morning. Each feeding experiment consisted of a 14-day preliminary period followed by a 10-day faeces and urine collection period. Urine was collected into 5 ml of concentrated H_2SO_4 using urinary funnels.

Chemical analysis

Nitrogen in feed, faeces and urine was determined by the Kjeldahl method. NDF was analysed according to Van Soest & Robertson (1985). *In vivo* true digestibility was determined by refluxion of faecal samples with NDS to separate microbial debris and endogenous matter from the undigested feed (Mason 1979). Faecal N was partitioned into total N and NDF-N by quantifying N bound to faecal NDF. This analysis was used to estimate metabolic and endogenous faecal N (MEFN = N minus NDF-N). Urine samples were analysed at the Rowett Research Institute for uric acid and the sum of hypoxanthine and xanthine by AutoAnalyser (Chen *et al.* 1990) and for analysis of allantoin by HPLC (Chen *et al.* 1993). The four metabolites are collectively referred to as 'purine derivatives' (PD). Intestinal flow of microbial protein to the animal was estimated based on total

PD excretion in the urine (Chen *et al*. 1992). These estimates were based on work with European sheep as were correction factors for endogenous PD excretion (Chen *et al*. 1992).

Statistical analysis

The SAS/STAT (1988) procedure was used to determine differences in the analysis of the various *in vitro* and chemical parameters. A paired *t*-test procedure was used for the examination of differences in *in vivo* parameters. Significant differences relate to the $P < 0{\cdot}05$ level unless stated otherwise.

RESULTS

Comparison of in vitro *parameters*

Significantly more gas was produced from the 24 h incubation of HSL than from LSL and the apparent substrate degradability was also higher ($P < 0{\cdot}05$) in the former treatment (Table 1). Apparent degradability was determined by centrifugation at 20000 ***g***, which spins down undegraded substrate and the microbial biomass generated in the fermentation. The other fermentative products, SCFAs and gases, are not recovered in the pellet. True degradabilities were analysed by NDS treatment of the apparently undegraded pellet and were practically identical between the HSL and LSL samples (Table 1). This is reflected in the significantly different PF values, where 1 ml of gas was generated from the degradation of 3·1 (328·7 mg : 107·2 ml) and 3·3 mg (327·3 mg : 100·7 ml) of substrate in the HSL and LSL respectively. The gravimetric estimation of biomass yield as based on the weight loss in the centrifuged and freeze-dried pellet after NDS treatment showed also a significantly higher biomass yield from LSL.

Comparison of in vivo *parameters*

Daily dry matter intakes (DMI) in both groups were *c*. 420 g, equivalent to 2% of the goats' body weight. Both groups refused *c*. 100 g of stover leaves. The LSL group consumed 0·35 g/day more N ($P < 0{\cdot}05$) than the HSL group (Table 2).

Both apparent (*c*. 60%) and true (*c*. 75%) *in vivo* digestibilities were very similar between groups and there was consequently no significant difference in the apparent and true digestible DMI. The N balance of both groups was negative but was higher by 0·66 g ($P < 0{\cdot}001$) in the LSL group. The daily excretion of total faecal N and MEFN was comparable ($P > 0{\cdot}05$) in both treatments, the latter presenting the bulk ($> 85\%$) of the total faecal N.

Daily PD excretion was higher by 0·5 mmol/day ($P < 0{\cdot}05$) in the LSL than in the HSL group (Table 3). The ratio of allantoin to total PD was 0·65 and 0·68 in the HSL and LSL group, respectively ($P > 0{\cdot}05$). Estimated daily microbial N supply to the host animal, assuming that all PD originated from rumen microbes, was 3·39 g in the LSL group and 2·95 g in the HSL group. Applying a correction factor for endogenous PD excretion reduced the mean estimated N supply by 0·44 and 0·55 g in the LSL and HSL groups, respectively (Table 3).

Table 1. *Comparison of hybrid* (*HSL*) *and local* (*LSL*) *maize stover leaves in 24 h* in vitro *gas production, concomitant apparent and true dry matter degradabilities, the partitioning factor* (*PF*; *mg truly degraded*: *ml gas produced*) *and parameters of microbial biomass yield. Data were obtained from the incubation of 500 mg of dry substrate*

Parameter	HSL	LSL	S.E.	D.F. error
Gas volume (ml)	107·2	100·7	0·8	4
Apparent degradability(mg)	227·3	212·4	2·8	4
True degradability (mg)	328·7	327·3	2·2	4
Partitioning factor (mg/ml)	3·1	3·3	0·04	4
Microbial biomass (mg)	101·4	114·9	2·8	4

DISCUSSION

The hypothesis that the *in vivo* microbial biomass production from both stover leaves would be different was based on the partitioning factor (PF) *in vitro* as suggested by Blümmel *et al*. (1997*b*). From the incubation of 500 mg (DM) substrate approximately 328 mg was truly degraded in both HSL and LSL but HSL produced significantly more gas from it (Table 1). This resulted in a significantly lower PF of 3·1 mg/ml (328·7 mg/107·2 ml) compared to 3·3 mg/ml (327·3 mg/100·7 ml) in the LSL incubation. Based on the stoichiometry of Wolin (1960) and data from Blümmel & Ørskov (1993), Blümmel *et al*. (1997*b*) calculated that in roughage fermentations the generation of 1 ml of gas is coupled to the requirement of 2·2 mg of the substrate for production of SCFA, and fermentative CO_2, CH_4 and H_2O. This stoichiometrically-derived factor is in good agreement with the ratio of substrate apparently degraded (Table 1) to volume of gas produced for both HSL (227·3 mg/107·2 ml = 2·1 mg/ml) and LSL (214·4 mg/100·7 ml = 2·1 mg/ml). The results presented in Table 1 show that in HSL, for 1 ml of gas, 3·1 mg of substrate was degraded of which 2·1 mg were used for SCFA and gases, while the respective values in LSL were 3·3–2·1 mg. Consequently, relative to 1 ml of gas, 1·0 mg (3·1–2·1) and 1·2 mg (3·3–2·1) of degraded substrate are unaccounted for by SCFA and gases in HLS and LSL respectively. These calculations suggest a conversion of degraded substrate to microbial biomass of 32·0 ($1{\cdot}0/3{\cdot}1 \times 100$) for HSL and of 36·0% ($1{\cdot}2/3{\cdot}3 \times 100$) for LSL. The assumption of

Table 2. *Mean daily dry matter and digestible dry matter intake, nitrogen (N) and metabolic and endogenous faecal nitrogen of hybrid (HSL) and local (LSL) maize stover leaves fed to Malawian goats*

Parameter	HSL	LSL	S.E.	D.F. error
Dry matter intake (g/d)	416	425	18·2	24
Apparent digestible dry matter intake (g/d)	253	254	8·0	24
True digestible dry matter intake (g/d)	315	320	10·0	24
Nitrogen intake (g/d)	5·44	5·79	0·2	24
Faecal N (g/d)	2·47	2·61	0·16	24
Metabolic and endogenous faecal N (g/d)*	2·24	2·19	0·11	24
Urinary nitrogen (g/d)	3·32	4·19	0·11	24
Nitrogen balance (g/d)	−0·35	−1·01	0·14	24

* Estimated as total faecal N–faecal NDF-N.

Table 3. *Daily excretion of purine derivatives (PD) of Malawian goats fed hybrid (HSL) and local (LSL) maize stover leaves. Daily microbial N supply to the host animal was estimated by the PD excretion before and after correction for endogenous PD excretions*

	PD excretion (mmol/d)		Corrected microbial N supply (g/d)		Uncorrected microbial N supply (g/d)	
Goat No.	HSL	LSL	HSL	LSL	HSL	LSL
1	2·69	2·66	1·76	1·73	2·33	2·30
2	3·67	4·26	2·73	3·32	3·18	3·69
3	2·91	3·94	1·90	2·99	2·52	3·41
4	4·05	4·70	3·00	3·66	3·51	4·07
5	4·37	4·53	3·32	3·48	3·78	3·92
6	3·32	4·95	2·19	3·91	2·87	4·28
7	2·92	3·42	1·89	2·43	2·53	2·96
8	4·19	4·20	3·18	3·20	3·63	3·64
9	3·15	3·90	2·17	2·95	2·73	3·38
10	2·89	4·12	1·76	3·10	2·50	3·57
11	3·40	3·27	2·39	2·25	2·94	2·83
12	3·35	3·72	2·49	2·78	2·90	3·22
13	3·44	3·17	2·40	2·21	2·98	2·74
Mean	3·41	3·91	2·40	2·92	2·95	3·39
S.E.	0·23 (24 D.F.)		0·17 (24 D.F.)		0·14 (24 D.F.)	

higher *in vitro* microbial biomass yield from LSL is also supported by the biomass yield directly determined as the difference in pellet weight before and after the NDS treatment. This treatment suggests a microbial biomass yield of 114·9 and 101·4 mg for LSL and HSL respectively (Table 1), the difference being significant. Biomass so determined was found to be in good agreement with ^{15}N studies (Blümmel *et al.* 1997*b*).

The hypothesis regarding differences in microbial biomass yield from the two stover leaves is supported by significantly higher purine derivative (PD) excretion in the LSL compared with the HSL treatment (Table 2) despite very similar intakes of truly digested feed (315 g for HSL and 320 g for LSL per day). These findings suggest that differences in microbial efficiencies as detected by the PF value *in vitro* also hold true *in vivo*. The microbial efficiencies in the two stover leaves, derived from the PF value, were 32·0 for HSL and 36·0 % for LSL. Based on these calculations, *in vivo* microbial biomass production might be estimated by considering the truly digestible feed intake and the proportion of this intake digested in the rumen. Assuming, in roughages, *c.* 90 % of the truly digested feed to be digested in the rumen (Südekum & Hasselmann 1989) a daily ruminal microbial biomass production of 90·7 (HSL) and 103·7 g (LSL) can be calculated.

When compared with these figures, the PD-derived estimations of microbial N supply to the host animal (Table 3) of 2·40 g/day for HSL and 2·92 g/day for LSL appear low. No figures for endogenous PD

excretion in Malawian goats are available. Assuming zero endogenous PD excretion in Malawian goats would increase these estimates by 22 and 16% to 2·95 and 3·39 g/d in the HSL and LSL group, respectively (Table 3). Taking an average N content in microbial biomass of 7·7% (Ørskov 1982), the daily microbial biomass yield estimated assuming zero endogenous PD excretion would be 38 ($100/7{\cdot}7 \times 2{\cdot}95$) in HSL and 44 g ($100/7{\cdot}7 \times 3{\cdot}39$) in LSL which is still only half the biomass yield estimated by the PF values. However, the daily N intakes (Table 2) of 5·44 (HSL) and 5·79 (LSL) have evidently been too low to support the maximum possible microbial biomass yield. This is in contrast with the *in vitro* incubations where ammonium bicarbonate from the buffer medium provided an additional 7 mg of N per incubation of 500 mg of substrate.

The DMI in the present work was *c*. 2% of the body weight of the goats while DMI was *c*. 3% in previous trials with sheep fed on maize stovers unfractionated and crudely chopped with urea supplementation (Chikagua & Blümmel 1996). It is unclear whether the observed lower DMI was due to the supplementation with soybean meal (SBM) or due to a dislike of the milled and somewhat dustlike stover leaves. Apart from possibly reducing the DMI of the stover leaves, SBM supplementation may have also affected the rumen fermentation of the basal diets as well as providing a microbial N source. Later incubations of mixtures of SBM and HSL (SBM *c*. 10%) and LSL (SBM *c*. 13%) *in vitro* in the proportions actually consumed showed higher PF values for the mixtures (3·41 mg/ml in the LSL + SBM and 3·24 mg/ml in the HSL + SBM incubation) than for the stover leaves alone (see Table 1). Since these incubations were also conducted in buffer medium containing 7 mg of buffer N, the higher PF values may have resulted from increased microbial efficiencies through the amino acids and peptides supplied by the SBM.

Even though the LSL group consumed 0·35 g/day more nitrogen than the HSL group, daily N losses were higher by 0·66 g in the LSL group. More microbial protein was produced in this group and more absorbed microbial protein may have been used to provide energy for maintenance requirements in the LSL group which would result in higher urinary N losses due to deamination. A higher PF value indicates proportionally higher microbial biomass yield and consequently lower SCFA production, and consequently less energy was provided by SCFAs in the LSL group. Thus, in feeding situations below the maintenance requirements, high PF values might not be beneficial.

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