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Supplementation of barley straw with *Sesbania pachycarpa* leaves *in vitro*: effects on fermentation variables and rumen microbial population structure quantified by ribosomal RNA-targeted probes

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Tropical livestock is often maintained on roughage-based diets deficient in N, and therefore requires supplementation with protein-rich substrates to achieve reasonable production levels. The optimum inclusion rate of a potential supplement is usually determined by *in vivo* feeding trials or by *in vitro* incubation of the diet components to estimate the feed value of the complete diet. The present work simulates a supplementation experiment *in vitro*, by incubating a pure roughage (barley straw), a pure supplement (*Sesbania pachycarpa* leaves) and mixtures of the two, with increasing inclusion levels of the supplement, in a short-term batch incubation system. Fermentation kinetics were followed by the release of fermentation endproducts (gas and short-chain fatty acids). Microbial biomass was estimated using ribosomal (r) RNA as internal marker for bacteria and eukaryotes separately. Cell-wall-degrading subpopulations were quantified by hybridisation with taxon-specific oligonucleotide probes targeting *Chytridiomycetes*, *Fibrobacter* spp., *Ruminococcus albus* and *R. flavefaciens*. Carboxymethylcellulase (CMCase) was assayed as an indicator for cell-wall-degrading activity. The addition of *S. pachycarpa* leaves stimulated fermentation in all cases. Gas production, and especially rRNA concentration, showed clear maxima at 40 % *S. pachycarpa* inclusion, rates that significantly exceeded the values interpolated from the incubations of the pure substrates. Short-chain fatty acid yield changed only slightly, but in the same way. The analysis of the microbial population structure showed that the positive effects were mainly mediated through enhanced growth of *Ruminococcus* spp. Increasing proportions of *S. pachycarpa* leaves in the diet led to a drastic decline in the total eukaryotic population. This points to a defaunation, which may also have added to the positive effects. The eukaryotic subpopulation of the rumen fungi were affected to a lesser degree. Although the cell-wall-degrading organisms showed positive responses to the supplementation, the CMCase activity was not affected significantly by the supplementation. The present work shows that it is possible to predict optimum inclusion levels for a new feed supplement *in vitro* and thus reduce *in vivo* experiments. It was also demonstrated that true supplementation effects occur particularly for the microbial biomass production, which is the primary source of amino acids for the ruminant animal. The analysis of microbial population structure in context with conventional metabolic measurements adds valuable information to interpret the observed effects on production-related variables.

Microbial biomass: Gas test: Ribosomal RNA probes

Cereal straws are an important source of feeds for ruminant animals in tropical countries, but their high cell-wall and low N contents limits the extent to which they can be fermented in the rumen, unless they are fed with a supplement. Agroindustrial by-products, such as brans or oil cakes, are widely used for supplementation, but their availability may be restricted, especially in rural areas. Naturally available sources of N in these regions, such as legumes, trees, shrubs or weeds, have been shown to increase animal performance *in vivo* (Odenyo *et al*. 1997; Khandaker *et al*. 1998), but feeding trials are laborious and time consuming, and large amounts of substrate are

Abbreviations: CMCase, carboxymethylcellulase; r, ribosomal; SCFA, short-chain fatty acid.

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needed. An initial screening *in vitro*, however, was employed to determine the 'feed value' of the supplement by incubating it as pure substrate.

An *in vitro* fermentation system, such as the Hohenheim gas test (Menke *et al*. 1979), can be applied in a much more versatile way. Besides the quantitative recovery of gas and short-chain fatty acids (SCFA), it allows the determination of the microbial biomass produced (Blümmel & Becker, 1997; Blümmel *et al*. 1997*a*). The rate of fermentation can be calculated quite easily (Wood & Manyuchi, 1997) and yields information about the potential intake of the test materials (Blümmel *et al*. 1996, 1997*b*). Sequential withdrawal of samples using parallel syringes and sampling for various biochemical and physiological assays adds kinetic information to any desired variable. It should thus be possible to test different supplementation strategies *in vitro* by incubating not only the pure components, but also the entire diet. The concept of supplementation implies that the supplement not only adds some nutrients to a poor substrate, but through the stimulation of the fermentation, also improves the utilisation of the substrates, so that the output of the combination is greater than the sum of the parts. In an *in vitro* system the pure substrates can be incubated as controls, and this 'sum of the parts' can be calculated as a reference value against which the measured output of the combinations can be evaluated. The results generated by this approach will predict an optimum inclusion level of the supplement and will allow greater precision in subsequent *in vivo* experiments. However *in vitro* methods are no replacement for *in vivo* feeding trials, because they cannot mimic the total tract digestibility or the influence of secondary plant metabolites on the animal.

From a practical, production-oriented point of view, it may be sufficient to determine the conditions for the highest increase in yield of endproducts; nevertheless, the underlying biological effects that bring about this increase are of great scientific interest. The advantages of the *in vitro* system described earlier also allow study of the effects of different substrates on the rumen microbial ecosystem (Dryhurst & Wood, 1998). Most published studies on the rumen microbial ecosystem have been based on methods that use isolation techniques and select for cultivable organisms. The hybridisation of ribosomal (r) RNA with taxonomic nucleic acid probes provides a new way of tracking rumen micro-organisms at different taxonomic levels within a complex microbial ecosystem (Olsen *et al*. 1986; Sayler & Layton, 1990; Raskin *et al*. 1995). So far, only a few studies have been done on rumen microbial ecology using these molecular techniques. Most researchers have worked on *Fibrobacter* spp., which is probably the best-examined group of rumen microbes. In an early experiment, Stahl *et al*. (1988) examined the influence of monensin on *Fibrobacter* spp. and *Lachnospira* spp. Results showed that the technique is suitable for detecting induced changes in the community structure. Briesacher *et al*. (1992) examined the proportion of *Fibrobacter* spp. within different compartments of the rumen, and members of the same group also worked on the effects of different N sources on *Fibrobacter* spp. (May *et al*. 1993). They reported marked inter-animal differences, and a strong influence of animal diet and time after feeding when the rumen contents were collected. Other rumen cell-wall-degrading organisms, like *Ruminococcus* spp., have only been addressed in a single study, by Krause *et al*. (1999), who followed the succession of the *Ruminococcus* population in immature lambs. The results of Muetzel *et al*. (2001) indicated that the microbial composition of the inoculum used for *in vitro* incubation experiments influenced both substrate degradation and the population structure of cell-wall-degrading organisms during fermentation of various substrates.

In the present work, the available probes were compiled to trace supplement-induced shifts in the population structure at different taxonomic levels, with a focus on fibrolytic organisms. The present study combines the quantitative approach for the determination of the optimum inclusion rate of *Sesbania pachycarpa* leaves on barley straw with a detailed analysis of the microbial population composition, endproduct formation and fibrolytic enzyme activity supported by the test diets. The aim was to demonstrate that supplementation does not only mean that the results are the sum of the individual components, but to show a true supplementary effect.

Experimental methods

The substrates incubated *in vitro* were a Syrian variety of barley straw (var. *Abiad*) grown in 1994 in Hohenheim, Germany, and *S. pachycarpa* leaves, obtained from the International Livestock Research Institute, Niamey, Niger. Air-dried samples were ground to pass a 1 mm sieve. DM and crude protein (N × 6·25) contents were determined according the methods of the Association of Official Analytical Chemists (1990). Fibre fractions were quantified by the method of Goehring & Van Soest (1970). Proximate composition and the fibre fractions are given in Table 1.

Saponins were extracted from an aqueous extract (30 mg/ml) of finely ground *S. pachycarpa* and *S. sesban* leaves. The suspension was then centrifuged (3500 ***g***, 15 min, 20°C). The supernatant fraction was collected

Table 1. Crude nutrient content and fibre fractions (g/kg DM) of the two substrates*

Substrates	Crude ash	Crude protein (N × 6·25)	Ethanol-insoluble residue	Neutral-detergent fibre	Acid-detergent fibre
Barley straw	68	42	895	724	577
Sesbania pachycarpa leaves	82	237	721	321	215

* Crude nutrients were determined according to the official methods of the Association of Official Analytical Chemists (1990) and fibre fractions according to Goehring & Van Soest (1970).

and an equal volume of *n*-butanol was added. The solution was stirred for 30 min and incubated overnight at room temperature for phase separation. The butanol phase was recovered and the butanol extraction was repeated. The butanol was evaporated from the combined fractions by flushing with N_2. The dried saponins were dissolved in distilled water (10 mg/ml). A sample of the solution (2 μl) was blotted on TLC plates covered with silica gel. Saponins were separated using chloroform–methanol–water (12:3:11, by vol.) as the mobile phase. Saponins were visualised by spraying the plates with concentrated sulfuric acid–ethylacetate–ethanol (5:5:9, by vol.). Saponins occur as violet spots (Fig. 1(a)). A separate plate was sprayed with sheep blood diluted 1:15 with PBS.

In vitro incubations were carried out in the Hohenheim gas test system according to Menke *et al*. (1979), as modified by Blümmel *et al.* (1997*b*). Rumen contents were obtained from a fistulated Hinterwaelder cow fed a hay–straw mixture at maintenance level. Barley straw and *S. pachycarpa* leaves were incubated as sole substrates and at four supplementation levels of barley straw–*S. pachycarpa* leaves (9:1, 8:2, 6:4 and 4:6 respectively). Substrate (12·5 mg/ml) was combined with 20 ml anaerobic medium and 10 ml rumen contents, strained through a 100 μm nylon net. Three independent *in vitro* incubations were performed with eight subreplicates each. At 3, 6, 9, 12, 18, 24, 36 and 48 h of incubation, gas production was recorded and a sample (one syringe-full, 30 ml) was removed for sampling. Three aliquots of 0·3 ml were collected from the whole incubation contents for the extraction of RNA and stored at −80°C until analysis. A sample of the remaining material (20 ml) was centrifuged (20 000 ***g***, 10 min, 4°C), and the supernatant fraction was recovered and stored at −20°C for the quantification of SCFA concentration. The pellet was used for the determination of carboxymethylcellulase (CMCase) activity.

Quantification of SCFA was by GC as described by Holtershinken *et al*. (1997). CMCase activity was determined by the release of reducing sugars as described in Groleau & Forsberg (1981).

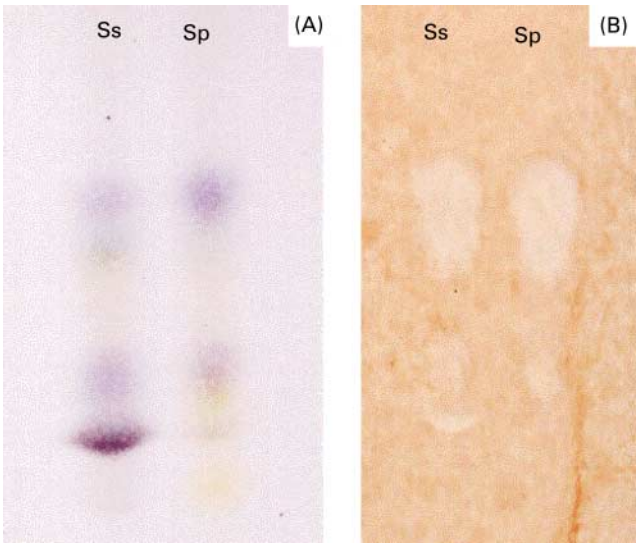

Fig. 1. Thin-layer chromatograms of saponins from *Sesbania sesban* (Ss) and *S. pachycarpa* (Sp) (A) leaves and their haemolytic activities (B). For details of procedures, see pp. 446–448.

Total RNA was extracted by a modification (Muetzel & Becker, 2002) of the low pH hot phenol extraction procedure described by Stahl *et al*. (1988). To 0·3 ml sample, 1 g zirconia silica beads, 0·6 ml phenol (pH 5·1), 0·27 ml buffer (50 mM-sodium acetate, 10 mM-EDTA, pH 5·1) and 30 μl SDS (200 g/l) were added. Samples were placed in a Beadbeater-8 (Biospec Products Inc., Bartlesville, OK, USA) and shaken at 50 Hz for 2 min. Samples were placed into a water-bath (60°C) for 10 min and the beating was repeated. After cooling on ice, 0·3 ml $CHCl_3$ was added and samples were shaken vigorously. Aqueous and organic phases were separated by centrifugation (10 000 ***g***, 5 min, 4°C), and the aqueous phase was transferred into a new vial containing 0·3 ml 7·5 M-ammonium acetate and 0·9 ml isopropanol. Nucleic acids were precipitated at −20°C overnight and recovered by centrifugation (16 000 ***g***, 10 min, 4°C). They were washed with 1 ml aqueous ethanol (800 ml/l), dissolved in 100 μl double-distilled water and stored at −80°C.

Slot blot hybridisations with radioactive-labelled oligonucleotide probes were performed according to the protocol of Stahl *et al*. (1988). The probes were custom synthesised by Amersham Pharmacia Biotec (Freiburg, Germany) and targeted total 16S rRNA (Zheng *et al*. 1996), the bacteria (Amann *et al*. 1990), the eukarya (Hicks *et al*. 1992), *Fibrobacter* spp. (Stahl *et al*. 1988), *Ruminococcus albus* and *R. flavefaciens* (Odenyo *et al*. 1994), and *Chytridiomycetes* (Dore *et al*. 1993) (Table 2). Approximately 100 ng total RNA was blotted on nylon membranes (Magna Charge; Micron Separation Inc., Westboro, MA, USA) under slight vacuum, using a Minifold II™ slot blotter (Schleicher and Schuell, Horb, Germany). Each membrane also contained a series of known concentrations of a reference organism for the respective probe. Membranes were baked (80°C, 1 h), and placed in 400 mm hybridisation bottles. For each membrane, 3 ml hybridisation buffer (0·9 M-NaCl, 50 mM-sodium phosphate, 5 mM-EDTA, 10 × Dehnhardts solution (2 g/l Ficoll 400, 2 g/l polyvinylpyrrollidone, 2 g/l BSA fraction V), 5 mg SDS/ml and 0·05 mg poly A/ml) was added and the bottles were incubated for 1 h at 40°C in a hybridisation oven. Probes were labelled with γ-adenosine [^{32}P]triphosphate using a T4 polynucleotide kinase (Amersham Pharamcia Biotech), purified with spin columns (QIA Quick spin, Qiagen GmbH, Hilden, Germany) according to the manufacturer's specifications and added to hybridisation buffer (3 ml per membrane). Prehybridisation buffer was discarded, the labelled probes were added to the membranes and hybridised at 40°C for 16 h. The hybridisation buffer was discarded and the membranes were washed for 15 min in 400 ml wash solution (0·15 M-NaCl, 0·015 M-sodium citrate and 10 g SDS/l) at the specified wash temperature (Table 2). Membranes were transferred into 400 ml fresh wash solution for another 15 min. Air-dried membranes were exposed to phosphor imaging screens (Fuji Photo Film Co., Ltd, Tokyo, Japan) to quantify the retained labelled probe using a phosphor imager (BAS 1000; Fuji Photo Film Co., Ltd). Image analysis was

Table 2. Target organisms, nucleotide sequence and wash temperature of the oligonucleotide probes used for membrane hybridisation

Target organisms	Sequence (5′–3′)	Wash temperature (°C)
All organisms	GAC GGG CGG TGT GTA CAA	44
Bacteria	GCT GCC TCC CGT AGG AGT	54
Eukarya	TAC AAA GGG CAG GGA C	42
Chytridiomycetes	GTA CAC ACA ATG AAG TGC ATA AAG G	43
Fibrobacter spp.	AAT CGG ACG CAA GCT CAT CCC	56
Ruminococcus albus	GTC AAC GGC AGT CCT GCT A	46
Ruminococcus flavefaciens	AAC GGC AGT CCC TTT AG	46

done using TINA 2·09 (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

Since the pure roughage and the pure supplement were incubated as controls, the expected concentration of end-products, or of total or group-specific rRNA, was calculated by multiplication of the data from the pure substrates with their inclusion levels. These interpolated values were then compared with the measured values from the incubations of the substrate mixtures. Data were analysed by a one-way ANOVA using the SAS package (SAS Institute Inc., Cary, NC, USA), treating the individual sampling times as repeated measurements. Values presented are means over the whole incubation period.

Results

Besides a higher protein and lower cell-wall content (Table 1) than barley straw, *S. pachycarpa* leaves are characterised by the presence of saponins. The presence of saponins in *S. pachycarpa* was shown by staining the chromatography plates (Fig. 1(a)). Its haemolytic activity was shown by the clearing zones on a separate plate after spraying with blood cells (Fig. 1 (b)).

During *in vitro* incubation gas and SCFA production for both substrates, i.e. barley straw and *S. pachycarpa* leaves, gas and SCFA production followed a sigmoidal curve that is typical for cumulative variables (Fig. 2 (a and b)). *S. pachycarpa* leaves were fermented more rapidly than barley straw, but more gas was produced from barley straw. The amount of SCFA produced after 48 h of incubation was similar for both feeds. Fatty acid composition varied only slightly. Acetate, propionate and butyrate proportions were 0·632, 0·259 and 0·082 for barley straw and 0·634, 0·262 and 0·047 for the *S. pachycarpa* leaves respectively. The fermentation of *S. pachycarpa* leaves led to an increase in minor SCFA

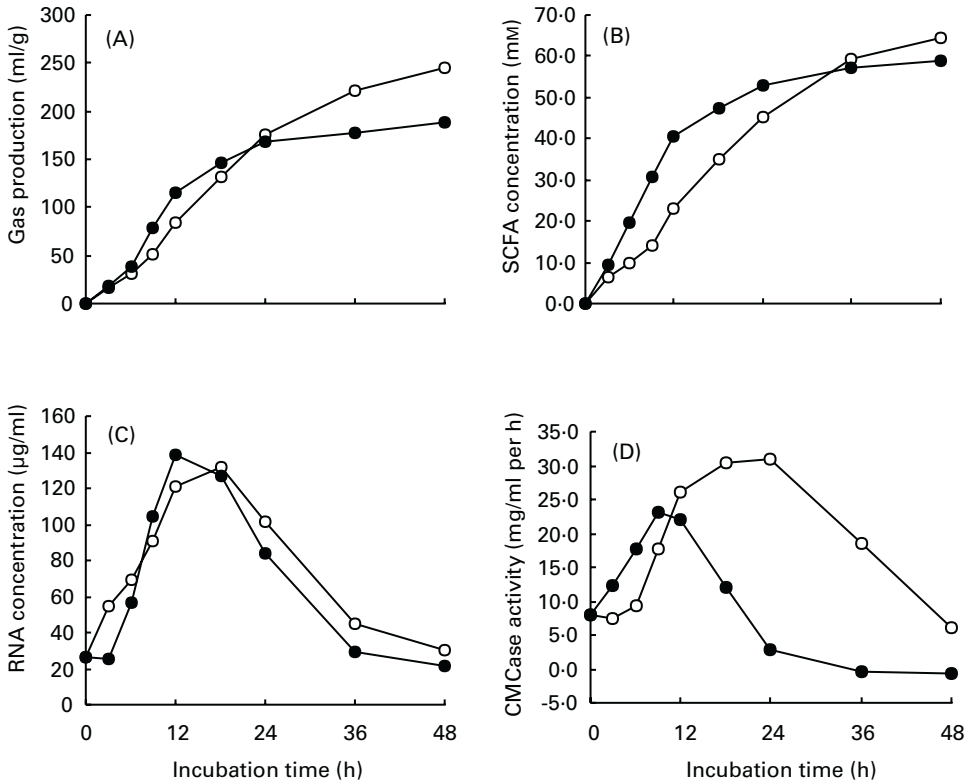

Fig. 2. Gas production (A), short-chain fatty acid (SCFA) production (B), RNA concentration (C) and carboxymethylcellulase (CMCase) activity (D) in barley straw (○) and *Sesbania pachycarpa* leaves (●) incubated in an *in vitro* batch culture with rumen contents. For details of procedures, see pp. 446–448.

(valerate, isobutyrate and isovalerate) concentrations at the expense of butyrate.

RNA concentration and CMC activity (Fig. 2(c and d)) produced bell-shaped curves in which concentration or activity increased to a definite maximum and then declined, indicating microbial growth followed by subsequent death and lysis. Although RNA concentration looked very similar for both substrates, the maximum concentration was reached at about 12 h with *S. pachycarpa* leaves, while the peak concentration with barley straw as substrate occurred at about 18 h.

Results for the mixed substrates are shown in Table 3. Some stimulatory effect of the *S. pachycarpa* inclusion on endproduct formation, microbial RNA yield, and CMCase activity was observed in almost all tested samples. Statistical significance was reached for gas production at the 8:2 and 6:4 ratios, where the measured results were 4·3 and 5·6 % greater than the interpolated reference values. The strongest effect occurred for total RNA concentrations, where the increase of the measured over the calculated value was 12·6 and 14·7 % at the 8:2 and 6:4 mixing ratios respectively, suggesting that the optimum inclusion level of *S. pachycarpa* leaves is between 20 and 40 %. Although not statistically significant, the CMCase activity was increased by 5·0 % at an inclusion level of 8:2, but did not show any increase at the 6:4 level, where microbial population, gas and SCFA production showed a maximum response.

When total RNA was quantified separately for bacteria and eukaryotes, it was evident that the positive effects of supplementation were mainly due to an increase in the bacterial population (Table 4). In the barley control and at the two lower *S. pachycarpa* inclusion rates, eukaryotic RNA concentration followed a bell-shaped curve with a maximum at 24 h (Fig. 3). At mixing ratios exceeding 8:2, however, it decreased in a dose-dependent manner to a level where no eukaryotic activity was detected when *S. pachycarpa* leaves were the only substrate.

Two groups of eukaryotes occur in the rumen, i.e. the rumen fungi (*Chytridiomycetes*) and the protozoa. Only the subpopulation of the *Chytridiomycetes* could be quantified with a separate probe (Fig. 4). The inhibition of fungal growth did not show a strict dose-dependent decline as observed for the total eukaryotic population. Nevertheless, when *S. pachycarpa* was incubated as the sole substrate, no fungal growth was observed. Generally, the *Chytridiomycetes* showed growth kinetics completely different from the total eukaryotes, with an increase up to 48 h of incubation and no definite peak in concentration. Since no probe for protozoa is available, information about this group of rumen organisms can be inferred only indirectly by subtracting fungal RNA concentrations from the total eukaryotic RNA concentration. The results show that after 48 h of incubation, the eukaryotic population was made up exclusively of the rumen fungi.

The *Chytridiomycetes* are known for their high activity in cell-wall breakdown. Within the bacterial population, three abundant fibrolytic taxa were quantified with separate probes, i.e. the genus *Fibrobacter* and two species of the genus *Ruminococcus*.

Table 5 shows the effects of increasing *S. pachycarpa* ratios on the respective population sizes. Again, an increase over the calculated reference values was observed in nearly all cases. For the *Chytridiomycetes* the effect was great in relative terms, but did not reach statistical significance. *Fibrobacter* spp., the most abundant bacterial cell-wall-degrading species, was affected to only a minor extent by the supplementation with *S. pachycarpa* leaves. The response of this organism was even less than that of the whole population. The greatest effect of supplementation was observed for the two *Ruminococcus* spp. Both species showed a statistically significant increase

Table 3. Differences in observed and interpolated values for different rumen *in vitro* fermentation variables of barley straw–*Sesbania pachycarpa* leaves at different inclusion levels†

Variable	Inclusion level‡	Interpolated	Observed	Difference (%)	Statistical significance of difference
Gas production (ml/g)	9:1	118·9	122·4	3·2	NS
	8:2	118·6	123·4	4·3	*
	6:4	117·9	124·2	5·6	*
	4:6	117·3	122·1	4·4	NS
SCFA concentration (mM)	9:1	32·9	33·8	2·6	NS
	8:2	33·6	34·5	2·5	NS
	6:4	35·1	36·2	3·1	NS
	4:6	36·6	37·6	2·8	NS
RNA concentration (μg/ml)	9:1	79·8	84·7	6·2	NS
	8:2	79·1	89·1	12·6	NS
	6:4	77·6	89·0	14·7	*
	4:6	76·2	85·6	12·4	NS
CMCase activity (mg/ml per h)	9:1	17·6	18·2	3·3	NS
	8:2	16·9	17·8	5·0	NS
	6:4	15·5	15·5	0·2	NS
	4:6	14·0	14·0	−0·4	NS

SCFA, short-chain fatty acid; CMCase, carboxymethylcellulase.
* $P < 0·05$.
† For details of procedures, see pp. 446–448.
‡ Barley straw–*Sesbania pachycarpa* leaves.

Table 4. Differences in observed and interpolated values of bacterial and eukaryotic RNA concentration during *in vitro* incubation with barley straw–*Sesbania pachycarpa* leaves at different inclusion levels†

Variable	Inclusion level‡	Interpolated (μg/ml)	Observed (μg/ml)	Difference (%)	Statistical significance of difference
Bacteria	9:1	66·7	70·4	5·5	NS
	8:2	67·0	75·0	12·0	*
	6:4	67·5	77·9	15·4	*
	4:6	68·0	77·3	13·7	*
Eukaryotes	9:1	10·2	11·2	9·7	NS
	8:2	9·3	10·8	16·5	NS
	6:4	7·3	7·5	2·9	NS
	4:6	5·4	5·0	−7·1	NS

* $P<0·05$.
† For details of procedures, see pp. 446–448.
‡ Barley straw–*Sesbania pachycarpa* leaves.

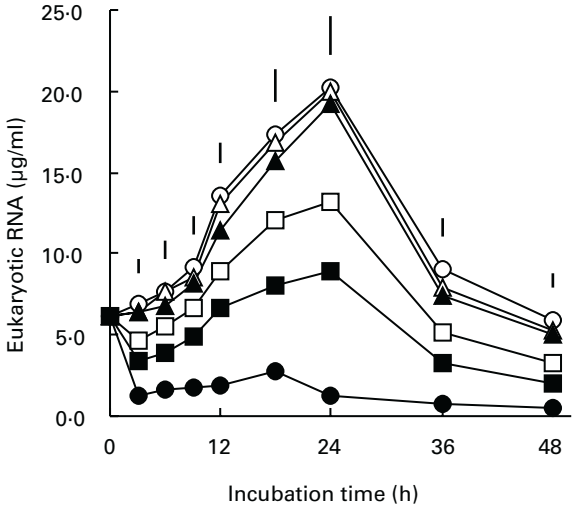

Fig. 3. Eukaryotic RNA concentration during 48 h *in vitro* incubations of barley straw (○), *Sesbania pachycarpa* leaves (■) or four mixtures (barley straw–*S. pachycarpa* leaves, 9:1 (●), 8:2 (△), 6:4 (▲), 4:6 (□) of the substrates. For details of procedures, see pp. 446–448. Values are means for three individual incubations with their standard errors of the differences (SED) for all data points at the given time, shown by vertical bars.

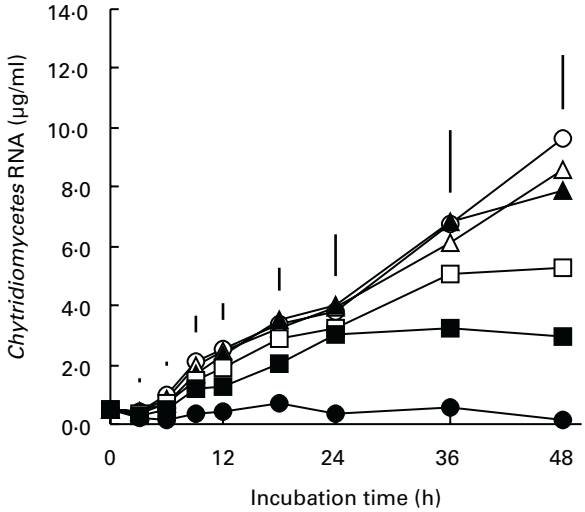

Fig. 4. *Chytridiomycetes* RNA concentration during 48 h *in vitro* incubations of barley straw (○), *Sesbania pachycarpa* leaves (■) or four mixtures (barley straw–*S. pachycarpa* leaves, 9:1 (●), 8:2 (△), 6:4 (▲), 4:6 (□) of the substrates. For details of procedures, see pp. 446–448. Values are means for three individual incubations with their standard errors of the differences (SED) for all data points at the given time, shown by vertical bars.

(42·7 and 34·5 %) at the 6:4 mixing ratio. This coincides with the optimum conditions for most of the other variables shown earlier.

Discussion

The fermentation endproducts, gas and SCFA, showed a very moderate response to the addition of the *S. pachycarpa* leaves. Even though gas and SCFA production are stochiometrically linked, according to Wolin (1960), these two variables differed markedly for the two pure substrates (Fig. 1). The slight differences in SCFA composition cannot fully explain this observation. The fermentation of protein supplied with the *S. pachycarpa* leaves would lead to greater SCFA and reduces gas production compared with carbohydrate fermentation (Groot *et al*. 1998; Cone & van Gelder, 1999), and therefore is the most likely explanation for the observed differences.

The strongest effect of supplementation was recorded for microbial biomass, estimated by total rRNA concentration. The results presented earlier were derived from hybridisation with the universal probe targeting small subunit rRNA from any organism. Estimation of rumen microbial biomass is problematic, since an ideal marker that occurs in all organisms at the same concentration and does not occur in the plant material is not available. Nucleic acids, however, have been successfully used as a marker to estimate microbial biomass. The concentration of nucleic acid in bacteria determined, however, varied between 4·0 % (Merry & McAllan, 1983), 11·2 % (John, 1984) and as high as 24·2 % (Hespell & Bryant, 1979). There is also a major difference in the nucleic acid content of bacteria and protozoa. Rumen bacteria contain 8·5 (SD 1·2) % RNA, whereas protozoa contain only 4·0 (SD 0·3) % RNA (Robinson *et al*. 1996). The nucleic acid content of a cell also varies with the growth stage. Dormant or starving cells contain much less RNA than growing cells (Nierlich, 1978) and there is discussion about the use of RNA as a marker for microbial biomass (Broudiscou & Jouany, 1995; Robinson *et al*. 1996). However, there

Table 5. Differences in observed and interpolated values of RNA concentrations (μg/ml rumen contents) of four cell-wall-degrading organisms during *in vitro* incubation with barley straw–*Sesbania pachycarpa* leaves at different inclusion levels†

Variable	Inclusion level‡	Interpolated (μg/ml)	Observed (μg/ml)	Difference (%)	Statistical significance of difference
Chytridiomycetes	9:1	3·37	3·53	4·8	NS
	8:2	3·03	3·83	26·3	NS
	6:4	2·36	2·87	21·2	NS
	4:6	1·70	1·94	14·3	NS
Fibrobacter spp.	9:1	8·72	8·73	0·1	NS
	8:2	8·35	8·68	3·9	NS
	6:4	7·61	8·18	7·4	NS
	4:6	6·88	7·57	10·1	NS
Ruminococcus albus	9:1	6·30	7·12	13·2	NS
	8:2	6·09	7·76	27·4	NS
	6:4	5·68	8·10	42·7	*
	4:6	5·26	7·31	38·9	NS
Ruminococcus flavefaciens	9:1	4·15	4·53	9·1	NS
	8:2	4·17	5·22	25·0	**
	6:4	4·22	5·68	34·5	**
	4:6	4·27	5·40	26·6	NS

*$P<0·05$, **$P<0·01$.
† For details of procedures, see pp. 446–448.
‡ Barley straw–*Sesbania pachycarpa* leaves.

are specific advantages of the use of intact ribosomal RNA as a microbial marker. In contrast to purines, there is no background from air-dried plant material, since RNA is rapidly degraded during the drying process. Bacterial and eukaryotic RNA can be quantified easily by densitometry after separation of the nucleic acids on polyacrylamide or agarose gels. Such a separation is also of nutritional importance to the ruminant animal, since protozoa contain a higher proportion of amino acids (Volden *et al*. 1999). The different RNA concentrations of bacteria and eukaryotes have also implications for the absolute amount of microbial biomass estimated. Applying the RNA concentrations proposed by Robinson *et al*. (1996) to the values measured for bacteria and eukaryotes, the maximum microbial yield is reached with the 8:2 inclusion level (Table 6). Neglecting the differences in RNA concentration for bacteria and eukaryotes indicates that there is no difference in microbial mass between the 8:2 and 6:4 inclusion levels.

The fact that supplementation increased the microbial biomass production to a greater degree than SCFA production indicates an increase of the efficiency of microbial growth through the supplementation. This effect could have been triggered by an increased amino acid supply from the *S. pachycarpa* leaves that stimulated the microbial growth (May *et al*. 1993; Atasoglu *et al*. 1999). The defaunating effect of *S. pachycarpa* leaves may be due to saponins present (Fig. 1) and may have been also contributed to the increased microbial efficiency due a decrease in protozoal predation as shown by Newbold *et al*. (1997) for *S. sesban* saponins. At greater levels, *S. pachycarpa* leaves also negatively influenced the growth of the anaerobic rumen fungi (Fig. 4), which are a major group of cell-wall-degrading organisms in the rumen. They can physically penetrate plant cell walls and possess very active cellulases (Li & Heath, 1993; Varga & Kolver, 1997). The reduced growth of anaerobic rumen fungi might have also contributed to the lack of a positive response of the CMCase activity.

In the present study, the enzyme CMCase was assayed as an indicator of fibrolytic activity (Huhtanen *et al*. 1998). This physiological variable was chosen, rather

Table 6. Estimates of *in vitro* rumen microbial biomass (mg/ml rumen contents) using separate RNA concentrations in bacteria and eukaryotes or only one marker for both the groups of organisms†

Substrate	Inclusion level‡	Separate markers§	One marker‖
Barley straw		1·07	0·92
Straw–leaves	9:1	1·11	0·97
Straw–leaves	8:2	1·16	1·01
Straw–leaves	6:4	1·11	1·01
Straw–leaves	4:6	1·04	0·97
Sesbania pachycarpa leaves		0·85	0·83

† For details of procedures, see pp. 446–448.
‡ Barley straw–*Sesbania pachycarpa leaves*.
§ 85 mg RNA/g bacterial and 40 mg RNA/g protozoal DM (Robinson *et al*. 1997).
‖ 85 mg RNA/g micro-organism DM applied to total RNA concentration.

than end-point digestibility, because it also yields kinetic information that could be related to the kinetics of endproducts and microbial growth. CMCase activity showed marked differences between the plants, which reflect the differences in cell-wall content of the two substrates (Table 1), but did not positively respond to the supplementation. However, the determination of only one cellulolytic enzyme activity does not necessarily reflect the degradation of the whole plant cell-wall, especially since *S. pachycarpa* leaves contain high levels of pectin, as indicated from the difference between the ethanol-insoluble residue and the neutral-detergent fibre fraction. Therefore, the decrease in cellulose content could be the reason for the lack of a positive response of the CMCase activity, which contrasts with the overall increase of the fibrolytic organisms. The difference in cell-wall composition may also explain the different response of the cell-wall-degrading organisms. *Fibrobacter*, a major cellulose-degrading organism (Malburg & Fosberg, 1993) did not respond to the supplementation with *S. pachycarpa* leaves. The decrease in cellulose and xylan (primary substrates for *Fibrobacter* spp.) contents of the diet (Stewart & Bryant, 1997) also partly explains the lack of a response of this organism to the supplementation. Growth of *Ruminococcus* spp., especially *R. flavefaciens*, on the other hand, was clearly positively affected by the supplementation. In contrast to *Fibrobacter* spp., *Ruminococcus* spp. have a wider substrate spectrum (Stewart & Bryant, 1997) and can also utilise pectin (Pettipher & Latham, 1979). As pectin concentration increased with the inclusion of *S. pachycarpa* leaves, pectin fermentation would explain the positive effect on *Ruminococcus* spp. Since pectinase activity was not assayed, no definite conclusions about the metabolic effects of the increased *Ruminococcus* spp. can be made.

In summary, the molecular evidence suggests that defaunation and an enhancement of *Ruminococcus* spp. due to pectin fermentation were two key mechanisms that mediated the observed supplementation effect. Results indicate that *in vitro* systems have the potential to predict an optimal supplementation strategy. It was also demonstrated that the combination of the substrates leads to true supplementation effects, with a higher biomass production than interpolated from the incubation of the pure substrates. The present results show that the interpolation of the results obtained from the incubation of pure substrates *in vitro* may underestimate the microbial biomass production of the whole diet. Although no *in vivo* validation was given in the context of the present study, the *in vitro* results are conclusive and correspond well with the work of Bonsi *et al.* (1995) and Khandaker *et al.* (1998). They reported significantly enhanced DM degradation and feed intake in sheep when *S. sesban* leaves were supplemented with teff (*Eragrostis tef*) and wheat straw respectively. The analysis of microbial population structure in combination with endproduct analysis and enzymatic activities adds valuable information on how the observed effects may be mediated and will eventually help to elucidate the complex relationships between substrate composition and the endproducts of fermentation.

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References

Amann RI, Krumholz L & Stahl DA (1990) Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology. *Journal of Bacteriology* **172**, 762–770.

Association of Official Analytical Chemists International (1990) *Official Methods of Analysis*, 15th ed., Arlington, VA: AOAC.

Atasoglu C, Valdes C, Newbold CJ & Wallace RJ (1999) Influence of peptides and amino acids on fermentation rate and *de novo* synthesis of amino acids by mixed micro-organisms from the sheep rumen. *British Journal of Nutrition* **81**, 307–314.

Blümmel M & Becker K (1997) The degradability characteristics of fifty-four roughages and roughage neutral-detergent fibres as described by *in vitro* gas production and their relationship to voluntary feed intake. *British Journal of Nutrition* **77**, 757–768.

Blümmel M, Makkar HS & Becker K (1997*a*) *In vitro* gas production – a technique revisited. *Journal of Animal Physiology and Animal Nutrition* **77**, 24–34.

Blümmel M, Paul C, Goodchild A & Becker K (1996) Grinding energy and in vitro gas technique for the assessment of Syrian barley straws – physical and microbial degradation and voluntary feed intake by sheep. *Journal of Animal Physiology and Animal Nutrition* **76**, 132–140.

Blümmel M, Steingass H & Becker K (1997*b*) The relationship between *in vitro* gas production, *in vitro* microbial biomass yield and ^{15}N incorporation and its implications for the prediction of voluntary feed intake of roughages. *British Journal of Nutrition* **77**, 911–921.

Bonsi MLK, Osuji PO, Tuah AK & Umunna NN (1995) Intake, digestibility, nitrogen balance and certain rumen characteristics of Ethiopian Menz sheep fed teff straw supplemented with cotton seed cake, dry *Sesbania*, dry *Leucaena* or fresh *Leucaena*. *Agroforestry Systems* **31**, 243–256.

Briesacher SL, May T, Grigsby KN, Kerley MS, Anthony RV & Paterson JA (1992) Use of DNA probes to monitor nutritional effects on ruminal prokaryotes and *Fibrobacter succinogenes* S85. *Journal of Animal Science* **70**, 289–295.

Broudiscou L & Jouany JP (1995) Reassessing the manipulation of protein synthesis by rumen microbes. *Reproduction Nutrition Development* **35**, 517–535.

Cone JW & van Gelder AH (1999) Influence of protein fermentation on gas production profiles. *Animal Feed Science and Technology* **7**, 251–264.

Dore J, Brownlee AG, Millet L, Virlogeux I, Saigne M, Fonty G & Gouet P (1993) Ribosomal DNA-targeted hybridization probes for the detection, identification and quantitation of anaerobic rumen fungi. *Proceedings of the Nutrition Society* **52**, 176.

Dryhurst N & Wood CD (1998) The effect of nitrogen source and concentration on *in vitro* gas production using rumen microorganisms. *Animal Feed Science and Technology* **71**, 131–143.

Groleau D & Forsberg CW (1981) Cellulolytic activity of the rumen bacterium *Bacteroides succinogenes*. *Canadian Journal of Microbiology* **27**, 517–530.

Goehring HK & Van Soest PJ (1970) *Forage Fiber Analyses Agricultural Handbook*, no. 379. pp. 20–52. Washington, DC: USDA.

Groot JCJ, Williams BA, Oostdam AJ, Boer H & Tamminga S (1998) The use of cumulative gas and volatile fatty acid production to predict *in vitro* fermentation kinetics of Italian

ryegrass leaf cell walls and contents at various time intervals. *British Journal of Nutrition* **79**, 519–525.
Hespell RB & Bryant MP (1979) Efficiency of rumen microbial growth: Influence of some theoretical and experimental factors in Y_{ATP}. *Journal of Animal Science* **49**, 1640–1659.
Hicks RE, Amann R & Stahl DA (1992) Dual staining of natural bacterioplankton with 4′, 6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. *Applied and Environmental Microbiology* **58**, 2158–2163.
Holtershinken M, Plitt U, Tammen FC, Hoffmann P & Scholz H (1997) Influence of mouldy grass on fermentation and thiamine metabolism in bovine rumen fluid (*in vitro*). *Deutsche Tierarztliche Wochenschrift* **104**, 17–22.
Huhtanen P, Vanhatalo A & Varvikko T (1998) Enzyme activities of rumen particles and feed samples incubated *in situ* with differing types of cloth. *British Journal of Nutrition* **79**, 161–168.
John A (1984) Effects of feeding frequency and level of feed intake on chemical composition of rumen bacteria. *Journal of Agricultural Science* **102**, 45–57.
Khandaker ZH, Steingass H & Drochner W (1998) Supplementation of wheat straw with *Sesbania* on voluntary intake, digestibility and ruminal fermentation in sheep. *Small Ruminant Research* **28**, 23–29.
Krause DO, Smith WJM, Ryan FME, Mackie RI & McSweeney CS (1999) Use of 16S-rRNA based techniques to investigate the ecological succession of microbial populations in the immature lamb rumen: Tracking of a specific strain of inoculated *Ruminococcus* and interactions with other microbial populations *in vivo*. *Microbial Ecology* **38**, 365–376.
Li J & Heath IB (1993) Chytridiomycetous gut fungi, oft overlooked contributors to herbivore digestion. *Canadian Journal of Microbiology* **39**, 1003–1013.
Malburg LM & Fosberg CW (1993) *Fibrobacter succinogenes* S85 possesses at least nine distinct glucanase genes. *Canadian Journal of Microbiology* **39**, 882–891.
May T, Kerley MS & Williams JE (1993) Supplemental protein influences on carbohydrate degradation and bacterial 16S ribosomal ribonucleic acid. *Journal of Dairy Science* **76**, 3479–3489.
Menke KH, Raab L, Salewski A, Steingass H, Fritz D & Schneider W (1979) The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. *Journal of Agricultural Science* **93**, 217–222.
Merry RJ & McAllan AB (1983) A comparison of the chemical composition of mixed bacteria harvested from the liquid and solid fractions of rumen digesta. *British Journal of Nutrition* **50**, 701–709.
Muetzel S & Becker K (2002) An optimised RNA extraction protocol for rumen samples. *Reproduction Nutrition Development* **42**, Suppl. 1, S8.
Muetzel S, Krishnamoorthy U & Becker K (2001) Effects of rumen fluid collection site on microbial population structure during in vitro fermentation of different substrates quantified by 16S rRNA hybridisation. *Archives of Animal Nutrition* **55**, 103–120.
Newbold CJ, Elhassan SM, Wang J, Ortega ME & Wallace RJ (1997) Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. *British Journal of Nutrition* **78**, 237–249.
Nierlich DP (1978) Regulation of bacterial growth, RNA, and protein synthesis. *Annual Reviews in Microbiology* **32**, 393–432.
Odenyo AA, Mackie RI, Stahl DA & White BA (1994) The use of 16S rRNA-targeted oligonucleotide probes to study competition between ruminal fibrolytic bacteria: Pure-culture studies with cellulose and alkaline peroxide-treated wheat straw. *Applied and Environmental Microbiology* **60**, 3697–3703.
Odenyo AA, Osuji PO & Karanfil O (1997) Effect of multipurpose tree (MPT) supplements on ruminal ciliate protozoa. *Animal Feed Science and Technology* **67**, 169–180.
Olsen GJ, Lane DJ, Giovannoni J & Pace NR (1986) Microbial ecology and evolution: a ribosomal RNA approach. *Annual Review of Microbiology* **40**, 337–365.
Pettipher GL & Latham MJ (1979) Characteristics of enzymes produced by *Ruminococcus flavefaciens* which degrade plant cell walls. *Journal of General Microbiology* **110**, 21–27.
Raskin L, Amann RI, Poulsen LK, Rittmann BE & Stahl DA (1995) Use of ribosomal RNA based molecular probes for characterization of complex microbial communities in anaerobic biofilms. *Water Science and Technology* **31**, 261–272.
Robinson PH, Fadel JG & Ivan M (1996) Critical evaluation of diaminopimelic acid and ribonucleic acid as markers to estimate rumen pools and duodenal flows of bacterial and protozoal nitrogen. *Canadian Journal of Animal Science* **76**, 587–597.
Sayler GS & Layton AC (1990) Environmental application of nucleic acid hybridization. *Annual Review of Microbiology* **44**, 625–648.
Stahl OA, Flesher B, Mansfield HA & Montgomery L (1988) Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Applied and Environmental Microbiology* **54**, 1079–1084.
Stewart CS & Bryant MP (1997) The rumen bacteria. In *The Rumen Microbial Ecosystem*, pp. 10–72 [PN Hobson, editor]. London: Blackie Academic & Professional.
Varga GA & Kolver ES (1997) Microbial and animal limitations to fiber digestion and utilization. *Journal of Nutrition* **127**, 5-S.
Volden H, Harstad OM & Mydland LT (1999) Amino acid content and profile of protozoal and bacterial fractions isolated from ruminal contents of lactating dairy cows fed diets differing in nitrogen supplementation. *Acta Agriculturae Scandinavica* **49A**, 245–250.
Wolin MJ (1960) A theoretical rumen fermentation balance. *Journal of Dairy Science* **40**, 1452–1478.
Wood CO & Manyuchi B (1997) Use of an *in vitro* gas production method to investigate interactions between Veld hay and Napier hay or groundnut hay supplements. *Animal Feed Science and Technology* **67**, 265–278.
Zheng DD, Aim EW, Stahl OA & Aaskin L (1996) Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. *Applied and Environmental Microbiology* **62**, 4504–4513.