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Repeated ruminal dosing of *Ruminococcus* spp. does not result in persistence, but changes in other microbial populations occur that can be measured with quantitative 16S-rRNA-based probes

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Digestibility of fibre in ruminants may be improved by the introduction of highly fibrolytic strains of ruminal bacteria. This approach may be feasible if, for example, strains of *Ruminococcus* that are significantly more fibrolytic than the normal population of *Ruminococcus* are used for inoculation purposes. Introduced strains of bacteria, irrespective of ecosystem, often decline after inoculation, and in this study, highly fibrolytic strains of *Ruminococcus* were continuously dosed to ensure that measurements of fibre digestion were made in the presence of significant numbers of the introduced bacteria. During dosing the total culturable count increased significantly ($P < 0.05$), but declined post-dosing. The level of dosed *Ruminococcus*, and total *Ruminococcus*, *Fibrobacter succinogenes* and eukaryotes measured by 16S rRNA probes increased significantly ($P < 0.05$) during the dosing period, but also declined post-dosing. When *in vitro* nylon bag digestibility, feed intake or whole-tract digestibility was measured, no improvement could be measured.

Keywords: *Fibrobacter succinogenes*, eukaryotes, ecology, fibre, cellulose digestibility

INTRODUCTION

Cellulose is one of the most abundant biopolymers on earth and is an important structural component of the plant cell wall (Forsberg *et al*., 1997). Ruminants have developed a unique microbial symbiosis in the foregut (rumen) to utilize this plant tissue (Hungate, 1984), but fermentable fibre can often be recovered from the faeces (Beever *et al*., 1972; Ulyatt & MacRae, 1974). Therefore, it can be hypothesized that the microbial population in the rumen is limiting. The view is often put forward that the physical and chemical properties of bacterial cellulases and hemicellulases (e.g. specific activity), or the number of highly cellulolytic bacteria, may limit digestion (Forsberg *et al*., 1997). These, or similar reasons, are often used to justify research on recombinant cellulolytic ruminal bacteria (Weimer, 1996; Forsberg *et al*., 1997). However, few *in vivo* experiments have examined the feasibility of this approach by giving due consideration to the inherent ecological and physiological constraints imposed upon fibre digestion by the rumen ecosystem.

The observations that faeces may contain potentially fermentable fibre were supported by the work of Van Gylswyk (1970), who demonstrated that nutritional inadequacy could limit ruminal fibre fermentation. *Ruminococcus* (one of the most fibrolytic rumen bacteria; Hespell *et al*., 1997) numbers were increased when poor quality forage was supplemented with urea and branched-chain volatile fatty acids (BCVFA). There was an associated improvement in feed intake and some improvement in forage digestibility, a result which suggested that the abundance of fibrolytic bacteria in the rumen was limiting cell-wall digestibility (Van Gylswyk, 1970). In contrast, Dehority & Tirabasso (1998) increased the numbers of fibrolytic bacteria in the rumen

Abbreviations: BCVFA, branched-chain volatile fatty acids; DDMI, digestible dry matter intake; DMD, dry matter digestibility; DMI, dry matter intake; TCC, total culturable count.

by feeding a high-cellulose diet composed of purified wood cellulose. There was a 10-fold increase in the number of cellulolytic bacteria, but no significant increase in the digestion of alfalfa (lucerne) cellulose when it was placed in a nylon bag and suspended in the rumen for 24 h.

If fibre digestibility is to be improved by microbial manipulation, then two questions need to be answered. The first is whether it is the cellulolytic bacteria or the actual nature of plant cell walls that are the limiting factor to improving fibre digestion. A second, and very significant, issue is whether introduced organisms can multiply and persist at levels in the rumen that are sufficient to improve fibre digestion. To address these questions, we increased the relative abundance of highly fibrolytic *Ruminococcus* by daily dosing over a period of 8 d. Introduced strains of ruminal bacteria often decline rapidly after dosing (Flint *et al*., 1989; Miyagi *et al*., 1995; Attwood *et al*., 1988), making it difficult to measure the effect of the dosed strains on fibre digestion. We hoped that our protocol would enable us to measure fibre digestion in the presence of high numbers of dosed strains and that repeated dosing would enable inoculants to establish in the rumen.

We designed and characterized unique 16S-rRNA-based oligonucleotide probes to each of the dosed strains in order to track these organisms in the rumen. In addition, we used higher-level probes to *Ruminococcus*, *Fibrobacter* and eukaryotes to explore the effects of dosing on these populations. Various physiological parameters of fibre digestion [*in situ* nylon bag digestibility, whole-tract dry matter digestibility (DMD), dry matter intake (DMI) and digestible dry matter intake (DDMI)] were used to measure fibrolytic activity in the rumen.

METHODS

General. Two separate animal experiments were conducted with adult sheep fitted with rumen cannulas (McSweeney, 1989). In experiment 1, six sheep (three sheep in each of the dosed and control groups) were fed at 80% of *ad libitum* intake and feed was dispensed with an automatic feeder which divided the daily ration into eight equal portions fed at 3 h intervals. Rumen samples were collected for measurement of microbiological (culturable and molecular) and functional parameters of fibre degradation *in vitro*. Measurements of *in situ* nylon bag digestibility, DMI and DDMI were also made. In experiment 2, ten sheep were included in each of the control and dosed groups, but only measurements of whole-tract DMI, DMD and DDMI were made. These animals were fed on an *ad libitum* basis and there was a slight difference in the dosing protocol (see below). Animals in both experiments were fed a daily ration of low-quality rhodes grass (*Chloris gayana*; 4·4% crude protein), supplemented with minerals (Fermafos; Rumevite) and cotton seed meal (100 g; 37% crude protein).

Housing and feeding

Experiment 1. Adult Merino sheep (30–35 kg) were maintained in individual metabolism crates in a containment animal house. Six sheep divided into dosing and control groups were housed in metabolism cages in separate rooms. The two groups were completely isolated from one another (separate feed and water supply and ventilation system) to prevent contamination of controls by dosed sheep. Dosing and sample collection was always carried out on control animals first, followed by the dosed animals.

In situ nylon bag DMD was only measured in experiment 1 and during the last 24 h of each of the pre-dosing, dosing and post-dosing periods. Air-dried rhodes grass was ground through a 2 mm mesh screen and 3 g was weighed into a Dacron bag (19 × 9·5 cm, pore size 50 μm). A single steel marble was inserted and the bag tied off before being suspended in the rumen. Bags were removed from the rumen and washed together with the control bag (not suspended in the rumen) in water while gently squeezing until no colour was visible in the wash. Bags were then dried at 65 °C and *in situ* nylon bag digestibility determined by difference.

Experiment 2. Ten adult cannulated sheep were used in the control and dosed groups. The number of animals was greater than that in experiment 1 to increase the possibility of obtaining statistically significant results for DMI, DMD and DDMI. Rotary feeders were not used and animals received feed *ad libitum*. Diets were identical to those in experiment 1.

Sheep were fitted with rubber rings glued to wool around the anus to allow attachment of faecal collection bags (Raabe, 1968). Total faecal collections were made for 10 d, after a training period of 2 weeks to accustom sheep to the diet and daily faecal collection procedures. There were three intake and collection periods of 10 d each, designated pre-dosing, dosing and post-dosing. During the dosing period, sheep were dosed daily (500 ml of dose) through the rumen fistula, and feed was immediately offered to provide 150–200 g in excess of the amount eaten during the previous day. Calculations of DDMI and DMI were made during the three periods and data from days 1 and 2 of each period were excluded to allow for adaptation to treatment. Total faecal output was collected from each sheep for each period, dried at 65 °C for 48 h and weighed. DMI was calculated as the total intake of rhodes grass corrected for feed refusal and wastage.

Medium composition. Basal medium composition was (per litre): 150 ml clarified rumen fluid, 150 ml mineral solution A (contents per 100 ml: 3 g $K_2HPO_4.3H_2O$), 150 ml minerals solution B (contents per 100 ml: 3 g KH_2PO_4, 6 g $(NH_4)_2SO_4$, 6 g NaCl, 1·23 g $MgSO_4.7H_2O$, 1·58 g $CaCl_2.2H_2O$), 2 ml trace mineral salts [contents per 100 ml: 0·5 mg $ZnSO_4.7H_2O$, 0·15 mg $MnCl_2.4H_2O$, 1·5 mg H_3BO_3, 1·0 mg $CoCl_2.6H_2O$, 0·05 mg $CaCl_2.2H_2O$, 0·1 mg $NiCl_2.H_2O$, 0·15 mg $Na_2MO_4.2H_2O$, 7·5 mg $FeCl_2.4H_2O$), 3·1 ml volatile fatty acid solution (contents per 100 ml: 0·68 ml acetic acid, 0·3 ml propionic acid, 0·18 ml butyric acid, 0·05 ml isobutyric acid, 0·06 ml methylbutyric acid, 0·06 ml valeric acid, 0·06 ml isovaleric acid, 0·1 g phenylacetic acid), 1 g L-cysteine.HCl and 0·01% resazurin. Media were prepared anaerobically according to the methods of Hungate (1950) as modified by Bryant (1972). The anaerobic gas was a 95% CO_2:5% H_2 mix, and 4 g Na_2CO_3 l^{-1} was included to buffer the medium at pH 6·7. Aliquots (9 ml) of anaerobically prepared medium were dispensed into 25 ml Balch tubes (18 mm × 250 mm) inside the anaerobic cabinet, stoppered and autoclaved for 15 min at 100 kPa. Medium for enumeration of total culturable counts (TCC) contained in addition to the basal medium (per litre): 150 ml clarified rumen fluid (total of 300 ml), 20 ml DL-lactic acid (10%, v/v), 0·4 g Casitone, 0·4 g cellobiose, 0·4 g soluble starch, 0·4 g maltose, 0·4 g birchwood xylan and 2·0 g agar. Anaerobic diluent was made up as described previously (Mackie & Wilkens, 1988).

Cultures used for dosing. Strains of *Ruminococcus albus* (SY3 and AR67) and *Ruminococcus flavefaciens* (Y1, LP9155 and AR72) were selected for dosing based on their superior ability to degrade dry matter and neutral detergent fibre of two tropical grasses [rhodes grass and spear grass (*Heteropogon contortus*)] and a temperate legume [lucerne (*Medicago sativa*)]. These were laboratory strains that had been in culture for at least 3 years. Their ability to degrade purified cellulose was also evaluated (Krause *et al*., 1999a).

Preparation of *Ruminococcus* spp. for dosing. *Ruminococcus* strains used for dosing were maintained as axenic cultures on basal medium plus 50 mg rhodes grass (in 10 ml medium) for at least 2 weeks to ensure that they were growing well on the same complex carbohydrate sources as fed to sheep. Medium for dosing was made up anaerobically in 2 l bottles and contained basal medium plus 10 g rhodes grass. In experiment 1, *Ruminococcus* strains were inoculated into the same 2 l medium bottle in an anaerobic chamber and were allowed to incubate at 39 °C for 24 h, at which time 0·1 % cellobiose was added to the medium. Bacteria were allowed to grow for an additional 24 h before dosing to sheep. Growth before the addition of cellobiose ensured that rhodes grass would be utilized, while growth on cellobiose increased cell yield. In experiment 2, the individual *Ruminococcus* strains were always grown separately, and were mixed together in an anaerobic cabinet immediately prior to dosing.

Sheep (experiments 1 and 2) were dosed with 500 ml medium containing the designated *Ruminococcus* strains. Control sheep received 500 ml fresh uninoculated medium. Direct microscopic counts indicated that *Ruminococcus* grew to approximately 1×10^{10} cells ml^{-1} (estimated from direct microscopic counts of several doses), so that each animal received approximately 5×10^{12} cells. Sheep were dosed consecutively for 9 d, 1 h after the morning feeding. Representative rumen digesta samples (approx. 100 g) were taken with a stomach tube (20 mm diameter) immediately prior to dosing, placed on ice and transported to the laboratory for further processing (only experiment 1).

Enumeration of TCC. Ten grams of rumen digesta was weighed out into a 300 ml beaker and diluted (1:10) with chilled anaerobic diluent. This mixture was blended for 1 min (Bamix) and serially diluted to the 10^{-10} dilution (Mackie & Wilkens, 1988). Droplets (20 µl) were pipetted onto TCC plates in an anaerobic chamber from the 10^{-5} to 10^{-9} dilutions. Plates were incubated for approximately 48 h before colonies were counted.

RNA extraction. RNA was extracted according to the procedure of Stahl *et al*. (1988) with some modifications. A 1 ml subsample of crude rumen digesta was taken with a wide-bore pipette (5 mm) so that sufficient plant material was included in the sample. The sample was pipetted into a 2 ml screw-cap tube containing 0·5 g zirconium beads (75–200 µm diameter). The tube was centrifuged at 10000 ***g*** for 1 min to pellet digesta, the supernatant was discarded and 700 µl phenol/chloroform (4:1, pH 5·1) was added. Mechanical disruption of microbial biomass was done by bead-beating (Biospec) for 5 min. The nucleic acid was precipitated with a one-tenth volume of sodium acetate (3 M), resuspended in RNase-free water and incubated at 39 °C with 1 µg ml^{-1} (final concentration) RNase-free DNase (Promega) to remove contaminating RNA. The RNA concentration was measured spectrophotometrically at 260 nm and adjusted to a final concentration of 100 ng μl^{-1}. At least two subsamples from each sample of ruminal digesta were extracted and pooled.

Probe hybridization protocol. Oligonucleotides were labelled with digoxigenin (Roche Diagnostics) and analysis was carried out as previously described (Krause & Russell, 1996). RNA was denatured by incubation for 10 min at 25 °C with 3 vols 2 % glutaraldehyde. A sample volume equivalent to 1 µg per slot was diluted with 0·0002 % bromophenol blue and 1 µg polyadenylic acid ml^{-1} before application to positively charged nylon membranes (Roche Diagnostics). The membranes were baked at 120 °C for 30 min to covalently cross-link the rRNA to the membrane. Prehybridization solution [25 % formamide, 5 × SSC (1 × SSC: 0·15 M sodium chloride and 0·015 sodium citrate), 50 mM Na_2HPO_4, 2 % blocking reagent (Roche Diagnostics), 2 % SDS, 0·1 % *N*-lauroylsarcosine] was incubated with membranes for at least 2 h before the addition of the labelled probe (10 ng ml^{-1}). Probes were allowed to hybridize overnight and membranes were then washed at the appropriate stringency for each probe with 1 × SSC. Membranes were subsequently processed according to the manufacturer's instructions (Roche Diagnostics). Total 16S rRNA was determined by hybridization with a universal eubacterial probe. To prevent contamination, pre-dosing samples from the dosed sheep and all the samples from the control sheep were blotted onto separate membranes. Blotting was done in duplicate, with each duplicate blotted onto a separate membrane. A dilution series of the reference organism was included on the membrane with the samples or alternatively the dilution series was done on a membrane with only selected samples and then cross-referenced to the master membrane.

Optimization of wash temperature. Denatured RNA samples (1000 ng) were applied by slot-blotting to positively charged nylon membranes (Roche Diagnostics) and hybridized as described above. After hybridization, the membranes were cut into individual slots and each membrane (consisting of duplicate hybridization slots) was washed in 1 × SSC for at least 10 min at 34 °C. This process was repeated 12 times at increasing temperatures (34, 37, 40, 43, 46, 49, 52, 55, 58, 61, 63 and 65 °C). Each membrane was then processed as described above. The hybridization intensity (probe remaining on blot) was plotted against the wash temperature and the dissociation temperature (T_d) was defined as the temperature at which 50 % of the duplex remained bound.

Cross-hybridization assay. Probes to the dosed ruminococci were synthesized and tested for specificity against a phylogenetically diverse group of ruminal bacteria. These bacteria included *R. flavefaciens* AR71, *R. flavefaciens* AR72, *R. flavefaciens* R13e2, *R. flavefaciens* LP-9155, *R. flavefaciens* RF1Ba, *R. flavefaciens* R1-addax, *R. flavefaciens* C14-addax, *R. flavefaciens* AR69, *R. flavefaciens* B146, *R. flavefaciens* AR46, *R. flavefaciens* AR47, *R. flavefaciens* Y1, *R. flavefaciens* FD-1, *R. albus* B199, *R. albus* Ra8, *R. albus* AR67, *R. albus* SY3, *Ruminococcus callidus* ATCC 27760, *Ruminococcus bromii* ATCC 27255, *Butyrivibrio fibrisolvens* OB156, *B. fibrisolvens* H17c, *Escherichia coli* K-12, *Eubacterium cellulosolvens* 5494, *Eubacterium ruminantium* GA195, *Fibrobacter succinogenes* S85, *Lactobacillus vitulinus* $B_2$6, *Megasphaera elsdenii* B159, *Prevotella ruminicola* 23, *Prevotella ruminicola* GA33, *Ruminobacter amylophilus* 70, *Selenomonas ruminantium* HD4, *Streptococcus bovis* JB1, *Streptococcus bovis* K11-21-09, *Succinimonas amylolytica* $B_2$4, *Succinivibrio dextrinosolvens* 22B, *Treponema bryantii* $B_2$5.

Statistical analysis. Experiments followed a repeated-measures factorial arrangement of treatments (dosed animal group and one control group) and time (levels) (Littell *et al*., 1998). Total sums of squares were partitioned between the sums of squares for treatments and time (level). Only *F*-values for

model effects with an alpha-value greater than 0·1 were considered significant. The standard error of the mean (SEM) was computed for each analysis. All statistical analyses were carried out with Statistica 5.0 (StatSoft, Tulsa, USA).

RESULTS

Specificity of oligonucleotide probes

For each oligonucleotide probe, an experimental (T_d) and actual (T_e) dissociation wash temperature is presented (Table 1). The amount of probe remaining bound to the nylon membrane had a sigmoid shape as the wash temperature increased (Fig. 1). The temperature at which 50% of the probe was washed off the membrane was defined as the T_d, and was 46·3, 58·1, 43·8, 56·7 and 46 °C for *Ruminococcus* strains SY3, AR67, Y1, LP9155 and AR72, respectively. However, in actual hybridization studies of ruminal samples, a compromise between specificity and sensitivity had to be made and the temperatures under experimental conditions were 50, 55, 46, 53 and 45 °C for strains SY3, AR67, Y1, LP9155 and AR72, respectively (Table 1, Fig. 1).

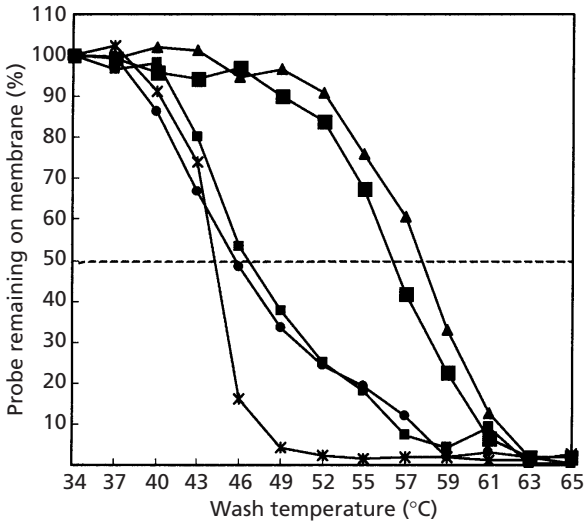

Fig. 1. Melting curves and dissociation temperatures (T_d) for probes targeting strains SY3 (small squares), AR67 (triangles), LP9155 (large squares), Y1 (crosses) and AR72 (circles).

Computer searches were made of oligonucleotide probes in GenBank and the closest small-subunit rRNA comparisons are shown in Table 2. S-Ss-Ralb SY3-0141-a-A-20 was homologous to *R. albus* OR108 and there were no other close matches. S-Ss-Ralb AR67-1214-a-A-23 had no homologues and there were no close 16S rDNA matches. S-Ss-Rflav Y1-0169-a-A-18 was 100% similar to *B. fibrisolvens* LP1265 and had four mismatches with the unidentified rumen bacterial clone RF4. S-Ss-Rflav LP9155-0160-a-A-28 was homologous to *R. flavefaciens* NJ and had one mismatch with the unidentified rumen bacterial clone 4C0d-11; the next closest match was with clone 4C0d-7 (10 mismatches). S-Ss-Rflav AR72-0186-a-A-18 was not 100% similar to any database sequence but had a 1 bp mismatch with *R. flavefaciens* strains FD-1, LP9155 and 4. There was also a 1 bp mismatch with clone AY006604 (GenBank accession number) and '*Clostridium phytofermentans*' ISDg.

Probes were hybridized with a phylogenetically diverse group of ruminal bacteria, as well as rRNA extracted from rumen fluid. All probes were reasonably specific,

Table 1. 16S-rRNA-based oligonucleotide probes used in this study

Target	Oligonucleotide†	Sequence (5′–3′)	T_e‡	T_d‡	Reference
R. albus SY3	S-Ss-Ralb SY3-0142-a-A-20	GATAAGCCGCATGACCTATC	50	46·3	This study
R. albus AR67	S-Ss-Ralb AR67-1213-a-A-23	GGGTAGCAAAGTGGTGACACGGA	55	58·1	This study
R. flavefaciens Y1	S-Ss-Rflav Y1-0189-a-A-18	GAGACCGCATGATTTCTG	46	43·8	This study
R. flavefaciens LP9155	S-Ss-Rflav LP9155-0128-a-A-28	CGGAGATATTTTGGTTTACTGAGATATC	53	56·7	This study
R. flavefaciens AR72	S-Ss-Rflav AR72-0193-a-A-18	CCGCATGATTTAGCTATC	45	46·0	This study
R. albus + *R. flavefaciens*	S-G-Rum IV-0132-a-A-17	TGKTAATACCYCATAAY	33	33·0	Krause *et al.* (1999b)
F. succinogenes	S-S-Fsucc-0650-a-A-20	TGCCCCTGAACTATCCAAGA	48	48	Lin *et al.* (1994)
Most bacteria	S-D-Bact-0338-a-A-18	GCTGCCTCCCGTAGGAGT	54	54	Amann *et al.* (1990)
Eukaryotes	S-D-Euka-1379-a-A-16	TACAAAGGGCAGGGAC	33	33	Hicks *et al.* (1992)
Most organisms	S-*-Univ-1390-a-A-18	GACGGGCGGTGTGTACAA	44	44	Zheng *et al.* (1996)

† Probe nomenclature according to Oligonucleotide Probe Database (Alm *et al.*, 1996).

‡ Wash temperature for slot-blot probes. T_e is the actual experimental wash temperature used in these experiments, and T_d is the experimentally determined wash temperature. Post-hybridization washes for slot-blotting were done with 1× SSC (0·15 M sodium chloride plus 0·015 sodium citrate).

Table 2. Comparison of oligonucleotide probes and their target small-subunit ribosomal rRNA sequences available from GenBank

Probe and target bacterium	Bacterial strain	5′ position of target	Probe and complementary target sequence (5′–3′)*	3′ position of target
S-Ss-Ralb SY3-0141-a-A-20			GATAAGCCGCATGACCTATC	
Ruminococcus albus	SY3	141	GATAAGCCGCATGACCTATC	160
Ruminococcus albus	OR108	174	GATAAGCCGCATGACCTATC	193
S-Ss-Ralb AR67-1214-a-A-23			GGGTAGCAAAGTGGTGACACGGA	
Ruminococcus albus	AR67	1214	GGGTAGCAAAGTGGTGACACGGA	1236
S-Ss-Rflav Y1-0169-a-A-18			GAGACCGCATGATTTCTG	
Ruminococcus flavefaciens	Y1	169	GAGACCGCATGATTTCTG	186
Butyrivibrio fibrisolvens	LP1265	158	GAGACCGCATGATTTCTG	175
Unidentified rumen bacterium	RF4	134	...ACCGCATGATTTCT.	151
S-Ss-Rflav LP9155-0060-a-A-28			CGGAGATATTTTGGTTTACTGAGATATC	
Ruminococcus flavefaciens	LP9155	60	CGGAGATATTTTGGTTTACTGAGATATC	87
Ruminococcus flavefaciens	NJ	60	CGGAGATATTTTGGTTTACTGAGATATC	87
Unidentified rumen bacterium	4C0d-11	13	CGGAGATATTTTGGTTTACTGAG.TATC	40
Unidentified rumen bacterium	4C0d-7	67	.......ATTTTGGTTTACTGAGAT...	94
S-Ss-Rflav AR72-0186-a-A-18			CCGCATGATTTAGCTATC	
Ruminococcus flavefaciens	AR72	186	CCGCATGATTTAGCTATC	203
Unidentified rumen bacterium	AY006604	175	CCGCATGATTT.GCTATC	192
Ruminococcus flavefaciens	FD-1	155	CCGCATGATTTAG.TATC	173
Ruminococcus flavefaciens	LP9155	183	CCGCATGATTTAG.TATC	200
Ruminococcus flavefaciens	4	181	CCGCATGATTTAG.TATC	198
'*Clostridium phytofermentans*'	ISDg	97	CCGCATGATTT.GCTATC	117

* 16S rDNA oligonucleotide probe sequence and matching rRNA sequence in GenBank are given. Stops indicate mismatches.

but all cross-reacted with rRNA extracted from control animals. However, the signal intensity in the dosed animals was sufficiently large to enable the tracking of dosed strains. When probes were hybridized with rRNA from individual strains cross-reactivity occurred. Probes to AR72 and LP9155 had 1 bp mismatches in comparison to their closest GenBank matches, and cross-reacted with AR71, and with AR64 and AR47, respectively. Probes to SY3 and AR67 were unique and no cross-reactivity with individual strains was detected.

One-quarter dilutions were made of 1000 ng rRNA extracted from strains SY3, AR67, Y1, LP9155 and AR72, and blotted onto nylon membranes. Hybridization with probes to SY3, AR67, Y1, LP9155 and AR72 indicated that as little as 0·9 ng 16S rRNA could be detected (Fig. 2). This value constituted approximately 10^6 cells ml^{-1} and represented the probe detection limit.

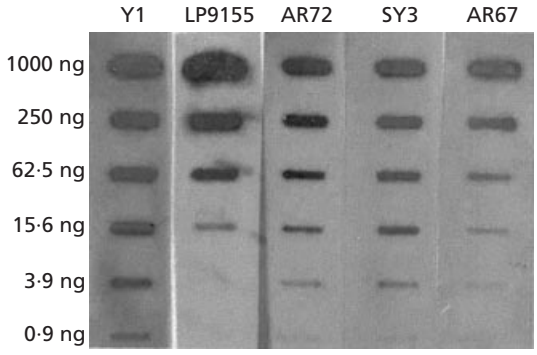

Fig. 2. One-quarter dilution series of rRNA extracted from strains SY3, AR67, LP9155, Y1 and AR72, hybridized with complementary probe and washed with 1× SSC at experimental wash temperatures (T_e; see Table 1).

Estimation of the relative abundance of different groups of bacteria

The relative abundance of individual strains increased during dosing and peaked at ∼ 1·9, 0·8 and 1·8 % for *R. flavefaciens* strains Y1, AR72 and LP9155, respectively (Fig. 3a). The levels of *R. albus* strains SY3 and AR67 peaked at ∼ 1·7 and 1·2 %, respectively (Fig. 3b). The effect of dosing was significant ($P < 0·05$ for Y1, LP9155, SY3 and AR67, and $P < 0·1$ for AR72) for all strains, and many time-point comparisons during the dosing and post-dosing periods were significant ($P < 0·05$). All five strains were grown together immediately prior to dosing and rRNA slot-blot analysis of the dose showed that the relative abundance of strains LP9155, Y1, SY3, AR67 and AR72 were 20 (±1·0), 19 (±2·0), 16 (±2·0), 9 (±0·8) and 6 (±0·7) %, respectively, of the total dose.

An oligonucleotide probe specific for most *R. albus* and

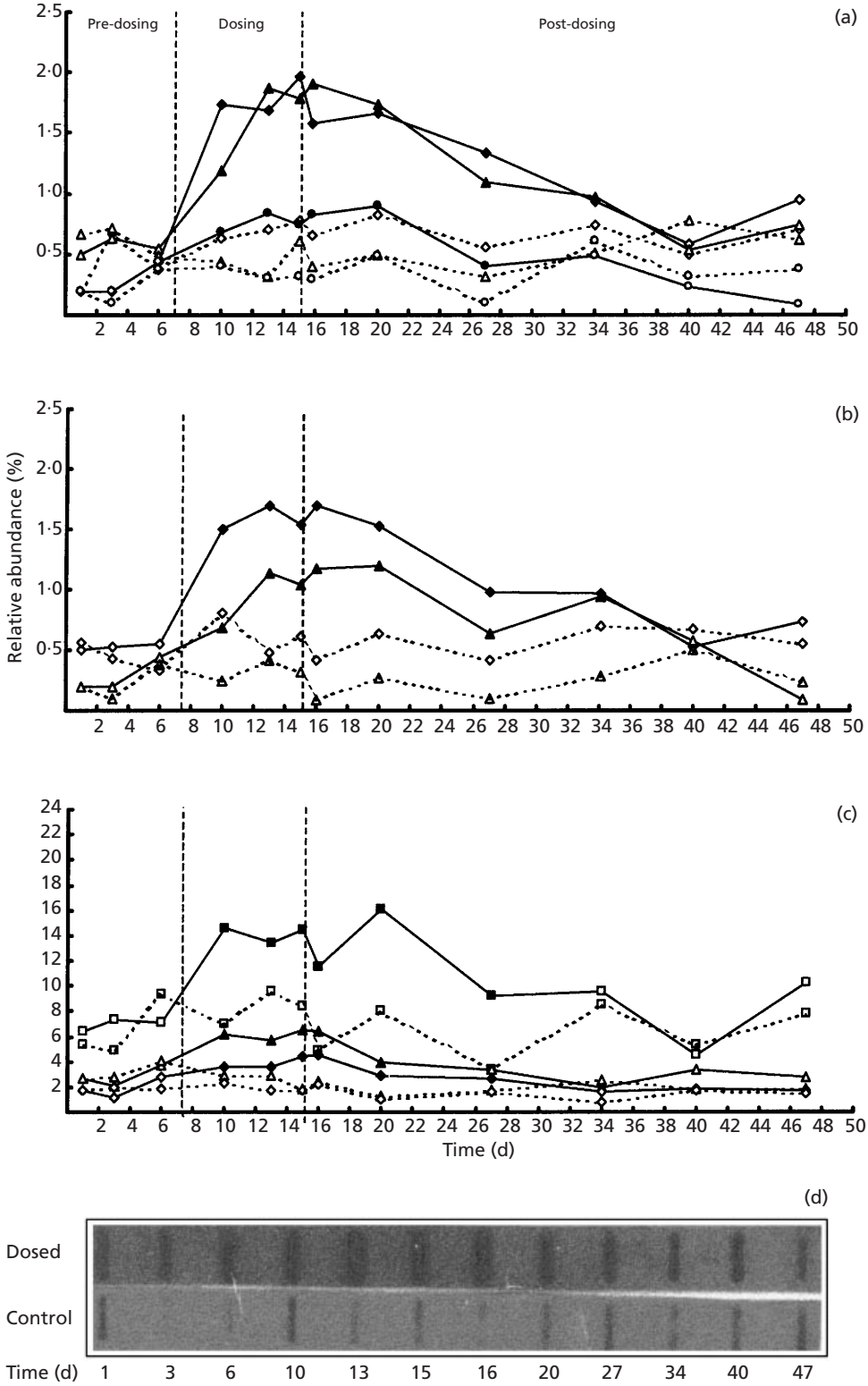

Fig. 3. (a–c) Small-subunit-rRNA-based relative abundances of specific organisms, or groups of organisms, in sheep dosed with *Ruminococcus*. (a, b) Relative abundance as a proportion of the total bacterial population for dosed *R. flavefaciens* strains Y1 (diamonds), LP9155 (triangles) and AR72 (circles) (a); and *R. albus* strains SY3 (diamonds) and AR67 (triangles) (b). (c) Total *Ruminococcus* (triangles) and *Fibrobacter* (diamonds) as a proportion of the bacterial population, and eukaryotes (squares) as a proportion of the total microbial population. In each panel (a, b or c), dotted lines represent undosed control animals and solid lines represent dosed animals; open symbols represent controls and values not significantly different ($P > 0{\cdot}05$) from controls; solid symbols represent values significantly different ($P < 0{\cdot}05$) from controls. (d) Typical hybridization results for an animal dosed with strains of ruminococci in comparison to an undosed control.

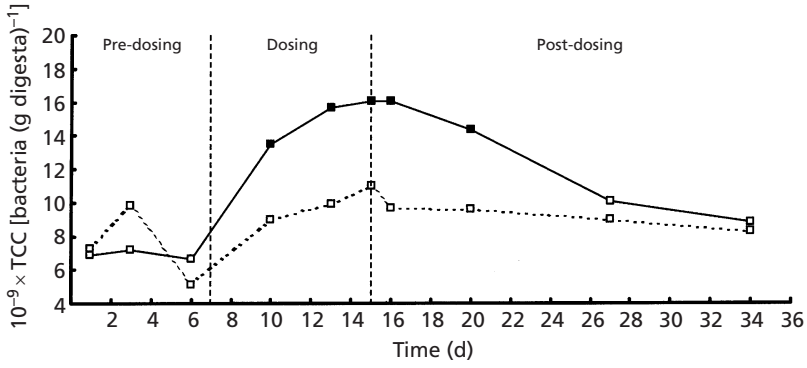

Fig. 4. Enumeration of the TCC. The dotted line represents undosed control animals and the solid line represents dosed animals; open symbols represent controls and values not significantly different ($P > 0{\cdot}05$) from controls; solid symbols represent values significantly different ($P < 0{\cdot}05$) from controls.

Table 3. Intake and *in situ* nylon bag digestibility values of sheep dosed with *Ruminococcus* spp. in experiment 1

Period	Treatments		SEM*
	Control	Dosed	
	DMI (g d^{-1})		
Pre-dosing	693·7	676·1	43·7
Dosing	794·1	793·0	64·9
Post-dosing	748·4	797·5	71·4
	***In situ* nylon bag digestibility (%)**		
Pre-dosing	34·7	33·9	1·5
Dosing	36·8	35·7	0·9
Post-dosing	36·7	36·7	1·4
	DDMI (g d^{-1})		
Pre-dosing	240·7	229·2	16·5
Dosing	292·2	283·1	26·4
Post-dosing	274·6	292·7	27·7
n†	3	3	

* Pooled standard error of the mean. Means were not significant at the $P < 0{\cdot}1$ or $P < 0{\cdot}05$ levels.

† Number of animals used.

Table 4. Intake and *in situ* nylon bag digestibility values of sheep dosed with *Ruminococcus* spp. in experiment 2

Period	Treatments		SEM*
	Control	Dosed	
	DMI (g d^{-1})		
Pre-dosing	768·9	800·8	48·4
Dosing	820·8	850·2	67·1
Post-dosing	863·5	860·5	73·4
	***In situ* nylon bag digestibility (%)**		
Pre-dosing	46·3	46·5	1·8
Dosing	45·5	45·5	1·1
Post-dosing	46·2	46·1	1·3
	DDMI (g d^{-1})		
Pre-dosing	352·1	372·2	22·6
Dosing	370·1	388·1	30·2
Post-dosing	385·7	399·4	35·6
n†	10	10	

* Pooled standard error of the mean. Means were not significant at the $P < 0{\cdot}1$ or $P < 0{\cdot}05$ levels.

† Number of animals used.

R. flavefaciens (Table 1) was used to monitor the total abundance of *Ruminococcus*. During the dosing period, ruminococci increased ($P < 0{\cdot}05$) to approximately 6·5 % of the total bacterial population, but declined to baseline levels by 19 d post-dosing (Fig. 3c). When the relative abundance of each individual dosed strain was summed, it correlated reasonably well ($r = 0{\cdot}58$) with that determined using the genus-specific *Ruminococcus* probe. It is likely that cross-reactivity between probe and non-specific target prevented a higher correlation from being obtained. When the *F. succinogenes* population size was estimated (Fig. 3c), there was an increase during the dosing period to approximately 4·7 % of the total bacterial 16S rRNA population and the effect of dosing was statistically significant ($P < {\cdot}05$). The *F. succinogenes* population declined post-dosing in a manner similar to that of the ruminococci population. Eukaryote abundance also increased ($P < 0{\cdot}1$) during dosing but was never greater than 16 % of the total 16S rRNA population (Fig. 3c).

During the dosing period, TCC increased significantly ($P < 0{\cdot}05$) from approximately 7×10^9 to $1{\cdot}6 \times 10^{10}$ ml^{-1} (Fig. 4). This was an increase of 128 % over that during the pre-dosing period. The increase in TCC was in general agreement with that of *Fibrobacter* and eukaryotic populations (Fig. 3c), which increased by approximately 100 %. In contrast, the *Ruminococcus* increased by almost 200 % (Fig. 3c).

Feed intake and *in situ* nylon bag digestibility

When animals were dosed there was an insignificant ($P > 0{\cdot}05$) increase in DMI in experiments 1 (Table 3) and 2 (Table 4). *In situ* nylon bag digestibility (experiment 1, Table 3) and whole-tract DMD showed no differences

between the control and dosed groups, but when digestibility was expressed as DDMI there appeared to be a slight, but insignificant ($P > 0{\cdot}05$) increase post-dosing (Tables 3 and 4).

DISCUSSION

Sequence variation of 16S rDNA among closely related strains is not great (Fox *et al*., 1992), and we sequenced the 23S–16S intragenic region (Vinuesa *et al*., 1998; Sawada *et al*., 1997; Rijpens *et al*., 1996) of 16 *R. albus* and *R. flavefaciens* strains to see if this area could be used as an alternative region for probe design (D. O. Krause, data not shown). Unfortunately, the sequence variation was no greater than that of the hypervariable regions of 16S rDNA. Probes were consequently derived from multiple alignments of 16S rDNA genes from 33 strains of *R. albus* and *R. flavefaciens*. In some cases, sequence variation was not sufficient for design of unique probes (e.g. probes to strains SY3 and Y1, Table 2) and stringency conditions in hybridization studies were relaxed to increase the degree of sensitivity (Table 1). This resulted in some cross-hybridization with rRNA from control sheep and with non-target ruminal strains. The hybridization intensity was, however, greater than background, which enabled the strain-specific probes to be useful tools for assessing the ecology of fibre digestion.

Probes to SY3, AR67 and Y1 performed well and there was very little cross-hybridization with non-target strains. In comparison, probes to LP9155 and AR72 cross-reacted and probe design relied, in some cases, on only 1 bp mismatches with non-target strains. A possible means of increasing probe specificity is the use of peptide nucleic acids (PNA), in which the sugar-phosphate backbone is replaced by peptide moieties (Nielsen, 1999; Von Wintzingerode *et al*., 2000). The stability of the PNA–DNA duplex is considerably greater than that of the RNA–DNA duplex (Nielsen, 1999).

Results from individual strain probes (Fig. 3a, b) showed that dosed organisms declined post-dosing, which is typical of dosing studies (Flint *et al*., 1989; Miyagi *et al*., 1995; Attwood *et al*., 1988), illustrating that ecological principles governing the persistence of bacterial inoculants in complex microbial communities are not well understood. It is, however, apparent that persistence is probably a consequence of community-level reproductive strategies in which community processes limit the relative abundance of individual organisms (Caldwell *et al*., 1997). These strategies are based on the evolution of cooperative networks of micro-organisms in which some members cleave specific bonds, others utilize particular substrates and still others produce inhibitors (Caldwell *et al*., 1997). A ruminal example is the production of cellodextrins by cellulolytic bacteria (Russell, 1985), which are utilized by non-structural carbohydrate-fermenting bacteria (NSC). The NSC in turn produce ammonia and BCVFA that are consumed by cellulolytic bacteria (Miller & Wolin, 1979; Wolin & Miller, 1988). It is unlikely that the inability to produce cellodextins or the lack of a requirement for BCVFA are the reason that the dosed strains did not persist. However, the long time that these strains have been in culture, inhibition by bacteriocin-producing organisms (Odenyo *et al*., 1994a, b) and protozoal predation (Sharp *et al*., 1994) may be significant factors in thwarting their successful reproduction in the rumen.

Establishment of an exogenous organism in the rumen is complicated by the fact that the rumen is not a closed ecosystem and micro-organisms are continually entering, making it a dynamic system that significantly impacts on the persistence of the inoculant. This was demonstrated by Varel *et al*. (1995) who removed the rumen contents from three cows and replaced it with 20 l medium buffer and 6 l '*Clostridium longisporum*'. At the initiation of the experiment, '*C. longisporum*' was the predominant cellulolytic bacterium, but it decreased to below the detection limit after 48 h. In similar experiments in our laboratory (D. O. Krause & C. S. McSweeney, unpublished) we grew *R. albus* AR67 in 20 l fermenters containing a nutrient medium and rhodes grass. The rumen contents of cannulated cattle were completely removed and replaced by the fermenter contents. Animals were immediately allowed to consume a rhodes grass diet, but we found that gastric stasis was often the consequence and the dosed strain declined rapidly. The reasons for the decline are not known, but the predatory behaviour of protozoa may be significant (Coleman & Sandford, 1979; Sharp *et al*., 1994; Newbold & Hillman, 1990).

Recent data suggest that dosing of ruminococci while the rumen is still immature does not allow the establishment of the introduced strains of bacteria (Krause *et al*., 1999c). When rRNA from the rumen of lambs was hybridized with a probe to the small-subunit rDNA of eukaryotes, there was a significant increase in the relative abundance of eukaryotic rRNA in the dosed groups, implying that protozoal predation might have a significant effect on persistence. In the current study, we observed an increase in the eukaryotic population during dosing to approximately 16% of the 16S rRNA population (Fig. 3c). In the non-dosed animals, eukaryotes ranged between approximately 3 and 9% (Fig. 3c). Direct observations of protozoal predation of the dosed strains in this study were not made, but *in vitro* experiments have shown that when a recombinant *Lactobacillus plantarum* was mixed with rumen fluid containing protozoa, the rate of decline of the recombinant bacterium was far greater than when the protozoa were absent (Sharp *et al*., 1994).

The increase in the eukaryote population may not have been specifically a predation response, but may simply have been a response to the nutrients present in the dose. The bacterial dose contained nitrogen (from bacteria) as well as fermentation acids, which have previously been shown to benefit ruminal fibre fermentation (Van Gylswyk, 1970). Lin *et al*. (1996), using a eukaryotic signature sequence (S-D-Euca-0502-a-A-16), probed rRNA extracted from rumen contents of animals con-

suming diets which differed in the proportion of forage and concentrate. When animals were on a 100% forage diet, the eukaryotic population varied between 16·9 and 18·8% of the total population. If concentrate was included in the diet (at least 40%), the eukaryotic population tended to decline.

The probe used to measure the increase in the eukaryote population will hybridize to rRNA from fungi as well as protozoa. Fungi are important inhabitants of the rumen and may make a significant contribution to fibre digestion (Hespell *et al*., 1997). In a previous study (Krause *et al*., 1999c), lambs were dosed with these same strains and we measured the fungal population separately from the eukaryotic populations. These data indicated that the fungal response to the dosing regimen was small in comparison to the total eukaryotic response. Future studies should design and validate 16S-like rRNA probes to ruminal protozoa so that this possibility can be examined specifically.

In dosed animals, the *Ruminococcus* population was as high as 6·5% and in the undosed group varied between 1·2 and 4·0% of the total bacterial population (Fig. 3c). *F. succinogenes* increased to 4·7% in dosed animals and was low as 0·9% of the bacterial population in control animals (Fig. 3c). Previous investigations with S-G-RumIV-0132-a-A-17 and S-S-Fsucc-0650-a-A-20 have shown that *Ruminococcus* and *F. succinogenes* vary as a proportion of the bacterial population depending on the diet. When animals were fed a diet of 100% rhodes grass, *Ruminococcus* was 1·3–1·9% of the population and *Fibrobacter* 0·8–2·7% (Krause *et al*., 1999b). In contrast, if sheep were fed a diet of 30% *Calliandra calothyrsus* (tannin-rich) and 70% rhodes grass, *Ruminococcus* and *Fibrobacter* were less than 2% of the bacterial population (McSweeney *et al*. 2001). When these same animals were placed on a diet of 70% rhodes grass and 30% lucerne (*Medicago sativa*) the populations rose to approximately 6% for both *Ruminococcus* and *Fibrobacter*. Lin *et al*. (1994), using S-S-Fsucc-0650-a-A-20, observed that *F. succinogenes* varied between approximately 0·5 and 6% of the population depending on the animal and diet consumed.

When cellulolytic bacteria are grown together in diculture, cellulose degradation is often below that of the pure culture alone (Dehority & Scott, 1967; Dehority, 1973), which is probably the result of competitive and non-competitive interactions between cellulolytic bacteria. Shi *et al*. (1997) demonstrated that cell numbers of individual species were approximately equal in cellulose-excess dicultures of *R. albus* plus *R. flavefaciens*, *R. albus* plus *F. succinogenes*, and *R. flavefaciens* plus *F. succinogenes*. However, when cellulose was limiting, *R. flavefaciens* > *R. albus*, *R. flavefaciens* > *F. succinogenes*, and *F. succinogenes* > *R. albus*. These competitive outcomes were likely the result of the superior ability of *R. flavefaciens* to adhere to cellulose (Shi & Weimer, 1996). It is interesting to note that *R. albus* survived under cellulose-limited conditions. This was probably a combination of its ability to utilize glucose (*R. flavefaciens* does not; Helaszek & White, 1991), to grow at low concentrations of cellobiose (Shi & Weimer, 1997) and to produce bacteriocins (Odenyo *et al*., 1994a).

A fuller understanding of how bacteria survive as members of consortia or cooperative networks is critical if we wish to advance the field of ecosystem biomodification. In relation to fibre degradation, the issue of bacteriocin production by certain cellulolytic bacteria has only recently being explored and is likely to be an essential component in the formation of cooperative microbial networks. *R. albus* strains can produce bacteriocin-like substances that inhibit the growth of *R. flavefaciens* but not of *F. succinogenes* (Odenyo *et al*., 1994a, b). There also appears to be an unusually high incidence of bacteriocin-like activity among *Butyrivibrio* isolates and butyrivibriocin has been isolated from *B. fibrisolvens* AR10 (Kalmokoff & Teather, 1997). How the ability to produce bacteriocins or resistance to bacteriocins are involved in the establishment and persistence of dosed ruminal bacteria is not known, but these compounds are likely to have important ecological consequences.

There were no significant improvements in DMI, DMD or DDMI (Tables 3 and 4). These results demonstrate that increasing the numbers of cellulolytic bacteria in the rumen to the extent that fibre digestion is improved is very difficult. In the dosing protocol used, we hoped that the microbial population would be perturbed to a sufficient extent to allow the introduced bacteria to establish and multiply. This proved not to be the case as shown by the molecular ecology measurements (Fig. 3). These data are confirmed by those of Dehority & Tirabasso (1998) who could not demonstrate any improvement in the proportion of cellulose digested with a 10-fold increase in the number of cellulolytic bacteria in the rumen.

It is clear from these studies that an improvement in fibre digestion *in vivo* is not a foregone conclusion simply because the dosed strains have been maintained at elevated levels in the rumen. The strains used for inoculation were selected using *in vitro* criteria and it is not known if these strains could be classified as 'superior' *in vivo*. For this to be done *in situ*, techniques for strain evaluation would have to be developed and a functional genomic approach could be taken in which the levels of expression of key enzymes are monitored. It is also known that fibrolytic strains can undergo subtle changes in phenotype because of repeated transfer under laboratory conditions. It is likely that key 'elements' are lost from the strains and many of these could be critical for the ability of strains to colonize and persist *in vivo*. Future studies should identify and evaluate these 'elements'.

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